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Insulin regulates the expression of several metabolism-related genes in the liver and primary hepatocytes of rainbow trout (*Oncorhynchus mykiss*)

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SUMMARY

Rainbow trout have a limited ability to use dietary carbohydrates efficiently and are considered to be glucose intolerant. Administration of carbohydrates results in persistent hyperglycemia and impairs post-prandial down regulation of gluconeogenesis despite normal insulin secretion. Since gluconeogenic genes are mainly under insulin control, we put forward the hypothesis that the transcriptional function of insulin as a whole may be impaired in the trout liver. In order to test this hypothesis, we performed intraperitoneal administration of bovine insulin to fasted rainbow trout and also subjected rainbow trout primary hepatocytes to insulin and/or glucose stimulation. We demonstrate that insulin was able to activate Akt, a key element in the insulin signaling pathway, and to regulate hepatic metabolism-related target genes both *in vivo* and *in vitro*. In the same way as in mammals, insulin decreased mRNA expression of gluconeogenic genes, including glucose 6-phosphatase (*G6Pase*), fructose 1,6-bisphosphatase (*FBPase*) and phosphoenolpyruvate carboxykinase (*PEPCK*). Insulin also limited the expression of carnitine palmitoyltransferase 1 (*CPT1*), a limiting enzyme of fatty acid β-oxidation. *In vitro* studies revealed that, as in mammals, glucose is an important regulator of some insulin target genes such as the glycolytic enzyme pyruvate kinase (*PK*) and the lipogenic enzyme fatty acid synthase (*FAS*). Interestingly, glucose also stimulates expression of glucokinase (*GK*), which has no equivalent in mammals. This study demonstrates that insulin possesses the intrinsic ability to regulate hepatic gene expression in rainbow trout, suggesting that other hormonal or metabolic factors may counteract some of the post-prandial actions of insulin.

Key words: insulin, liver, hepatocytes, gene expression, rainbow trout.

INTRODUCTION

Carnivorous fish such as rainbow trout (*Oncorhynchus mykiss* Walbaum) are characterized by a limited ability to use dietary carbohydrates efficiently (Wilson, 1994) and are thus considered to be 'glucose intolerant' (Hemre et al., 2002; Moon, 2001). Oral or intravenous administration of glucose and a carbohydrate-rich diet result in persistent hyperglycemia in various fish species, including rainbow trout (Bergot, 1979; del sol Novoa et al., 2004; Legate et al., 2001; Palmer and Ryman, 1972; Parrizas et al., 1994b), and are also associated with an increase in insulin levels. The magnitude of the insulin response is very fish-species dependent and less than that in mammals (Furuichi and Yone, 1981). Insulin receptors are also present in major insulin-responsive tissues in fish, i.e. white muscle, liver and adipose tissues (Gutierrez et al., 1991; Navarro et al., 1999). Up-regulation of insulin binding and tyrosine kinase activity have been observed after insulin treatment and a carbohydrate-rich diet, respectively (Banos et al., 1998; Gutierrez et al., 1991; Parrizas et al., 1994a; Parrizas et al., 1994b). The persistent hyperglycemia in rainbow trout fed with carbohydrates despite the existence of insulin secretion and receptors suggests an insulin resistance state comparable to that observed in humans with type II diabetes.

Glucose uptake mediated by the facilitative insulin-regulated glucose transporter GLUT4 homologue in white muscle and fat tissues and by GLUT2 in the liver has been reported in trout (Capilla et al., 2002; Diaz et al., 2007a; Diaz et al., 2007b; Krasnov et al., 2001). Fish glucose transporters GLUT2 and GLUT4 are characterized by a lower affinity for glucose, which may at least partly explain the persistent post-prandial hyperglycemia observed in trout fed with carbohydrates (Capilla et al., 2004a; Krasnov et al., 2001). At the metabolic level, most of the key enzymes involved in carbohydrate metabolism have been described in fish (Cowey and Walton, 1989). For some of these, including glucokinase (liver), phosphofructokinase (liver and muscle) and pyruvate kinase (liver and muscle), their induction by dietary carbohydrates is similar to that described in mammalian systems (Fideu et al., 1983; Panserat et al., 2001a; Panserat et al., 2000b). However, findings concerning other metabolic mediators of glucose metabolism suggest the existence of atypical regulation after carbohydrate intake by trout, i.e. a lower capacity for glucose phosphorylation by hexokinases in the muscle of fish than in mammalian species, as confirmed by the poor role of exogenous glucose as a glycogenic substrate in trout muscle (Kam and Milligan, 2006; Kirchner et al., 2005; Wilson, 1994). Moreover, in rainbow trout a carbohydrate-rich diet does not affect the activity or gene expression of key enzymes of gluconeogenesis such as glucose 6-phosphatase (G6Pase), fructose 1,6-bisphosphatase (FBPase) and phosphoenolpyruvate carboxykinase (PEPCK) (Panserat et al., 2001b; Panserat et al., 2000a; Panserat et al., 2001c; Tranulis et al., 1991). Such impaired post-prandial down regulation of gluconeogenesis in rainbow trout fed with carbohydrates is similar to that observed in human patients with type II diabetes. In healthy patients insulin lowers plasma

glucose levels by inducing plasma glucose uptake into skeletal muscle and adipose tissues and by inhibiting endogenous hepatic production of glucose. In mammals, gluconeogenic genes are mainly under insulin control (Barthel and Schmoll, 2003b). Insulin inhibits expression of PEPCK and G6Pase enzymes at the transcriptional level (Barthel and Schmoll, 2003a) through the activation of the protein kinase Akt (also known as protein kinase B) (Liao et al., 1998; Schmoll, 2000), a critical node in the insulin signaling pathway (Taniguchi et al., 2006). Because of insulin resistance, patients with type II diabetes present fasting hyperglycemia that is correlated with an increased glucose production by the liver. This is illustrated by the higher level of expression of two key enzymes of hepatic gluconeogenesis, PEPCK and G6Pase. As observed in these diabetic patients, the absence of post-prandial inhibition of hepatic gluconeogenesis in rainbow trout probably contributes to persistent hyperglycemia. The reason for the persistent post-prandial endogenous production of glucose in rainbow trout therefore remains to be elucidated.

Thus, we put forward the hypothesis that the glucose intolerance observed in rainbow trout may be related to the impaired ability of insulin to regulate mRNA levels of hepatic target genes. In order to test this hypothesis, we performed intraperitoneal administration of bovine insulin ($10\,\mathrm{u\,kg^{-1}}$) to 48 h-fasted rainbow trout and analyzed the activation of Akt and the mRNA levels of genes involved in gluconeogenesis (PEPCK, G6Pase and FBPase). Since regulation of target gene expression by insulin may be more widely impaired in rainbow trout, the study was enlarged to include other hepatic genes identified as being under insulin control in mammals. We therefore also analyzed the insulin regulation of the mRNA expression of glucokinase (GK) and pyruvate kinase (PK), which catalyse the phosphorylation of glucose and the conversion of phosphoenolpyruvate to pyruvate, the first and the final steps in glycolysis, respectively (Towle et al., 1997). We also investigated the regulation of mRNAs encoding two proteins involved in fatty acid metabolism, i.e. fatty acid synthase (FAS), which promotes the synthesis of fatty acids, primarily palmitate, through the condensation of malonyl-CoA and acetyl-CoA (Smith et al., 2003), and carnitine palmitoyltransferase 1 (CPT1), which is a limiting enzyme of fatty acid β-oxidation responsible for the entry of long chain fatty acids into the mitochondria (Bartlett and Eaton, 2004).

Despite the well-established and fully described potential and advantages of fish hepatocyte systems (Moon et al., 1985; Scholz et al., 1998; Segner, 1998a), few studies have used cultured trout hepatocytes to analyze the regulation of intermediary metabolism (Alvarez et al., 2000). In order to rule out the possibility of an indirect effect of insulin and of possible interactions with other endocrine mediators on target gene expression *in vivo*, we used a primary cell culture of rainbow trout hepatocytes to analyze the transcriptional effects of insulin in this model.

MATERIALS AND METHODS

Animals

In vivo and *in vitro* experiments were performed using sexually immature rainbow trout (250–300 g) obtained from the INRA experimental fish farm facilities of Donzacq, France. Fish were maintained in tanks kept in open circuits with 18°C well-aerated water. Fish were fed with a commercial diet (T-3P classic, Trouw, France). All experiments were carried out in accordance with legislation governing the ethical treatment of animals, and investigators were certified by the French Government to carry out animal experiments.

Experimental procedure for *in vivo* studies

To study the regulation of hepatic gene expression by insulin *in vivo*, rainbow trout were placed in two separate tanks. Fish were kept unfed for 48 h before the day of the experiment in order to obtain fish with empty digestive tracts but without onset of body protein degradation. Fasting also allowed us to obtain basal plasma glucose levels and to limit individual variability. On the day of the experiment, fish received an intraperitoneal injection of bovine insulin ($10\,\mathrm{u\,kg^{-1}}$ body weight; Sigma, St Louis, MO, USA) as previously described (Salgado et al., 2004) or were sham treated with vehicle (saline). Four fish per tank were left untreated and unfed, and served as controls. At 2, 4 and 6 h after treatment, fish (N=8) were killed by cervical section. Blood was collected from the dorsal aorta using a syringe pre-treated with a solution containing 4% NaF and 4% $K_2C_2O_4$, and centrifuged, and plasma was kept frozen at –20°C until analysis. Liver samples were collected, snap frozen in liquid nitrogen then stored at –80°C prior to further mRNA and protein analyses.

Animals and experimental procedure for *in vitro* studies

Hepatocyte isolation and culture

Isolated liver cells were prepared from 4 day-fasted rainbow trout as previously described by Mommsen and colleagues (Mommsen et al., 1994). Fish were anesthetized by placing them in water containing $60\,\mathrm{mg\,l^{-1}}$ aminobenzoic acid. After *in situ* perfusion using liver perfusion medium (1×; 17701-038, Invitrogen, Carlsbad, CA, USA), livers were excised, minced with a razor blade and immediately digested in a liver digest medium (17703-034, Invitrogen) for 20 min at 18°C. After filtration and centrifugation (120 ***g***, 2 min), the resulting cell pellet was resuspended three successive times in modified Hanks' medium ($136.9\,\mathrm{mmol\,l^{-1}}$ NaCl, $5.4\,\mathrm{mmol\,l^{-1}}$ KCl, $0.81\,\mathrm{mmol\,l^{-1}}$ $MgSO_4$, $0.44\,\mathrm{mmol\,l^{-1}}$ KH_2PO_4, $0.33\,\mathrm{mmol\,l^{-1}}$ Na_2HPO_4, $5\,\mathrm{mmol\,l^{-1}}$ $NaHCO_3$ and $10\,\mathrm{mmol\,l^{-1}}$ Hepes) supplemented with $1.5\,\mathrm{mmol\,l^{-1}}$ $CaCl_2$ and 1.5% defatted bovine serum albumin (BSA; Sigma), and centrifuged (70 ***g***, 2 min). Cells were finally taken up in modified Hanks' medium supplemented with $1.5\,\mathrm{mmol\,l^{-1}}$ $CaCl_2$, 1% defatted BSA, $3\,\mathrm{mmol\,l^{-1}}$ glucose, MEM essential amino acids (1×; Invitrogen), MEM non-essential amino acids (1×; Invitrogen) and antibiotic antimycotic solution (1×; Sigma). Cell viability (>98%) was assessed using the Trypan Blue exclusion method (0.04% in $0.15\,\mathrm{mol\,l^{-1}}$ NaCl) and cells were counted using a hemocytometer. The hepatocyte cell suspension (CS) was plated in a six well Primaria culture dish (BD Biosciences, NJ, USA) at a density of 3×10^6 cells per well and incubated at 18°C. The incubation medium was replaced every 24 h over the 72 h of primary cell culture. Microscopic examination ensured that hepatocytes progressively re-associated throughout culture to form two-dimensional aggregates, in agreement with earlier reports (Ferraris et al., 2002; Segner, 1998b). Cell viability and cytotoxicity were monitored throughout culture using CellTiter 96® aqueous one solution cell proliferation assay (Promega, Madison, WI, USA) and CytoTox 96® non-radioactive cytotoxicity assay (Promega) respectively, according to the manufacturer's recommendations.

Insulin treatment of hepatocyte primary cell culture

For analysis of Akt phosphorylation, 48 h-cultured hepatocytes were stimulated using $4\times10^{-9}\,\mathrm{mol\,l^{-1}}$ bovine insulin (Sigma) for 5, 15, 30 and 60 min [$4\times10^{-9}\,\mathrm{mol\,l^{-1}}$ insulin corresponds to the post-prandial level of insulin irrespective of dietary carbohydrate level (del sol Novoa et al., 2004)]. At the end of the stimulation period, cells were collected for protein extraction.

Table 1. Primers used to amplify rainbow trout target genes by conventional RT-PCR

Gene	Primer sequences	Hybridization temperature (°C)	Amplicon size (bp)
InsR (c form)	F: 5′-TCATTTGGTGTGGTGCTG-3′ R: 5′-CCCGCTTCGTCCCTCTCC-3′	56	413
HNF4	F: 5′-TGCAGGTTTAACCGTCAGTG-3′ R: 5′-AGAGTTAGTGCTGGGCTGGA-3′	55	192
AP	F: 5′-ACGAGCCAGGCCACTTCATC-3′ R: 5′-TCAGGGCCCATGTGGAAGTT-3′	60	220
TAT	F: 5′-GTCTTCTGCCTGCCCGCTAC-3′ R: 5′-GGTCTAGCTCGTTGCTGTCTTGG-3′	60	157
STP	F: 5′-CAGCAGCACAGATGGCGTTT-3′ R: 5′-GGCCTTTGAGGAGAGCCACA-3′	60	186
GLUT2	F: 5′-CCGTCTTTACCATGGTGTCG-3′ R: 5′-CCACAATGAACCAGGGGATG-3′	57	222

R, reverse primer; F, forward primer.

GenBank accession no. or sigenae accession no.: *InsR* (c form; insulin receptor), AF062498; *HNF4* (hepatic nuclear factor 4), tcbk0002c.b.02_5.1.s.om.8; *AP* (serum albumin 1 precursor), tcaa0001c.c.01_5.1.s.om.8; *TAT* (tyrosine aminotransferase), tcay0014b.e.19_3.1.s.om.8; *STP* (serotransferin precursor), tcay0003b.j.14_3.1.s.om.8; *GLUT2* (glucose transporter 2), AF246147.

For gene expression analysis, 48 h-cultured cells were exposed for an additional 24 h to 4×10^{-9} mol l^{-1} bovine insulin in the presence of 3 or 20 mmol l^{-1} glucose, the mean glucose concentrations measured in fasted trout and trout fed the carbohydrate-rich diet, respectively (Bergot, 1979; del sol Novoa et al., 2004; Hemre et al., 2002; Panserat et al., 2001c). Osmotic pressures of insulin–glucose-supplemented cell culture media were verified before each treatment and calibrated to 300 mosmol l^{-1} using a K7400 osmometer (Knauer, Berlin, Germany). At the end of the stimulation period, cells were collected for total RNA extraction and subsequent RT-PCR analysis.

Determination of plasma glucose levels

Plasma glucose levels were measured with the Glucose RTU kit from BioMerieux (Marcy l'Etoile, France) according to the manufacturer's recommendations.

Gene expression analysis

Total RNA samples were extracted from livers and hepatocytes using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations and treated with DNAse to avoid any genomic DNA contamination. A 1 μg sample of the resulting total RNA was reverse transcribed into cDNA using the SuperScript III RnaseH$^-$ reverse transcriptase kit (Invitrogen) and oligo dT primers (Promega) according to the manufacturer's instructions.

Conventional RT-PCR analysis

In order to confirm that the cultured cells might be considered as hepatocyte-like cells, expression of hepatocyte-specific genes such as albumin precursor (*AP*), tyrosine amino transferase (*TAT*), hepatic nuclear factor 4 (*HNF4*) and serotransferine precursor (*STP*) was examined by conventional RT-PCR. We also checked the mRNA expression of the glucose transporter (*Glut2*) and the insulin receptor (*InsR*) for further stimulation of these cells by glucose and/or insulin. Expression of these genes was studied in cell suspension as well as after 4, 24, 48 and 72 h of culture. For this purpose, cDNA samples were amplified by PCR using specific primers chosen from the Sigenae rainbow trout cDNA sequence public database (http://www.sigenae.org; Table 1). The PCR reaction was carried out using 2 μl of cDNA as template in a final reaction volume of 25 μl containing 1.5 mmol l^{-1} $MgCl_2$, 0.16 μmol l^{-1} of each primer, and 1 U of Taq polymerase (Promega). Samples were subjected to 35 amplification cycles of a standard PCR protocol (94°C for 20 s, specific primer hybridization temperature for 20 s, and 72°C for 20 s). The different PCR products were monitored by sequencing (GENOME Express, Meylan, France) to confirm the nature of the amplicon.

Real time RT-PCR analysis

Gene expression analyses were performed on samples from the livers of control and treated fish 6 h after intraperitoneal administration as well as from hepatocytes after 24 h of stimulation by insulin and glucose. Target gene expression levels were determined by real-time quantitative RT-PCR (qRT-PCR) using specific real-time PCR primers (Table 2). To avoid amplification of genomic DNA, when possible primer pairs included one intron-spanning oligonucleotide. Real-time RT-PCR was carried out on an iCycler iQ™ real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using iQ™ SYBR® Green Supermix. mRNA levels of *GK*, *PK*, *G6Pase* isoforms 1 and 2, *PEPCK*, *FBPase*, *CPT1* isoforms A and B, and *FAS* were evaluated. Elongation factor 1α (*EF1α*) was employed as a non-regulated reference gene as previously used in rainbow trout (Gabillard et al., 2003; Kamangar et al., 2006). The absence of a change in *EF1α* gene expression was also observed in our *in vivo* and *in vitro* studies (data not shown). PCR was performed using 10 μl of the diluted cDNA mixed with 5 pmol of each primer in a final volume of 25 μl. The PCR protocol was initiated at 95°C for 3 min for initial denaturation of the cDNA and hot-start iTaq™ DNA polymerase activation, and continued with a two-step amplification program (20 s at 95°C followed by 30 s at specific primer hybridization temperature) repeated 40 times. Melting curves were systematically monitored (temperature gradient at 0.5°C/10 s from 55 to 94°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. The different PCR products were initially checked by sequencing to confirm the nature of the amplicon. Each PCR run included replicate samples (duplicate of reverse transcription and duplicate of PCR amplification) and negative controls (reverse transcriptase-free samples, RNA-free samples).

Relative quantification of target gene expression was performed using the mathematical model described by Pfaffl (Pfaffl, 2001). The relative expression ratio (R) of a target gene was calculated on the basis of real-time PCR efficiency (E) and the CT deviation (ΔCT) of the unknown sample *versus* a control sample, and expressed in comparison to the *EF1α* reference gene:

$$R = \frac{(E_{\text{target gene}})^{\Delta \text{CT}_{\text{target gene}}(\text{control}-\text{sample})}}{(E_{\text{EF1}\alpha})^{\Delta \text{CT}_{\text{EF1}\alpha}(\text{control}-\text{sample})}} .$$

PCR efficiency (E) was measured by the slope of a standard curve using serial dilution of cDNA. All PCR efficiency values were between 85 and 100%.

Protein extraction and Western blotting

Frozen liver (300 mg) and hepatocytes were homogenized on ice with an Ultraturrax homogenizer (IMLAB sarl, Lille, France) in a

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} sodium fluoride, 4 mmol l^{-1} sodium pyrophosphate, 2 mmol l^{-1} sodium orthovanadate, 1% Triton X-100, 0.50% NP40-IGEPAL and a protease inhibitor cocktail (Roche, Basel, Switzerland). Homogenates were centrifuged at 1000 ***g*** for 30 min at 4°C and supernatants were then centrifuged for 45 min at 150 000 ***g***. The resulting supernatants were aliquoted and stored at –80°C. Protein concentrations were determined using the Bio-Rad protein assay kit. Liver and cell lysates (20 μg of protein) were subjected to SDS-PAGE and Western blotting using anti-phospho-Akt Ser473 and anti-Akt antibodies (Cell Signaling Technology, Ozyme, St Quentin-en-Yvelines, France), consecutively. Rabbit phospho-Akt Ser473 and Akt antibodies were directed against synthetic peptides corresponding to residues surrounding phosphorylated Ser473 of mouse Akt and the carboxyterminal sequence of mouse Akt, respectively. These antibodies have been shown to successfully cross-react with rainbow trout Akt protein (Seiliez et al., 2008). After washing, protein detection was performed by chemiluminescence using LumiGLO reagents (Cell Signaling Technology) and horseradish peroxidase-conjugated anti-IgG as the secondary antibody (Cell Signaling Technology).

Table 2. Primers used to amplify rainbow trout target genes by real-time RT-PCR

Gene	Primer sequences	Hybridization temperature (°C)	Amplicon size (bp)
EF1α	F: 5′-TCCTCTTGGTCGTTTCGCTG-3′ R: 5′-ACCCGAGGGACATCCTGTG-3′	59	159
GK	F: 5′-TGAAGGATCAGAGGTGGGTGATT-3′ R: 5′-GAAGGTGAAACCCAGAGGAAGC-3′	59	253
PK	F: 5′-CCATCGTCGCGGTAACAAGA-3′ R: 5′-ACATAGGAAAGGCCAGGGGC-3′	59	158
mPEPCK	F: 5′-GTTGGTGCTAAAGGGCACAC-3′ R: 5′-CCCGTCTTCTGATAAGTCCAA-3′	59	149
G6Pase 1	F: 5′-CTCAGTGGCGACAGAAAGG-3′ R: 5′-TACACAGCAGCATCCAGAGC-3′	55	77
G6Pase 2	F: 5′-TAGCCATCATGCTGACCAAG-3′ R: 5′-CAGAAGAACGCCCACAGAGT-3′	55	82
FBPase	F: 5′-GCTGGACCCTTCCATCGG-3′ R: 5′-CGACATAACGCCCACCATAGG-3′	59	182
FAS	F: 5′-TGATCTGAAGGCCCGTGTCA-3′ R: 5′-GGGTGACGTTGCCGTGGTAT-3′	60	161
CPT1A	F: 5′-TCGATTTTCAAGGGTCTTCG-3′ R: 5′-CACAACGATCAGCAAACTGG-3′	55	166
CPT1B	F: 5′-CCCTAAGCAAAAAGGGTCTTCA-3′ R: 5′-CATGATGTCACTCCCGACAG-3′	55	149

R, reverse primer; F, forward primer.

GenBank accession no. or sigenae accession no.: *EF1α* (elongation factor 1α), AF498320; *GK* (glucokinase), AF135403; *PK* (pyruvate kinase), AF 246146; *mPEPCK* (phosphoenolpyruvate carboxykinase, mitochondrial isoform), AF246149; *G6Pase 1* (glucose 6-phosphatase 1), tcay0019b.d.18_3.1.s.om.8.1-1693; *G6Pase 2* (glucose 6-phosphatase 2), AF120150; *FBPase* (fructose 1,6-bisphosphatase), AF333188; *FAS* (fatty acid synthase), tcab0001c.e.06_5.1.s.om.8; *CPT1A* (carnitine palmitoyltransferase 1 A), AF 327058; *CPT1* (carnitine palmitoyltransferase 1 B), AF606076.

Statistical analysis

The results of *in vivo* mRNA expression analyses are expressed as means ± s.e.m. (*N*=8) and were analyzed by one-way ANOVA. Results from plasma glucose levels (*N*=8) and mRNA expression in primary hepatocyte cell culture (*N*=6) are expressed as means ± s.e.m. and were analyzed by two-way ANOVA. Means were compared by Student–Newman–Keuls multiple comparison test. The level of significance was set at $P<0.05$.

RESULTS

Hypoglycemic effect of insulin administration

Data on plasma glucose levels measured 2, 4 and 6 h after intraperitoneal administration of insulin or saline solution are presented in Fig. 1. Administration of insulin gradually and significantly decreased plasma glucose levels to reach 1.78±0.18 mmol l^{-1} 6 h after intraperitoneal injection compared with saline-treated trout (4.49±0.19 mmol l^{-1}). A significant increase in plasma glucose levels was recorded 2 h after administration of saline solution but no further difference between control and vehicle-treated fish was observed 4 and 6 h after the intraperitoneal administration.

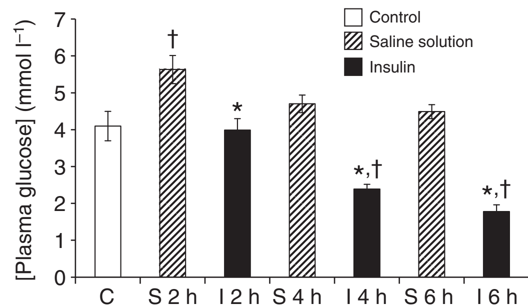

Fig. 1. Plasma glucose levels 2, 4 and 6 h after intraperitoneal administration of insulin (10 u kg^{-1}; I) or saline solution (S) to 48 h-fasted control rainbow trout (C). Results are expressed as means ± s.e.m. (*N*=8) and were analyzed by two-way ANOVA followed by Student–Newman–Keuls multiple comparison test. *Significant difference between insulin- and saline-treated fish ($P<0.05$). †Significant difference between 48 h-fasted control fish (C) and insulin (I)- or saline (S)-treated fish ($P<0.05$).

Akt phosphorylation after *in vivo* insulin administration

Since insulin signaling is mediated through Akt phosphorylation on threonine 308 and serine 473 in mammals, Western blot analysis was performed to study the phosphorylation of Akt on serine 473 compared with endogenous levels of total Akt in rainbow trout. As illustrated in Fig. 2A, insulin induced Akt phosphorylation whereas administration of saline solution did not. The phosphorylation of Akt was observed as early as 2 h after intraperitoneal administration of insulin and was maintained until at least 6 h after insulin administration.

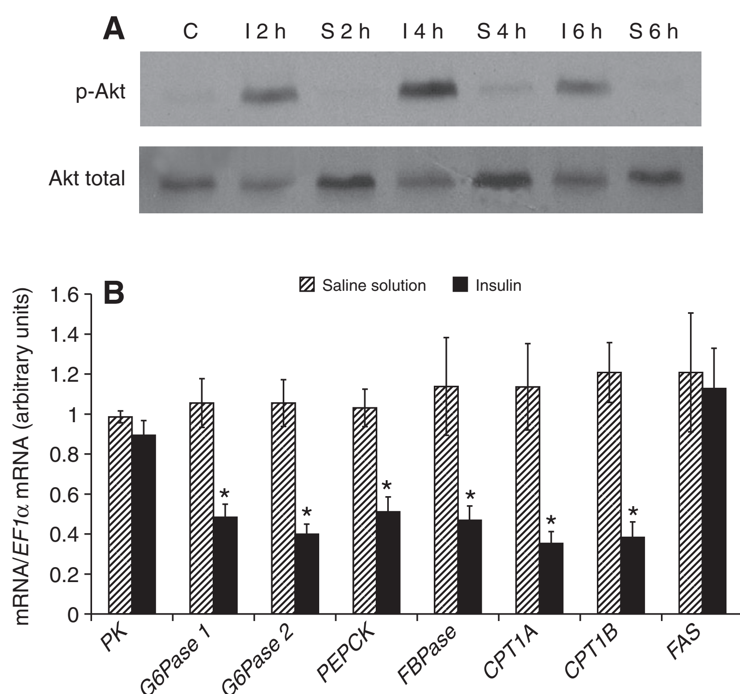

Fig. 2. (A) Western blot analysis of Akt phosphorylation in rainbow trout liver 2, 4 and 6 h after intraperitoneal administration of insulin (I) or saline solution (S) as well as in 48 h-fasted control rainbow trout (C). p-Akt, phosphorylated form of Akt. The gel was loaded with 20 μg of total protein per lane. The figure is a representative blot. Western blots were performed on six individual samples and similar results were obtained. (B) Effects of intraperitoneal administration of insulin (I) or saline solution (S) on the level of expression of mRNA encoding hepatic genes. Pyruvate kinase (*PK*), glucose 6-phosphatase isoforms 1 and 2 (*G6Pase 1* and *2*), phosphoenolpyruvate carboxykinase (*PEPCK*), fructose 1,6-bisphosphatase (*FBPase*), carnitine palmitoyltransferase 1 isoform A and B (*CPT1 A* and *B*) and fatty acid synthase (*FAS*) mRNA levels were estimated using real-time RT-PCR. Expression values were normalized with elongation factor 1α (*EF1α*)-expressed transcripts and are indicated as fold variation of the saline solution-treated group. Results are expressed as means ± s.e.m. (N=6) and were analyzed by one-way ANOVA followed by Student–Newman–Keuls comparison test. *Significant difference (P<0.05).

Insulin regulation of hepatic gene expression *in vivo*

As illustrated in Fig. 2B, insulin administration significantly decreased the expression of gluconeogenic genes, including both isoforms of *G6Pase* (2.1- and 2.6-fold decrease for *G6Pase 1* and *2*, respectively), *PEPCK* (2.0-fold) and *FBPase* (2.4-fold). Expression of both isoforms of *CPT1* also decreased about 3-fold under insulin treatment. Expression of *FAS* and *PK* mRNA was not affected by administration of insulin or saline solution. *GK* mRNA levels were also measured but were mostly undetectable in either saline or insulin-treated fish (data not shown).

Molecular characterization of hepatocyte primary cell culture

Using end-point RT–PCR we monitored the expression of hepatocyte-specific markers (such as albumin precursor, serotransferin precursor and tyrosine aminotransferase), the hepatocyte nuclear factor 4 (HNF4) required for hepatocyte differentiation, the hepatocyte-specific glucose transporter GLUT2, and the c form insulin receptor (Fig. 3). Fragments of the expected length were amplified for the albumin precursor, serotransferin precursor, tyrosine aminotransferase, *HNF4* and *GLUT2* genes in cell suspension and in cultured cells over 4 to 72 h. For the c form insulin receptor, the expected 413 bp amplification fragment was detected in cell suspension as well as during the full time course of incubation. However, we also detected the amplification of a larger fragment in cell cultures during the first 24 h. Sequencing of this larger fragment revealed that it corresponded to an alternatively spliced form (data not shown).

Akt phosphorylation after insulin treatment of hepatocyte primary cell culture

To analyze the effects of insulin on the phosphorylation of Akt *in vitro*, 48 h-incubated cells were treated with 4×10^{-9} mol l^{-1} insulin for 5, 15, 30 and 60 min. After Western blot analysis of phospho-Akt (serine 473), we observed that phosphorylation of Akt occurred as early as 5 min after insulin exposure, while the phosphorylated form of Akt was undetectable in untreated control cells (Fig. 4). Akt phosphorylation was detected until 60 min of incubation with insulin but the signal declined progressively.

Insulin and glucose regulation of gene expression in cultured hepatocytes

As shown in Fig. 5, we observed that the mRNA level of *GK* was significantly enhanced by both insulin and glucose (average of 1.7- and 1.4-fold, respectively. *PK* and *FAS* gene expression were found

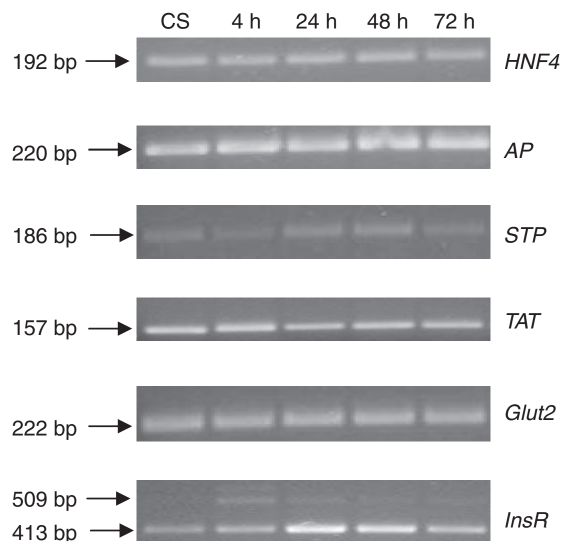

Fig. 3. Expression of hepatic nuclear factor 4 (*HNF4*), albumin precursor (*AP*), serotransferin precursor (*STP*), tyrosine aminotransferase (*TAT*), glucose transporter 2 (*GLUT2*) and insulin receptor (*InsR*) assessed by conventional RT-PCR in cell suspension (CS) and hepatocytes after 4, 24, 48 and 72 h of culture. Expected lengths of the fragments are indicated in the left margin. For InsR, a larger fragment (509 bp) was also detected.

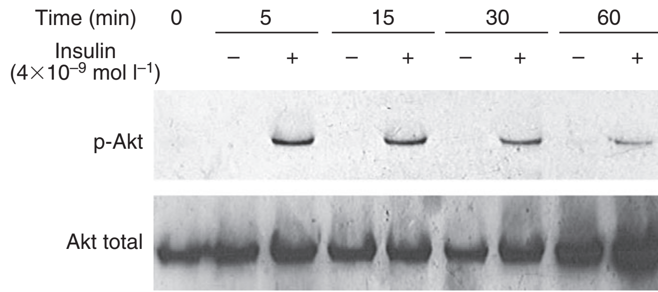

Fig. 4. Induction of Akt phosphorylation in rainbow trout hepatocyte cell culture 5, 15, 30 and 60 min after insulin stimulation. The gel was loaded with 20 μg of total protein per lane. The figure is a representative blot. Western blots were performed on six individual samples and similar results were obtained. p-Akt, phosphorylated form of Akt.

to be regulated in the same way: insulin increased the expression of *PK* and *FAS* genes but the insulin stimulatory effect was enhanced in the presence of 20 mmol l^{-1} glucose, as indicated by the significant statistical interaction between insulin and glucose. We found that insulin and glucose have opposite effects on the regulation of expression of both isoforms of *G6Pase*. While glucose significantly increased the expression of *G6Pase*, *G6Pase* mRNA levels declined in the presence of insulin. In the absence of insulin, the effect of glucose on *G6Pase* mRNA levels was more pronounced on the first isoform of this gene (3.7-fold increase) than on the second isoform (1.1-fold increase). The level of expression of the *FBPase* gene was not affected by the glucose concentrations tested in this experiment. We recorded a significant increase (1.6-fold) in *FBPase* mRNA level only when cells were incubated with insulin in the presence of 20 mmol l^{-1} glucose. On investigating the regulation of expression of *PEPCK* (mitochondrial isoform), we found that the *PEPCK* mRNA level was slightly suppressed by glucose in the absence of insulin but strongly reduced by insulin (5.5- and 2-fold, respectively) when cells were incubated with 3 and 20 mmol l^{-1} glucose. Expression of *CPT1* (isoforms A and B) was almost undetectable in hepatocyte cultures.

DISCUSSION

Unlike in mammals, expression of the gluconeogenic genes is poorly regulated by carbohydrate intake in rainbow trout (Panserat et al., 2001b; Panserat et al., 2000b), suggesting a potentially impaired ability of insulin to regulate the expression of gluconeogenic genes and, more widely, hepatic target genes in teleost carnivorous fish. However, combining both *in vivo* and *in vitro* experiments, we have clearly demonstrated here that insulin is able to regulate the expression of hepatic target genes in rainbow trout.

The *in vivo* study based on intraperitoneal administration of insulin confirmed the ability of exogenous and heterologous insulin to induce hypoglycemia in rainbow trout, as already shown (Albalat et al., 2006; Salgado et al., 2004). *In vivo* insulin administration led not only to the reduction of plasma glucose levels but also to changes in other metabolic and hormonal signals (fatty acids, glucagons, etc.) potentially involved in the regulation of hepatic gene expression. In mammals, glycolytic and lipogenic genes are reciprocally regulated by glucose and fatty acids in liver through molecular mechanisms involving the recently discovered glucose signaling transcription factor carbohydrate-responsive element binding protein (ChREBP) (for a review, see Dentin, 2006). Glucagon increases glucose output by dephosphorylating the CREB (cAMP-responsive element binding protein) coactivator TORC2 (transducer of regulated CREB activity), which is transported to the nucleus where it enhances CREB-dependent transcription of gluconeogenic genes such as *PEPCK* and *G6Pase* (Alan Cheng, 2006). We therefore used primary cell culture of rainbow trout hepatocytes to eliminate the relative contributions of other confounding factors. The study with primary cultured hepatocytes was focused on the contribution of glucose and insulin in the regulation of expression of insulin target genes. Displaying both the expression of genes representative of liver gene expression and the expression of the first mediators of glucose and insulin signaling, this primary hepatocyte culture thus provides a good model for this purpose.

Insulin regulates the transcription of target genes by controlling Akt activation of transcription factors such as Forkhead transcription factors of the FoxO subfamily and transcription factors belonging to the family of sterol regulatory element binding proteins (SREBPs) (Foufelle and Ferre, 2002). Our findings clearly show that insulin was also able to induce Akt phosphorylation in the liver and primary hepatocyte cell culture from rainbow trout, confirming activation of the insulin signaling pathway after insulin treatment both *in vivo* and *in vitro*. These results are in agreement with the recent demonstration that insulin and insulin-like growth factors (IGFs) are able to activate the phosphorylation of Akt in zebra fish embryonic cells (Pozios et al., 2001) as well as in primary cell culture of muscle cells from two different fish species, i.e. rainbow trout and gilthead sea bream (*Sparus aurata*) (Castillo et al., 2006; Montserrat et al., 2007).

We also demonstrated that activation of the insulin signaling pathway was associated with the regulation of hepatic gene expression both *in vivo* and *in vitro*. For instance, G6Pase, PEPCK and FBPase, three key enzymes of endogenous glucose production (Granner and Pilkis, 1990), presented a significantly lowered gene expression level after insulin treatment. This inhibition was observed not only *in vivo* following intraperitoneal administration of insulin but also *in vitro*, at least for G6Pase and PEPCK. This suggests a direct effect of insulin on the transcription of these genes that probably involves the activation of FoxO1 and PGC1α transcription factors, as has been described in mammals (Puigserver et al., 2003). However, the effects of insulin on the regulation of gluconeogenic gene expression were studied in animals that presented plasma glucose levels (1.78±0.18 mmol l^{-1}) far below those normally measured in fasted animals (4.10±0.40 mmol l^{-1}) or even in rainbow trout fed with 28% carbohydrates (12.2 mmol l^{-1}) (del sol Novoa et al., 2004). In this context, we also analyzed the effects of insulin on target gene expression in primary hepatocytes in relation to glucose concentrations corresponding to plasma glucose levels of fasted and carbohydrate-fed trout, respectively. Our findings demonstrate that *G6Pase* and *PEPCK* gene expression are subject to regulation by glucose but in opposite ways. Glucose stimulated *G6Pase* gene expression in rainbow trout hepatocytes independently of insulin, as in diabetic rats *in vivo* (Massillon et al., 1996) and *in vitro* (Argaud et al., 1997). Although paradoxical, this may contribute to the maintenance of an appropriate level of G6P, a key regulator of glycogen synthesis and glycolysis, in the face of increased GK activity (Aiston et al., 1999). Glucose also inhibited *PEPCK* gene expression in rainbow trout hepatocytes independently of insulin, but the down-regulating effect of insulin was above that of glucose. The significant inhibition of *PEPCK* gene expression by glucose may be related to the stimulatory effect of glucose on *GK* gene expression. The inhibition of *PEPCK* gene expression by glucose in mammals is mediated through the activation of GK (Scott et al., 1998). However, instead of confirming the inhibition of

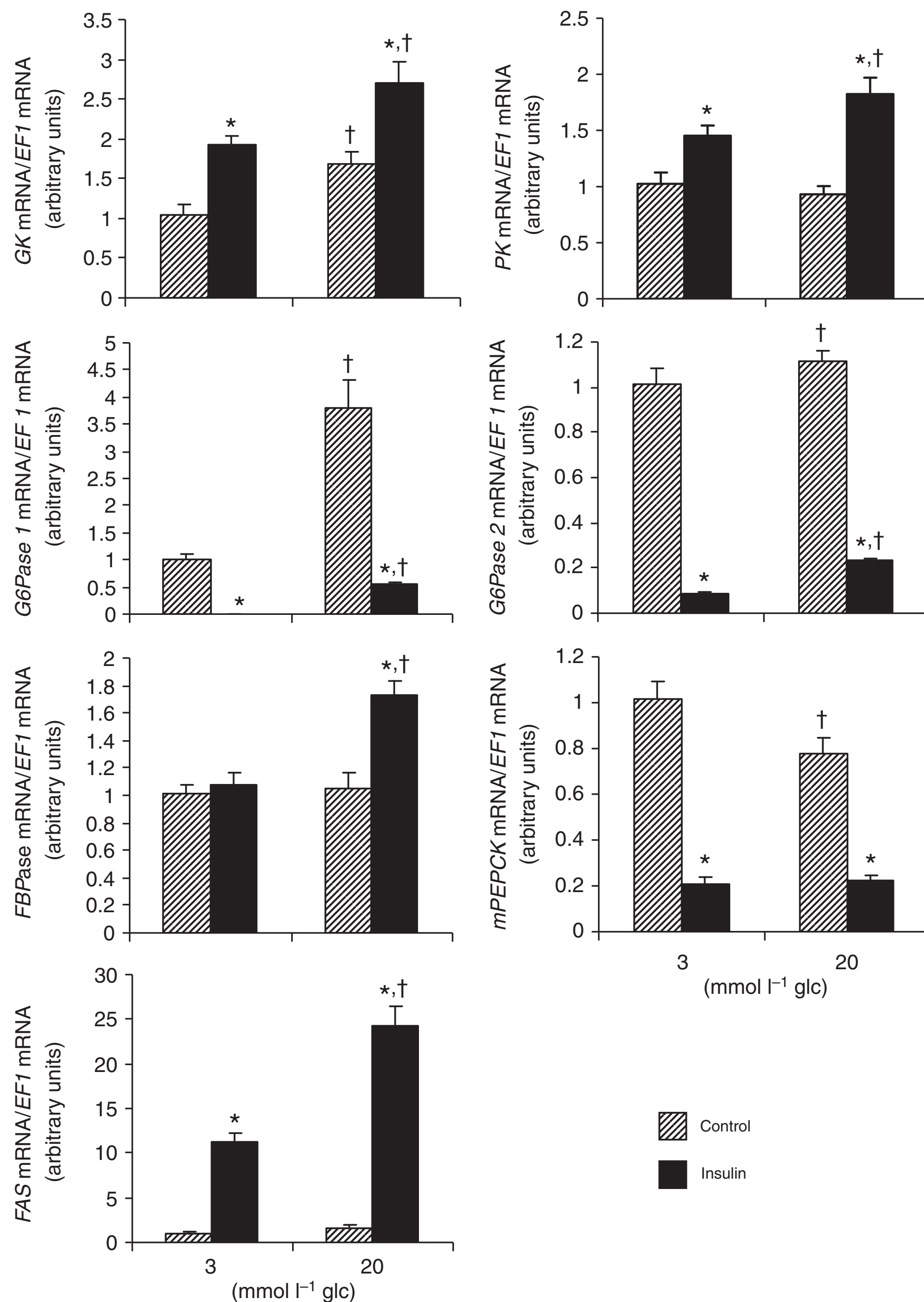

Fig. 5. Effects of insulin and glucose on the expression level of mRNA encoding hepatic genes. Glucokinase (*GK*), *PK*, *G6Pase 1* and *2*, *PEPCK*, *FBPase* and *FAS* mRNA levels were estimated using real-time RT-PCR. The experiment was conducted in 48 h-cultured hepatocytes of rainbow trout incubated for an additional 24 h with (Insulin, filled bars) or without (Control, hatched bars) insulin (4×10^{-9} mol l^{-1}) and in the presence of 3 or 20 mmol l^{-1} glucose (glc). Expression values are normalized with elongation factor 1α EF1α)-expressed transcripts and are indicated as fold variation of 3 mmol l^{-1} glucose control condition. Results are expressed as means +s.e.m. (N=6) and were analyzed by one-way ANOVA followed by Student–Newman–Keuls multiple comparison test (P<0.05). *Significant effect of insulin. †Significant effect of glucose.

FBPase gene expression by insulin *in vitro* we observed an up regulation of *FBPase* mRNA expression under insulin stimulation at high glucose concentrations, whereas insulin clearly decreased the expression of the endogenous *FBPase* gene in diabetic rats and rat primary hepatocyte cell culture (El-Maghrabi et al., 1991; El-Maghrabi et al., 1988). Further studies are needed to investigate the potential role of glucose and insulin in *FBPase* gene expression in the rainbow trout liver. Except for *FBPase*, our *in vivo* and *in vitro* findings suggest that, unlike what happens in the post-prandial state, insulin is clearly able to down regulate the expression of the gluconeogenic genes in rainbow trout as previously demonstrated in mammals.

Control of blood glucose levels is closely related to the efficiency of the glycolytic pathway and this, like gluconeogenesis, is under insulin control. Upon entering the hepatocyte, glucose is phosphorylated to G6P by glucokinase (Granner and Pilkis, 1990) before being stored as glycogen, transformed into fatty acids or catabolized. Insulin is a major regulator of *GK* gene expression in the mammalian liver (Iynedjian et al., 1989). In our study *GK* mRNA was undetectable in fasted rainbow trout, in accordance with previous findings in the same species (Panserat et al., 2001c) and in gilthead sea bream (Caseras et al., 2000). However, intraperitoneal administration of insulin failed to induce *GK* mRNA expression. In contrast, both glucose and insulin were able to increase *GK* gene expression in hepatocytes *in vitro* and their effects were additive. This represents the first demonstration that in rainbow trout *GK* gene expression may be directly regulated by glucose, as to our knowledge this does not occur in mammals. The potential control of *GK* gene expression by glucose in rainbow trout may provide an explanation for the absence of GK regulation *in vivo* following insulin administration to fasted rainbow trout. Indeed, plasma glucose levels were so drastically reduced 6 h after intraperitoneal administration of insulin (1.78±0.18 mmol l^{-1}) that the expected effect of insulin was counteracted by the inhibitory effect of the very low glucose level. These results are in agreement with the previous demonstration that *GK* gene expression was only enhanced when rainbow trout were fed with a single meal of glucose or a carbohydrate-rich diet (Panserat et al., 2000b; Panserat et al., 2001c) whereas insulin secretion was similar in trout fed with high or low carbohydrate diets (Capilla et al., 2004b; del sol Novoa et al., 2004). The regulation of the second glycolytic enzyme that we studied – PK, the last enzyme of glycolysis (Towle et al., 1997) – exhibited a different response under insulin stimulation in *in vivo* and *in vitro* studies. While insulin had no effect on *PK* gene expression *in vivo*, it enhanced *PK* mRNA accumulation in the primary hepatocytes. *In vitro*, it was noted that glucose was essential

to stimulate the positive effect of insulin on *PK* gene expression. Since fish were hypoglycemic 6 h after insulin administration, insulin was not able to stimulate PK gene expression. In fact, in mammals *PK* gene transcription is induced by insulin-stimulated glucose metabolism (Alam and Saggerson, 1998; Towle, 2005). For example, *PK* gene expression is stimulated by glucose in cultured hepatocytes expressing *GK*, independently of insulin (Alam and Saggerson, 1998; Doiron et al., 1994). Finally, we demonstrated that insulin is involved in the control of gene expression of at least the first and last enzymes of glycolysis. However, in contrast to the gluconeogenic genes, it seems that glucose represents an important co-factor in the regulation of expression of *GK* and *PK* genes by insulin in the rainbow trout.

The liver also plays a central role in fatty acid metabolism, and interactions between glucose and lipid metabolism are well characterized (Weickert and Pfeiffer, 2006). Lipogenesis in fish mainly occurs in the liver, which is also a site of lipid catabolism – fatty-acid β-oxidation (Gutieres et al., 2003). Both mechanisms are known to be under insulin control in mammals. We focused this study on FAS and CPT1, which are involved in lipid synthesis and degradation, respectively, and have been identified as insulin targets in mammals. Insulin regulation of *FAS* transcription in mammals is mediated by the PI3-kinase/Akt signaling pathway (Sul et al., 2000). In the present study, insulin regulation of rainbow trout *FAS* gene expression was in every way similar to that observed for the *PK* gene. As reported in mammals (Doiron et al., 1994; Sul and Wang, 1998), our results showed that glucose was essential for insulin stimulation of *FAS* gene expression. On the other hand, we demonstrated that *CPT1* gene expression was down regulated in the rainbow trout liver. This down regulation was only shown *in vivo* following administration of insulin since expression of the two isoforms of *CPT1* was undetectable in rainbow trout hepatocytes. Chatelain and colleagues demonstrated that long chain fatty acids markedly increased *CPT1* mRNA level in primary culture of fetal rat hepatocytes (Chatelain et al., 1996). This accumulation resulted from two mechanisms: stimulation of gene transcription and stabilization of the mRNA. Thus the undetectable expression of *CPT1* might be related to the total absence of fatty acids in our culture medium. However, our *in vivo* results were consistent with a previous study showing that insulin decreased the *CPT1* mRNA level in rat H4IIE hepatoma cells (Park et al., 1995). Finally, the regulation of *FAS* and *CPT1* gene expression by insulin and glucose in fish was on the whole consistent with what happens in mammals.

Perspective and significance

Based on earlier findings showing the absence of post-prandial inhibition of gluconeogenic gene expression (Panserat et al., 2001b; Panserat et al., 2000b), and since insulin represents the main regulator of the expression of these genes, we investigated whether the ability of insulin to control gluconeogenic gene expression was impaired in fish. As we thought that the transcriptional effect of insulin may be affected in its entirety, we enlarged the study to other hepatic insulin target genes. By combining *in vivo* and *in vitro* approaches, we clearly demonstrated that in the rainbow trout insulin possesses the intrinsic ability to activate its signaling pathway and regulate expression of hepatic target genes, including gluconeogenic genes. The absence of post-prandial regulation of hepatic gluconeogenic gene expression cannot therefore be directly attributed to a fault in insulin signaling *per se*. However, the mechanisms involved in the regulation of metabolism often depend on the cross-talk between nutritional and hormonal signals. This study revealed the importance that other factors, including nutrients such as glucose, may have in the insulin regulation of target gene expression. In mammals, excessive dietary proteins and amino acids have detrimental effects on glucose homeostasis by promoting insulin resistance and increasing gluconeogenesis (Tremblay et al., 2005). One particular feature of the rainbow trout diet is that total protein content may exceed 45% of the dry matter. This consistently high dietary amino acid intake can thus have undesirable effects on insulin sensitivity, particularly on insulin-regulated gene expression. This could explain the absence of post-prandial down regulation of the expression of insulin target genes such as *G6Pase* and *PEPCK* and the restoration of their inhibition by reducing dietary protein levels (Kirchner et al., 2003). Further experiments are needed to examine this hypothesis and to analyze the effects of excessive amino acids on the regulation of the hepatic target gene by insulin.

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REFERENCES

Aiston, S., Trinh, K. Y., Lange, A. J., Newgard, C. B. and Agius, L. (1999). Glucose-6-phosphatase overexpression lowers glucose 6-phosphate and inhibits glycogen synthesis and glycolysis in hepatocytes without affecting glucokinase translocation. Evidence against feedback inhibition of glucokinase. *J. Biol. Chem.* **274**, 24559-24566.

Alam, N. and Saggerson, E. D. (1998). Malonyl-CoA and the regulation of fatty acid oxidation in soleus muscle. *Biochem. J.* **334**, 233-241.

Alan Cheng, A. R. S. (2006). More TORC for the gluconeogenic engine. *BioEssays* **28**, 231-234.

Albalat, A., Sanchez-Gurmaches, J., Gutierrez, J. and Navarro, I. (2006). Regulation of lipoprotein lipase activity in rainbow trout (*Oncorhynchus mykiss*) tissues. *Gen. Comp. Endocrinol.* **146**, 226-235.

Alvarez, M. J., Diez, A., Lopez-Bote, C., Gallego, M. and Bautista, J. M. (2000). Short-term modulation of lipogenesis by macronutrients in rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Br. J. Nutr.* **84**, 619-628.

Argaud, D., Kirby, T. L., Newgard, C. B. and Lange, A. J. (1997). Stimulation of glucose-6-phosphatase gene expression by glucose and fructose-2,6-bisphosphate. *J. Biol. Chem.* **272**, 12854-12861.

Banos, N., Baro, J., Castejon, C., Navarro, I. and Gutierrez, J. (1998). Influence of high-carbohydrate enriched diets on plasma insulin levels and insulin and IGF-I receptors in trout. *Regul. Pept.* **77**, 55-62.

Barthel, A. and Schmoll, D. (2003a). Novel concepts in insulin regulation of hepatic gluconeogenesis. *Am. J. Physiol.* **285**, E685-E692.

Barthel, A. and Schmoll, D. (2003b). Novel concepts in insulin regulation of hepatic gluconeogenesis. *AACN Clin. Issues* **285**, E685-E692.

Bartlett, K. and Eaton, S. (2004). Mitochondrial beta-oxidation. *Eur. J. Biochem.* **271**, 462-469.

Bergot, F. (1979). Effects of dietary carbohydrates and of their mode of distribution on glycaemia in rainbow trout (Salmo gairdneri richardson). *Comp. Biochem. Physiol. A* **64**, 543-547.

Capilla, E., Diaz, M., Gutierrez, J. and Planas, J. V. (2002). Physiological regulation of the expression of a GLUT4 homolog in fish skeletal muscle. *Am. J. Physiol.* **283**, E44-E49.

Capilla, E., Diaz, M., Albalat, A., Navarro, I., Pessin, J. E., Keller, K. and Planas, J. V. (2004a). Functional characterization of an insulin-responsive glucose transporter (GLUT4) from fish adipose tissue. *Am. J. Physiol.* **287**, E348-E357.

Capilla, E., Medale, F., Panserat, S., Vachot, C., Rema, P., Gomes, E., Kaushik, S., Navarro, I. and Gutierrez, J. (2004b). Response of hexokinase enzymes and the insulin system to dietary carbohydrates in the common carp, Cyprinus carpio. *Reprod. Nutr. Dev.* **44**, 233-242.

Caseras, A., Meton, I., Fernandez, F. and Baanante, I. V. (2000). Glucokinase gene expression is nutritionally regulated in liver of gilthead sea bream (*Sparus aurata*). *Biochim. Biophys. Acta* **1493**, 135-141.

Castillo, J., Ammendrup-Johnsen, I., Codina, M., Navarro, I. and Gutierrez, J. (2006). IGF-I and insulin receptor signal transduction in trout muscle cells. *Am. J. Physiol.* **290**, R1683-R1690.

Chatelain, F., Kohl, C., Esser, V., Mcgarry, J. D., Girard, J. and Pegorier, J.-P. (1996). Cyclic AMP and fatty acids increase carnitine palmitoyltransferase I gene transcription in cultured fetal rat hepatocytes. *Eur. J. Biochem.* **235**, 789-798.

Cowey, C. and Walton, M. (1989). Intermediary metabolism. In *Intermediary Metabolism* (ed. Cowey, C. and Walton, M.), pp. 259-329. New York: Academic Press.

del sol Novoa, M., Capilla, E., Rojas, P., Baro, J., Gutierrez, J. and Navarro, I. (2004). Glucagon and insulin response to dietary carbohydrate in rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **139**, 48-54.

Dentin, R., Denechaud, P.-D., Benhamed, F., Girard, J. and Postic, C. (2006). Hepatic gene regulation by glucose and polyunsaturated fatty acids: A role for ChREBP. *J. Nutr.* **136**, 1145-1149.

Diaz, M., Capilla, E. and Planas, J. V. (2007a). Physiological regulation of glucose transporter (GLUT4) protein content in brown trout (*Salmo trutta*) skeletal muscle. *J. Exp. Biol.* **210**, 2346-2351.
Diaz, M., Antonescu, C. N., Capilla, E., Klip, A. and Planas, J. V. (2007b). Fish glucose transporter (GLUT)-4 differs from rat GLUT4 in its traffic characteristics but can translocate to the cell surface in response to insulin in skeletal muscle cells. *Endocrinology* **148**, 5248-5257.
Doiron, B., Cuif, M., Kahn, A. and Diaz-Guerra, M. (1994). Respective roles of glucose, fructose, and insulin in the regulation of the liver-specific pyruvate kinase gene promoter. *J. Biol. Chem.* **269**, 10213-10216.
El-Maghrabi, M. R., Pilkis, J., Marker, A. J., Colosia, A. D., D'Angelo, G., Fraser, B. A. and Pilkis, S. J. (1988). cDNA sequence of rat liver fructose-1,6-bisphosphatase and evidence for down-regulation of its mRNA by insulin. *Proc. Natl. Acad. Sci. USA* **85**, 8430-8434.
El-Maghrabi, M., Lange, A., Kummel, L. and Pilkis, S. (1991). The rat fructose-1,6-bisphosphatase gene. Structure and regulation of expression. *J. Biol. Chem.* **266**, 2115-2120.
Ferraris, M., Radice, S., Catalani, P., Francolini, M., Marabini, L. and Chiesara, E. (2002). Early oxidative damage in primary cultured trout hepatocytes: a time course study. *Aquat. Toxicol.* **59**, 283-296.
Fideu, M. D., Soler, G. and Ruiz-Amil, M. (1983). Nutritional regulation of glycolysis in rainbow trout (*Salmo gairdneri* R.). *Comp. Biochem. Physiol. B* **74**, 795-799.
Foufelle, F. and Ferre, P. (2002). New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. *Biochem. J.* **366**, 377-391.
Furuichi, M. and Yone, Y. (1981). Change of blood sugar and plasma insulin levels of fishes in glucose tolerance test. *Bull. Jpn. Soc. Sci. Fish.* **47**, 761-764.
Gabillard, J. C., Weil, C., Rescan, P. Y., Navarro, I., Gutierrez, J. and Le Bail, P. Y. (2003). Environmental temperature increases plasma GH levels independently of nutritional status in rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **133**, 17-26.
Granner, D. and Pilkis, S. (1990). The genes of hepatic glucose metabolism. *J. Biol. Chem.* **265**, 10173-10176.
Gutieres, S., Damon, M., Panserat, S., Kaushik, S. and Medale, F. (2003). Cloning and tissue distribution of a carnitine palmitoyltransferase I gene in rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. B* **135**, 139-151.
Gutierrez, J., Asgard, T., Fabbri, E. and Plisetskaya, E. M. (1991). Insulin-receptor binding in skeletal-muscle of trout. *Fish Physiol. Biochem.* **9**, 351-360.
Hemre, G.-I., Mommsen, T. P. and Krogdahl, A. (2002). Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. *Aquac. Nutr.* **8**, 175-194.
Iynedjian, P. B., Jotterand, D., Nouspikel, T., Asfari, M. and Pilot, P. R. (1989). Transcriptional induction of glucokinase gene by insulin in cultured liver cells and its repression by the glucagon-cAMP system. *J. Biol. Chem.* **264**, 21824-21829.
Kam, J. C. and Milligan, C. L. (2006). Fuel use during glycogenesis in rainbow trout (*Oncorhynchus mykiss* Walbaum) white muscle studied *in vitro*. *J. Exp. Biol.* **209**, 871-880.
Kamangar, B. B., Gabillard, J. C. and Bobe, J. (2006). Insulin-like growth factor-binding protein (IGFBP)-1, -2, -3, -4, -5, and -6 and IGFBP-related protein 1 during rainbow trout postvitellogenesis and oocyte maturation: molecular characterization, expression profiles, and hormonal regulation. *Endocrinology* **147**, 2399-2410.
Kirchner, S., Kaushik, S. and Panserat, S. (2003). Low protein intake is associated with reduced hepatic gluconeogenic enzyme expression in rainbow trout (*Oncorhynchus mykiss*). *J. Nutr.* **133**, 2561-2564.
Kirchner, S., Seixas, P., Kaushik, S. and Panserat, S. (2005). Effects of low protein intake on extra-hepatic gluconeogenic enzyme expression and peripheral glucose phosphorylation in rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. B* **140**, 333-340.
Krasnov, A., Teerijoki, H. and Molsa, H. (2001). Rainbow trout (*Onchorhynchus mykiss*) hepatic glucose transporter. *Biochim. Biophys. Acta* **1520**, 174-178.
Legate, N.J., Bonen, A. and Moon, T. W. (2001). Glucose tolerance and peripheral glucose utilization in rainbow trout (*Oncorhynchus mykiss*), American eel (*Anguilla rostrata*), and black bullhead catfish (*Ameiurus melas*). *Gen. Comp. Endocrinol.* **122**, 48-59.
Liao, J., Barthel, A., Nakatani, K. and Roth, R. A. (1998). Activation of protein kinase B/Akt is sufficient to repress the glucocorticoid and cAMP induction of phosphoenolpyruvate carboxykinase gene. *J. Biol. Chem.* **273**, 27320-27324.
Massillon, D., Barzilai, N., Chen, W., Hu, M. and Rossetti, L. (1996). Glucose regulates *in vivo* glucose-6-phosphatase gene expression in the liver of diabetic rats. *J. Biol. Chem.* **271**, 9871-9874.
Mommsen, T. P., Moon, T. W. and Walsh, T. J. (1994). Hepatocytes: isolation, maintenance and utilization. In *Biochemistry and Molecular Biology of Fishes* (ed. Hochachka, P. W. and Mommsen, T. P.). Amsterdam: Elsevier Science B.V.
Montserrat, N., Gabillard, J. C., Capilla, E., Navarro, M. I., Gutierrez, J. and Kamangar, B. B. (2007). Role of insulin, insulin-like growth factors, and muscle regulatory factors in the compensatory growth of the trout (*Oncorhynchus mykiss*). Coordinated regulation of the GH/IGF system genes during refeeding in rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **150**, 462-472.
Moon, T. W. (2001). Glucose intolerance in teleost fish: fact or fiction? *Comp. Biochem. Physiol. B* **129**, 243-249.
Moon, T. W., Walsh, T. J. and Mommsen, T. P. (1985). Fish hepatocytes: a model metabolic system. *Can. J. Fish. Aquat. Sci.* **42**, 1772-1782.
Navarro, I., Leibush, B., Moon, T. W., Plisetskaya, E. M., Banos, N., Mendez, E., Planas, J. V. and Gutierrez, J. (1999). Insulin, insulin-like growth factor-I (IGF-I) and glucagon: the evolution of their receptors. *Comp. Biochem. Physiol. B* **122**, 137-153.
Palmer, T. N. and Ryman, B. E. (1972). Studies on oral glucose intolerance in fish. *J. Fish Biol.* **4**, 311-319.
Panserat, S., Medale, F., Breque, J., Plagnes-Juan, E. and Kaushik, S. (2000a). Lack of significant long-term effect of dietary carbohydrates on hepatic glucose-6-phosphatase expression in rainbow trout (*Oncorhynchus mykiss*). *J. Nutr. Biochem.* **11**, 22-29.
Panserat, S., Medale, F., Blin, C., Breque, J., Vachot, C., Plagnes-Juan, E., Gomes, E., Krishnamoorthy, R. and Kaushik, S. (2000b). Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. *Am. J. Physiol.* **278**, R1164-R1170.
Panserat, S., Plagnes-Juan, E. and Kaushik, S. (2001a). Nutritional regulation and tissue specificity of gene expression for proteins involved in hepatic glucose metabolism in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **204**, 2351-2360.
Panserat, S., Plagnes-Juan, E., Breque, J. and Kaushik, S. (2001b). Hepatic phosphoenolpyruvate carboxykinase gene expression is not repressed by dietary carbohydrates in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **204**, 359-365.
Panserat, S., Capilla, E., Gutierrez, J., Frappart, P. O., Vachot, C., Plagnes-Juan, E., Aguirre, P., Breque, J. and Kaushik, S. (2001c). Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (Oncorhynchus mykiss) by a single meal with glucose. *Comp. Biochem. Physiol. B* **128**, 275-283.
Park, E. A., Mynatt, R. L., Cook, G. A. and Kashfi, K. (1995). Insulin regulates enzyme activity, malonyl-CoA sensitivity and mRNA abundance of hepatic carnitine palmitoyltransferase-I. *Biochem. J.* **310**, 853-858.
Parrizas, M., Planas, J., Plisetskaya, E. M. and Gutierrez, J. (1994a). Insulin binding and receptor tyrosine kinase activity in skeletal muscle of carnivorous and omnivorous fish. *Am. J. Physiol.* **266**, R1944-R1950.
Parrizas, M., Banos, N., Baro, J., Planas, J. and Gutierrez, J. (1994b). Up-regulation of insulin binding in fish skeletal muscle by high insulin levels. *Regul. Pept.* **53**, 211-222.
Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, E45-E45.
Pozios, K. C., Ding, J., Degger, B., Upton, Z. and Duan, C. (2001). IGFs stimulate zebrafish cell proliferation by activating MAP kinase and PI3-kinase-signaling pathways. *Am. J. Physiol.* **280**, R1230-R1239.
Puigserver, P., Rhee, J., Donovan, J., Walkey, C. J., Yoon, J. C., Oriente, F., Kitamura, Y., Altomonte, J., Dong, H., Accili, D. and Spiegelman, B. M. (2003). Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1[alpha] interaction. *Nature* **423**, 550-555.
Salgado, M. C., Meton, I., Egea, M. and Baanante, I. V. (2004). Transcriptional regulation of glucose-6-phosphatase catalytic subunit promoter by insulin and glucose in the carnivorous fish, *Sparus aurata*. *J. Mol. Endocrinol.* **33**, 783-795.
Schmoll, D. (2000). Regulation of glucose-6-phosphatase gene expression by protein kinase B[alpha] and the forkhead transcription factor FKHR. Evidence for insulin response unit-dependent and -independent effects of insulin on promoter activity. *J. Biol. Chem.* **275**, 36324-36333.
Scholz, S., Braunbeck, T. and Segner, H. (1998). Viability and differential function of rainbow trout liver cells in primary culture: coculture with two permanent fish cells. *In Vitro Cell. Dev. Biol. Anim.* **34**, 762-771.
Scott, D. K., O'Doherty, R. M., Stafford, J. M., Newgard, C. B. and Granner, D. K. (1998). The repression of hormone-activated PEPCK gene expression by glucose is insulin-independent but requires glucose metabolism. *J. Biol. Chem.* **273**, 24145-24151.
Segner, H. (1998a). Fish cell lines as a tool in aquatic toxicology. In *Fish Ecotoxicology* (ed. Braunbeck, T., Hinton, D. E. and Streit, D. E.), pp. 1-38. Basel: Birkhäuser Verlag.
Segner, H. (1998b). Isolation and primary culture of teleost hepatocytes. *Comp. Biochem. Physiol. A* **120**, 71-81.
Seiliez, I., Panserat, S., Skiba-Cassy, S., Fricot, A., Vachot, C., Kaushik, S. and Tesseraud, S. (2008). Feeding status regulates the polyubiquitination step of the ubiquitin-proteasome-dependent proteolysis in rainbow trout (*Oncorhynchus mykiss*) muscle. *J. Nutr.* **138**, 487-491.
Smith, S., Witkowski, A. and Joshi, A. K. (2003). Structural and functional organization of the animal fatty acid synthase. *Prog. Lipid Res.* **42**, 289-317.
Sul, H. S. and Wang, D. (1998). Nutritional and hormonal regulation of enzymes in fat synthesis: studies of fatty acid synthase and mitochondrial glycerol-3-phosphate acyltransferase gene transcription. *Annu. Rev. Nutr.* **18**, 331-351.
Sul, H. S., Latasa, M.-J., Moon, Y. and Kim, K.-H. (2000). Regulation of the fatty acid synthase promoter by insulin. *J. Nutr.* **130**, 315-320.
Taniguchi, C. M., Emanuelli, B. and Kahn, C. R. (2006). Critical nodes in signalling pathways: insights into insulin action. *Nat. Rev. Mol. Cell Biol.* **7**, 85-96.
Towle, H. C. (2005). Glucose as a regulator of eukaryotic gene transcription. *Trends Endocrinol. Metab.* **16**, 489-494.
Towle, H. C., Kaytor, E. N. and Shih, H. (1997). Regulation of the expression of lipogenic enzyme genes by carbohydrate. *Annu. Rev. Nutr.* **17**, 405-433.
Tranulis, M. A., Christophersen, B., Blom, A. K. and Borrebaek, B. (1991). Glucose dehydrogenase, glucose-6-phosphate dehydrogenase and hexokinase in liver of rainbow trout (*Salmo gairdneri*). Effects of starvation and temperature variations. *Comp. Biochem. Physiol. B* **99**, 687-691.
Tremblay, F., Jacques, H. and Marette, A. (2005). Modulation of insulin action by dietary proteins and amino acids: role of the mammalian target of rapamycin nutrient sensing pathway. *Curr. Opin. Clin. Nutr. Metab. Care* **8**, 457-462.
Weickert, M. and Pfeiffer, A. (2006). Signalling mechanisms linking hepatic glucose and lipid metabolism. *Diabetologia* **49**, 1732-1741.
Wilson, R. P. (1994). Utilization of dietary carbohydrate by fish. *Aquaculture* **124**, 67-80.