Acute rapamycin treatment improved glucose tolerance through inhibition of hepatic gluconeogenesis in rainbow trout (*Oncorhynchus mykiss*)

Weiwei Dai¹, Stéphane Panserat¹, Frédéric Terrier¹, Iban Seiliez¹ and Sandrine Skiba-Cassy¹,²

¹ INRA, UR 1067 Nutrition Métabolisme, Aquaculture, Pole d’hydrobiologie, CD 918, F-64310 Saint-Pée-sur-Nivelle, France.

AUTHOR CONTRIBUTIONS

W. D. performed data acquisition, data analysis and wrote the manuscript. F. T. contributed in the intraperitoneal administration and fish rearing. S. S.-C. and S. P. developed the study design. S. S.-C., S. P. and I. S. contributed to the manuscript correcting. S. S.-C. is the guarantor of this study and take full responsibility for the data, analysis, statistical analysis and manuscript submission.

Running head: Acute rapamycin treatment improved glucose tolerance in trout

² Corresponding author:

Sandrine SKIBA-CASSY
INRA, UR1067 Nutrition, Métabolisme, Aquaculture
F-64310 Saint-Pée-sur-Nivelle, France
Tel: (33) 5 59 51 59 93
Fax (33) 5 59 54 51 52
E-mail: skiba@st-pee.inra.fr
ABSTRACT

Our aim was to investigate the potential role of TOR (target of rapamycin) signaling pathway in the regulation of hepatic glucose metabolism in rainbow trout. Fasted fish were firstly treated with a single intraperitoneal (IP) injection of rapamycin or vehicle and then submitted to a second IP administration of glucose 4 h after. Our results revealed that IP administration of glucose induced hyperglymia for both vehicle and rapamycin treatments, which peaked at 2 h. Plasma glucose level in vehicle-treated fish was significantly higher than in rapamycin-treated fish at 8 and 17 h whereas it remained at the basal level in rapamycin-treated fish. Glucose administration significantly enhanced the phosphorylation of Akt and ribosomal protein S6 kinase (S6K1) in vehicle treated fish, while rapamycin completely abolished the activation of S6K1 in rapamycin-treated fish, without inhibiting the phosphorylation of Akt on Thr308 or Ser473. Despite the lack of significant variation in phosphoenolpyruvate carboxykinase (mPEPCK) mRNA abundance, mRNA abundance for glucokinase (GK), glucose 6-phosphatase (G6Pase) I and II, and fructose 1,6-bisphosphatase (FBPase) was reduced by rapamycin 17 h after glucose administration. The inhibition effect of rapamycin on GK and FBPase was further substantiated at the activity level. The suppressions of GK gene expression and activity by rapamycin provided the first in vivo evidence in fish that glucose regulates hepatic GK gene expression and activity through a TORC1 dependent manner. Unlike in mammals, we observed that acute rapamycin treatment improved glucose tolerance through the inhibition of hepatic gluconeogenesis in rainbow trout.

Key words: TOR, rapamycin, gluconeogenesis, glucokinase, glucose homeostasis, rainbow trout
INTRODUCTION

Plasma glucose level is the result of glucose intake, utilization and endogeneous production. As regards glucose homeostasis, carnivorous animals like salmonids are particular model. In their natural habitat, carnivorous animals such as cat, mink, trout, salmon, sea bass and sea bream consume prey high in protein but low in carbohydrates and are therefore metabolically adapted to lower glucose but higher protein utilization (4, 26, 73).

The presence of high levels of carbohydrates in fish diet is highly problematic since carnivorous fish are recognized for their low efficiency in using digestible carbohydrates and are typically classified as “glucose intolerant” (11, 39, 40). Oral, intravenous or intraperitoneal (IP) administration of glucose or a carbohydrate-rich diet result in persistent hyperglycemia in various fish species, including rainbow trout (3, 32, 40, 41). Several parameters may contribute to this phenotype. Glucokinase (GK), also known as hexokinase IV, plays a pivotal role in blood glucose homeostasis by catalyzing the phosphorylation of glucose, providing metabolites for glycolysis, glycogenesis and pentose phosphate pathway in hepatocytes (1, 5), and sensing glucose for insulin secretion in pancreatic β-cells (35). The rate of gluconeogenesis is mainly controlled by the activities of unidirectional enzymes including phosphoenolpyruvate carboxykinase (mPEPCK), fructose-1, 6-bisphosphatase (FBPase), and glucose-6-phosphatase (G6Pase). G6Pase catalyzes the last step, which is shared by gluconeogenesis and glycogenolysis. Unlike cat, a mammalian carnivorous model, where hepatic GK expression and activity are lacking (26, 73) and hepatic de novo lipogenesis does not use carbon backbones from glucose as substrate (71), carnivorous fish has inducible GK (43) and can use exogenous glucose for de novo lipogenesis (4). In trout, hepatic GK gene expression and activity are highly induced by dietary carbohydrates (24, 43) or glucose (54) instead of insulin; the persistent hyperglycemia is often associated with low utilization of glucose by the peripheral tissues and impaired post-prandial down-regulation of
gluconeogenesis in liver (50, 52), similar to those typical features in human type II diabetes (56).

Recently, TOR (target of rapamycin) signaling pathway has emerged as a potential mediator of the regulation of glucose homeostasis (6). The TOR kinase exists as two physically and functionally distinct protein complexes, TORC1 and TORC2, which differ in their regulation, downstream targets, and sensitivity to the allosteric TOR inhibitor rapamycin (16). TORC1 integrates signals from growth factors, amino acids, and cellular energy status, while TORC2 is activated by growth factors only (6). TORC1 controls cell growth and protein translation through the subsequent activation of the p70 ribosomal S6 protein kinase 1 (S6K1) and the ribosomal protein S6 (74). TORC1 is also involved in the regulation of metabolism related gene expression. It enhances lipogenic gene expression by regulating the expression and processing of sterol regulatory element-binding protein 1c (SREBP1c) (72, 77) and promotes the expression of genes that regulate glucose transport and glycolysis (8, 31). TORC2 responds to the presence of growth factors such as insulin by phosphorylating several members of the AGC kinase subfamily such as Akt (30). In the liver, TORC2 also controls glucose homeostasis via activation of glycolysis and inhibition of gluconeogenesis (17). TORC2 stimulates glycolysis through activation of GK (17, 27).

Despite the well-studied nature of TOR in mammals, the physiological role(s) of TOR in the regulation of glucose utilization and gluconeogenesis in fish remains to be fully explored. Acute inhibition of TORC1 activity by rapamycin in trout liver and primary hepatocytes has shown that GK expression and activity were controlled by the TOR pathway (7, 29). However, contradictory results were obtained for genes involved in gluconeogenesis. Experiments performed in primary cell culture of hepatocytes tend to indicate that the regulation of expression of G6Pase is dependent upon the TOR pathway (7, 29), while acute administration of rapamycin in vivo failed to modify the post-prandial expression of G6Pase,
FBPase or mPEPCK in trout liver (7) despite effective inhibition of TORC1 activity. Moreover, abundant investigations in fish indicated that hepatic GK expression and activity are mainly induced by dietary carbohydrate or glucose (10, 42, 43, 55), whereas in mammalian liver GK gene expression is strictly controlled by insulin (22, 23, 36, 57). We thus hypothesized that dietary carbohydrate/glucose regulates hepatic GK activity through a TORC1 dependent manner. To test this hypothesis, we pre-treated trout with IP administration of rapamycin or not to achieve an acute inhibition of the TORC1 signaling pathway (7) followed by an IP injection of glucose to investigate the consequences on plasma glucose levels as well as on the regulation of expression of main genes involved in hepatic glucose utilization and production, which were glucokinase (GK) for glucose phosphorylation, glucose-6-phosphatase (G6Pase) for glucose dephosphorylation, fructose-1,6-bisphosphatase (FBPase) and phosphoenolpyruvate carboxykinase (mPEPCK) for gluconeogenesis.

MATERIAL AND METHODS

Experimental and sampling procedure

Juvenile rainbow trout (*Oncorhynchus mykiss*) 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 body mass 140 g) were distributed into six tanks (12 fish per 70 L tank). Prior to the intraperitoneal (IP) administration, fish were food deprived for 72 h (time required to ensure the complete emptying of the digestive tract). After this period, trout were sedated with benzocaine (10 mg/L) and treated with a single IP injection of rapamycin (0.5 mg/kg) or
vehicle (75% DMSO and 25% saline solution, NaCl 0.9%) at 100 µL/100 g body mass. Four
hours after the first injection, six fish per treatment (vehicle or rapamycin, two fish per tank)
were sampled, while all remaining fish were sedated and subjected to the second IP
administration with 250 mg D-glucose/kg body weight based on previous studies in fish (40).
Two fish per tank (three tanks per treatment) were randomly sampled at 2, 4, 6, 8 and 17 h
after glucose injection (N=6). Trout were anaesthetized with benzocaine (30 mg/L) 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. 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 metabolites analysis**

Plasma glucose (Glucose RTU, bioMérieux, Marcy l’Etoile, France) levels were
determined using commercial kits adapted to a microplate format, according to the
recommendations of the manufacturer.

**Western blot analysis**

Frozen livers (N=6; 200 mg) from fasted fish (T0) and 2 h glucose injected fish (T2)
were homogenised on ice with an ULTRA-TURRAX® homogenizer (IKA®-WERKE,
Staufen, Germany) in 2 mL of buffer containing 150 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EGTA, 1 mmol/L EDTA (pH 7.4), 100 mmol/L NaF, 4 mmol/L sodium pyrophosphate, 2 mmol/L 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 the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with BSA as standard. Lysates (10 µg of total protein for Akt and 20 µg for S6K1) 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), antiphospho-S6 protein kinase 1 (Thr389) (no. 9205) and anti-S6 protein kinase 1 (no. 9202) were purchased from Cell Signaling Technologies (Ozyme, Saint Quentin Yvelines, France). All of these antibodies were successfully cross-reacted with rainbow trout proteins (7, 60, 62). Membranes were washed and 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 (Version 3.0, LI-COR Biosciences).

**Gene expression analysis: real-time PCR**

Liver samples (N=6) for gene expression were collected from fasted fish (T0) and 17 h glucose injected fish (T17), which was chosen based on the hepatic gene expression peak or trough of rainbow trout (38). 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
was 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, Carlsbad, CA, USA) and random primers (Promega, Charbonnières, France) according to the manufacturers’ instructions. Target gene expression abundance was determined by quantitative real-time (q)RT-PCR, using specific primers (63).

qRT-PCR was carried out on a LightCycler® 480 II (Roche Diagnostics, Neuilly sur Seine, France) using LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany). qRT-PCR analyses were focused on several key enzymes of hepatic metabolism, which were glucokinase (GK; EC 2.7.1.2) for glucose phosphorylation, 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 (mPEPCK; EC 4.1.1.32) for gluconeogenesis. When different isoforms of a gene were known in rainbow trout (as for G6Pase), 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 µM), 3 µL LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) 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 Tm (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 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 \( \Delta \Delta CT \) method by the software of LightCycler® 480 (Version SW 1.5, Roche Diagnostics). PCR efficiency, which was assessed 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 fasted (T0) and 17 h glucose injected trout (T17). Liver samples for GK enzyme activities were homogenized in four volumes of ice-cold buffer (50mmol/l TRIS, 5mmol/l EDTA, 2 mmol/l DTT and a protease inhibitor cocktail (Sigma, St Louis, MO; P2714), pH 7.4) and centrifuged at 900 \( g \) at 4°C for 10 min. GK activities were assayed immediately in the supernatant as described elsewhere (13, 43). Livers for G6Pase enzyme activities were homogenized in eight volumes of ice-cold buffer (as described for GK), then centrifuged and the supernatant were assayed following the protocol of Alegre et al. (2). Samples for FBPase enzyme activities were homogenized in seven volumes of ice-cold buffer (as described for GK), centrifuged at 900 \( g \) at 4°C for 10 min and the supernatant were assayed immediately for FBPase enzyme activity in pre-established conditions (65). Enzyme activity is defined as \( \mu \)moles of substrate converted to product, per minute, at 37°C and is expressed per mg of liver protein.

**Statistical analysis**

The data for plasma glucose, western blot, gene expression and enzyme activity are expressed as means + s.e.m. \( (N=6) \). The effects of time (T), IP administration of vehicle or Rapamycin (I) and their interaction were analyzed using two-way ANOVA, followed by a Tukey test when the interaction was significant. In cases where data were nonparametric or not
homoscedastic, data transformations, such as logarithms, square roots and reciprocals, were used to meet ANOVA criteria. Normality was assessed using the Shaprio–Wilk test, while homoscedasticity was determined using Levene’s test. For all statistical analyses, the level of significance was set at $P \leq 0.05$.

**RESULTS**

**Plasma glucose levels**

Plasma glucose levels peaked at 2 h after the glucose administration then returned to their initial level from 4 h until 8 h for both vehicle and rapamycin treatments (Fig. 1). In vehicle treated fish, plasma glucose level measured at 17 h in vehicle treatment was not significantly different compared to its peak level, while it stayed lower than its peak level in rapamycin treatment. As indicated by the statistical interaction between time and IP administration of vehicle or rapamycin ($P \leq 0.01$), plasma glucose level in vehicle-treated fish was significantly higher than in rapamycin-treated fish at 8 h and 17 h after glucose administration ($P \leq 0.01$).

**Akt/TOR signaling pathway**

Two-way ANOVA statistical analysis showed that the phosphorylation of Akt on Thr308 and Ser473 was not affected by glucose administration ($P > 0.05$) or IP injection of vehicle or rapamycin ($P > 0.05$) (Fig. 2A and 2B). Based on the significant interaction between glucose administration and injection of vehicle or rapamycin ($P \leq 0.05$), we further analyzed the data and found that glucose administration significantly induced the phosphorylation of Akt on Ser473 in vehicle treated fish ($P \leq 0.05$) but not in rapamycin treated fish. Moreover, rapamycin significantly increased Akt Ser473 phosphorylation level.
before glucose injection ($P \ 0.05$). Phosphorylation of S6K1 was significantly enhanced by glucose administration in vehicle treatment, while this enhancement was totally abolished by rapamycin treatment ($P \ 0.01$; Fig. 2C).

**Messenger RNA levels of target genes**

To determine the potential involvement of the TOR signaling pathway in the regulation of hepatic glucose metabolism-related gene expression, we performed qRT-PCR analysis (Fig. 3). GK mRNA abundance was significantly up-regulated by glucose administration in vehicle treated fish, while rapamycin treatment inhibited this up-regulation (Fig. 3A). As regards gluconeogenesis, both glucose administration and rapamycin treatment inhibited mRNA abundance of G6Pase I ($P \ 0.01$) (Fig. 3B) and G6Pase II ($P \ 0.01$) (Fig. 3C) whereas only rapamycin treatment down-regulated FBPase gene expression ($P \ 0.01$) (Fig 3D). No significant variation was observed for mPEPCK mRNA abundance (Fig. 3E).

**Enzyme activity analysis**

To substantiate the results from gene expression analysis, hepatic enzyme activities of GK, G6Pase and FBPase, were determined before and 17 h after glucose injection (Fig. 4). Glucose injection significantly increased GK enzyme activity in vehicle-treated fish, while this increase was blocked in rapamycin-treated fish (Fig. 4A). G6Pase enzyme activity remained unchanged regardless of time or IP administration of vehicle or rapamycin (Fig. 4B) whereas FBPase enzyme activity was significantly decreased by IP administration of rapamycin ($P \ 0.01$, Fig. 4C).

**DISCUSSION**
In the present study, we investigated the role of TOR signaling pathway in the regulation of glycemia in rainbow trout. For that purpose, we performed IP administration of rapamycin, followed by IP administration of glucose 4 h after the first injection. Glucose administration has been reported to cause hyperglycemia in a number of teleost species (18, 20, 25, 40). In the present experiment, plasma glucose levels exhibited a biphasic response. Consistent with previous trout observations (18, 32), the first phase consisted of a peak of plasma glucose occurring 2 h after glucose injection with a recovery of the basal level (0 h) at least 4 h after glucose injection in both vehicle and rapamycin treated fish. It is worthwhile to note that in the second phase, plasma glucose level in vehicle-treated fish was significantly higher than in rapamycin-treated fish between 8 and 17 h and displayed an increasing tendency whereas it remained at the basal level in rapamycin-treated fish indicating that, unlike mammals (19, 28, 75), acute rapamycin treatment improved glucose homeostasis in rainbow trout.

Investigation of TOR signaling pathway reveals that glucose administration significantly enhanced the phosphorylation of S6K1 in vehicle treated fish, while rapamycin completely abolished this activation, confirming the efficiency of rapamycin in inhibiting TORC1 (7). Considering Akt Ser473, glucose administration significantly activates Akt signaling pathway, suggesting efficient induction of insulin secretion (39, 52). However, due to high level of Akt phosphorylation in rapamycin treated fish before glucose administration, no further enhancement of Akt phosphorylation was recorded after glucose administration. This absence of significant difference could be due to acute induction of insulin release by rapamycin treatment (14) or rapamycin inhibition the S6K1-induced negative feedback loop on early events of insulin signaling (19, 66, 68-70).

The regulation of GK expression in healthy mammals is dual: in the pancreatic β-cells, it is controlled by the blood glucose level, while in the liver it is strictly controlled by
insulin (22, 36, 57) through PI3-kinase/Akt pathway (34). In cultured rat hepatocytes, the
effect of insulin as inducer of GK was shown to be primarily at a transcriptional level, taking
place in glucose free as well as glucose containing medium without any synergistic effect of
glucose supplement (21). In fish, the regulation of GK gene expression seems to be clearly
dependent on carbohydrate supplement (10, 42, 43, 49, 55). The present study clearly
confirms this feature at both gene expression and activity levels. The suppression of GK gene
expression and activity by rapamycin provided the first in vivo evidence in fish that glucose
regulates hepatic GK gene expression and activity through a TORC1 dependent manner
confirming previous data indicating that GK gene expression may be inhibited by rapamycin
in rainbow trout hepatocytes (29). Given the key role of GKR P (glucokinase regulatory
protein) in the allosterical regulation of GK activity and subcellular localization (48), further
investigations are needed to explore its potential involvement in the nutritional regulation in
fish.

Hepatic endogenous production of glucose is driven by the availability of
gluconeogenic substrates and the activity of three key gluconeogenic enzymes, mPEPCK,
FBPase, and G6Pase (10, 15). Among these three enzymes, only G6Pase gene expression
(G6Pase I and II) was reduced by glucose injection in both rapamycin and vehicle treated fish,
whereas mPEPCK and FBPase mRNA abundance remained stable. Moreover, neither
G6Pase nor FBPase activities were controlled by glucose. This phenotype is in agreement
with the well-known glucose intolerant phenotype of carnivorous fish (42, 44, 45, 64), in
which carbohydrate-rich diets do not affect the activity or gene expression of the three
hepatic key enzymes of gluconeogenesis. While G6Pase is poorly regulated at the activity
level in fish, G6Pase gene expression showed more sensitive response to feeding (7, 42),
macronutrients composition of the diets (61), and in vitro insulin treatment (29, 52), even
though its expression is poorly regulated by dietary carbohydrate levels (46, 47). Thus, it is
consistent to record an inhibitory effect by glucose on hepatic G6Pase at gene expression
level but no regulation at its activity level. This discrepancy between gene expression and
enzyme activity may be attributed to post-transcriptional regulation. MicroRNAs are small
regulatory molecules which post-transcriptionally regulate mRNA stability and translation.
They represent one mechanism that may finely regulate enzyme activities. Indeed, recent
findings indicate that miR-29a-c can negatively regulate hepatic gluconeogenesis in mice
through direct targeting of G6Pase mRNA (33). In trout, the hepatic expression of miR29a
has been shown to be regulated by feeding (37), however further experiment are needed to
investigate the functional interaction between miR29 and G6Pase mRNA.

In terms of rapamycin effect, only FBPase was reduced at both gene expression and
activity levels, while G6Pase I and G6Pase II merely decreased at the expression level and
mPEPCK remained unaffected. Thus we concluded that acute administration of rapamycin
inhibited hepatic gluconeogenesis at the level of FBPase by limiting both its gene expression
and activity. This inhibition of gluconeogenesis by rapamycin may finally explain why
plasma glucose level remains at the basal level in rapamycin-treated fish between 8 and 17 h
after glucose administration. Therefore the absence of inhibition of hepatic gluconeogenesis
in vehicle-treated fish might lead to the higher glycemia between 8 and 17 h after glucose
administration despite a significant induction of hepatic GK in gene expression and activity.
However, GK activity measured at 17 h after glucose administration was relatively low
compared to the levels that could be reached after glucose administration in trout (24, 42),
suggesting that gluconeogenesis may be predominant upon glucose utilization at this time
point. Interestingly, the effects caused by acute rapamycin treatment in fish are opposite to
the effects caused by chronic rapamycin treatment in mice (12, 19, 28, 75). While acute
rapamycin treatment in trout improved glucose intolerance through the down-regulation of
FBPase, a rate-limiting enzyme of gluconeogenesis, chronic administration of rapamycin in
mice resulted in glucose intolerance and hyperlipidemia as a result of insulin resistance and enhanced hepatic gluconeogenesis (19, 28, 75).

Since TORC1 inhibition is the main consequence of acute rapamycin administration (59), our results support the idea that TORC1 activity positively contributes to G6Pase gene expression, which in line with our previous demonstration in primary cell culture of hepatocytes (29). The mechanism linking rapamycin to the down-regulation of FBPase gene expression and activity is less clear than for G6Pase since rapamycin does not regulate FBPase gene expression in hepatocytes (29). Notably, rapamycin not only suppressed gluconeogenesis but also slightly enhanced Akt phosphorylation, these associated effects tend to indicate that hepatic gluconeogenesis and FBPase may be negatively controlled by TORC2 in rainbow trout, through enhanced phosphorylation of Akt on Ser473, in agreement with previous in vivo demonstrations in mice (28).

**Perspective and Significance**

The present study highlighted specific features of rainbow trout glucose homeostasis including TORC1 dependent regulation of GK regulation and predominant role of gluconeogenesis in the control of glucose homeostasis. In mammals, TORC1 activation is sufficient to stimulate glucose uptake and glycolysis through the transcriptional factor HIF1α (9, 76). Further investigations are now needed to explore the involvement of HIF1α in the TORC1 dependent control of GK in fish. Furthermore, F-2,6-bisphosphate is known as a potent allosteric activator of 6PFIK and a competitive inhibitor of FBPase (51, 58). F-2,6-bisphosphatase expression and activity are warranted to further explore its role as a potential regulator of FBPase. Moreover, current hyperglycemia and impaired down-regulation of gluconeogenesis in rainbow trout may attribute to defective insulin actions (52, 53). A potential inducer for insulin resistance is amino acid (AA), which is thought to play a
significant role in the pathogenesis of insulin resistance by modulating the endocrine function of the pancreas, acting as gluconeogenic precursors, stimulating hexosamine biosynthesis, or activating the S6K1-induced feedback loop toward insulin receptor substrate 1 (IRS-1) (67). It would be interesting to perform an *in vivo* investigation with a pair-feeding method and two diets containing high protein/low carbohydrate (HPLC) or high protein/high carbohydrate (HPHC), or an *in vitro* experiment with high AA level and graded levels of glucose to test this hypothesis in the future.
ACKNOWLEDGMENTS

We thank K. Dias, V. Véron, A. Herman, E. Plagnes-Juan, A. Surget and M. Cluzeaud for technical assistance in the laboratory. We also acknowledge the technical staff of the INRA experimental fish farm at Donzacq (Y. Hontang, and F. Sandres) for fish rearing.

FUNDING

W. Dai gratefully acknowledges the financial assistance provided by the China Scholarship Council (CSC, File No. 2011633111) for his doctoral fellowship.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

W. D. performed data acquisition, data analysis and wrote the manuscript. F. T. contributed in the intraperitoneal administration and fish rearing. S. S.-C. and S. P. developed the study design. S. S.-C., S. P. and I. S. contributed to the manuscript correcting. S. S.-C. is the guarantor of this study and take full responsibility for the data, analysis, statistical analysis and manuscript submission.


46. Panserat S, Plagnes-Juan E, and Kaushik S. Gluconeogenic enzyme gene expression is decreased by dietary carbohydrates in common carp (Cyprinus carpio) and gilthead seabream (Sparus aurata). *Biochim Biophys Acta* 1579: 35-42, 2002.


Fig. 1 Plasma glucose concentrations in rainbow trout subjected to intraperitoneal (IP) administration of vehicle or rapamycin (Rapa), 0, 2, 4, 6, 8 and 17 h after IP injection of 250mg D-glucose/kg body weight (0 corresponds to fasted fish or non-glucose injected fish). Data are means ± s.e.m. (N=6). The effects of time (T), IP administration of vehicle or Rapamycin (I) and their interaction were analyzed using two-way ANOVA (shown in the upper right table, \( P<0.05 \)), followed by a Tukey test when the interaction was significant. Letters, “A” and “B” or ‘a’ and ‘b’ indicate significant differences between each time point for vehicle or Rapa treatment, respectively. “*” indicates significant difference between the injection of vehicle and rapamycin at a given time (\( P<0.05 \)). “LG10” indicates data were transformed and statistical analyzed with log transforms. “I” represents IP administration of vehicle or rapamycin, “T” represents different times, “I*T” represents interaction between I and T.

Fig. 2 Western blot analysis of hepatic (A) protein kinase B (Akt Thr\(^{308}\)), (B) Akt (Ser\(^{473}\)), and (C) ribosomal protein S6 kinase (S6K1) protein phosphorylation in rainbow trout subjected to intraperitoneal (IP) administration of vehicle or rapamycin (Rapa), 0 and 2 h after IP injection of 250mg D-glucose/kg body weight (0 corresponds to fasted fish or non-glucose injected fish). Gels were loaded with 10 µg of total protein per lane for Akt Thr\(^{308}\)/Akt Ser\(^{473}\) and 20 µg for S6K1, respectively. 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 analyzed using two-way ANOVA, followed by a Tukey test when the interaction was significant. “*” indicates significant effect of glucose injection in vehicle fish. “#” indicates significant effect of rapamycin
administration before glucose injection. Values with different superscript letters are significantly different at $P<0.05$. See Fig. 1 legend for details.

**Fig. 3** Gene expression of selected glycolytic and gluconeogenic enzymes in the liver of rainbow trout subjected to IP administration of vehicle or rapamycin (Rapa), 0 and 17 h after IP injection of 250mg D-glucose/kg body weight (0 corresponds to fasted fish or non-glucose injected fish). (A) Glucokinase (GK), (B) glucose-6-phosphatase isoform 1 (G6Pase I), (C) G6Pase II, (D) fructose 1,6-bisphosphatase (FBPase) and (E) phosphoenolpyruvate carboxykinase (mPEPCK) mRNA abundance were assessed using quantitative real-time (q)RT-PCR. Expression values are normalized with elongation factor-1 alpha (EF1α)-expressed transcripts. Results are means ± s.e.m. ($N=6$) and were analyzed using two-way ANOVA, followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at $P<0.05$. See Fig. 1 legend for details.

**Fig. 4** Enzyme activity of (A) glucokinase (GK), (B) glucose-6-phosphatase (G6Pase) and (C) fructose 1,6-bisphosphatase (FBPase) in the liver of rainbow trout subjected to IP administration of vehicle or rapamycin (Rapa), 0 and 17 h after IP injection of 250mg D-glucose/kg body weight (0 corresponds to fasted fish or non-glucose injected fish). Enzyme activity is defined as μmoles of substrate converted to product, per minute, at 37°C and is expressed per mg of liver protein. Results are means ± s.e.m. ($N=6$) and were analyzed using two-way ANOVA, followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at $P<0.05$. “SQRT” indicates data were transformed and statistical analyzed with square roots. See Fig. 1 legend for details.
Fig. 1

Plasma Glucose

<table>
<thead>
<tr>
<th>LG10</th>
<th>I</th>
<th>T</th>
<th>I*T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Glucose concentration (mmol/L) vs. Time (h)
Fig. 2

A

\[
\begin{array}{c}
\text{Vehicle T0} & \text{Vehicle T2} & \text{Rapa T0} & \text{Rapa T2} \\
0.64 & 0.11 & 0.10
\end{array}
\]

B

\[
\begin{array}{c}
\text{Vehicle T0} & \text{Vehicle T2} & \text{Rapa T0} & \text{Rapa T2} \\
0.28 & 0.31 & <0.05
\end{array}
\]

C

\[
\begin{array}{c}
\text{Vehicle T0} & \text{Vehicle T2} & \text{Rapa T0} & \text{Rapa T2} \\
<0.01 & <0.01 & <0.01
\end{array}
\]
Fig. 3

A

B

C

D

E
Fig. 4

A

Glucokinase Activity (mU/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle T0</th>
<th>Vehicle T17</th>
<th>Rapa T0</th>
<th>Rapa T17</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

G6Pase Activity (mU/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle T0</th>
<th>Vehicle T17</th>
<th>Rapa T0</th>
<th>Rapa T17</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

FBPase Activity (mU/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle T0</th>
<th>Vehicle T17</th>
<th>Rapa T0</th>
<th>Rapa T17</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>