Amino Acids (2010) 39:801–810
DOI 10.1007/s00726-010-0533-3

ORIGINAL ARTICLE

Integration of insulin and amino acid signals that regulate hepatic metabolism-related gene expression in rainbow trout: role of TOR

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Received: 27 November 2009 / Accepted: 12 February 2010 / Published online: 6 March 2010

Abstract Amino acids are considered to be regulators of metabolism in several species, and increasing importance has been accorded to the role of amino acids as signalling molecules regulating protein synthesis through the activation of the TOR transduction pathway. Using rainbow trout hepatocytes, we examined the ability of amino acids to regulate hepatic metabolism-related gene expression either alone or together with insulin, and the possible involvement of TOR. We demonstrated that amino acids alone regulate expression of several genes, including glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, pyruvate kinase, 6-phospho-fructo-1-kinase and serine dehydratase, through an unknown molecular pathway that is independent of TOR activation. When insulin and amino acids were added together, a different pattern of regulation was observed that depended upon activation of the TOR pathway. This pattern included a dramatic up-regulation of lipogenic (fatty acid synthase, ATP-citrate lyase and sterol responsive element binding protein 1) and glycolytic (glucokinase, 6-phospho-fructo-1-kinase and pyruvate kinase) genes in a TOR-dependent manner. Regarding gluconeogenesis genes, only glucose-6-phosphatase was inhibited in a TOR-dependent manner by combination of insulin and amino acids and not by amino acids alone. This study is the first to demonstrate an important role of amino acids in combination with insulin in the molecular regulation of hepatic metabolism.

Keywords Amino acids · Insulin · TOR · Hepatic gene expression · Metabolism

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Introduction

Amino acids are not only important substrates for the synthesis of proteins and other nitrogenous compounds, but also are now widely considered to be key regulators of flux through the control of major metabolic pathways (Jobgen et al. 2006; Meijer 2003). There is increasing evidence that amino acids regulate intracellular signalling pathways. Through an unknown pathway, probably involving Rag GTPase (Sancak et al. 2008), amino acids regulate protein synthesis by activating the target of rapamycin (TOR) nutrient signalling pathway in common with insulin (Avruch et al. 2009; Kimball and Jefferson 2006). In mammals, TOR is part of a molecular complex known as mTOR complex 1 (TORC1) and contains the WD40 repeat-containing subunit raptor (a scaffold for TOR substrates), GBL (or mLST8, a positive effector of TOR) and PRAS40 (a negative effector of TOR). This complex is a nutrient sensor regulated by both insulin (or other growth factors) and amino acids, and is a key regulator of energy homeostasis, cell growth and protein translation thought the subsequent activation of p70 ribosomal S6 protein kinase 1 (S6K1) and ribosomal protein S6 (Wullschleger et al. 2006).

The mechanism leading to activation of TORC1 by insulin is already well known. Insulin acts through a tyrosine kinase membrane receptor. When activated by insulin binding, tyrosine kinase recruits and phosphorylates intracellular substrates known as IRS (insulin receptor substrates). Phosphorylated IRS are further used as docking sites for proteins which then transmit the insulin signal through several molecules, including successively phosphatidylinositol 3 kinase (PI3K) and Akt (also known as protein kinase B), a critical node of the insulin signalling pathway leading to at least the regulation of glucose

transport, glycogen synthesis, mRNA translation and gene expression (Taniguchi et al. 2006). Indeed, Akt regulates intermediary metabolism-related gene expression via phosphorylation and nuclear exclusion of Forkhead-box Class O1 (FoxO1) transcription factor and controls protein synthesis by the inactivation of the tuberous sclerosis complex (TSC), leading to the activation of mTORC1.

Increasing evidence has emerged in recent years to show that amino acids may interfere with insulin function. For example, excessive levels of amino acids have detrimental effects on glucose homeostasis by promoting insulin resistance and increasing gluconeogenesis (Tremblay et al. 2007b), which may be due to the activation of TORC1. Recent findings have also indicated that activation of TORC1 contributes to the regulation of fatty acid biosynthesis (Porstmann et al. 2008). For example, Porstman et al. demonstrated that Akt-induced nuclear accumulation of mature sterol responsive element binding protein (mSREBP1) and expression of SREBP target genes such as fatty acid synthase (FAS) and ATP-citrate lyase (ACLY) require TORC1 activity. Moreover, they showed that induction of FAS and ACLY gene expression in response to Akt activation was blocked in the presence of rapamycin, indicating that TORC1 is involved in the regulation of SREBP-dependent transcription. This provides an example of the possible involvement of the TOR signalling pathway in the regulation of lipid metabolism-related gene expression.

The main aim of the present study was thus to investigate more widely the specific role of amino acids in the regulation of mRNA levels encoding genes involved in carbohydrate and lipid metabolism, and to evaluate the possible involvement of the TORC1 signalling pathway in such regulation. For this purpose, we chose to work on primary cell cultures of hepatocytes prepared from the livers of rainbow trout (*Oncorhynchus mykiss*), a carnivorous fish with high dietary protein requirement (1993) compared to mammals. Total protein content of the rainbow trout diet may exceed 45% of the dry matter, and it seems that this high dietary intake of amino acids may have undesirable effects on insulin sensitivity, particularly on insulin-regulated gene expression (Kirchner et al. 2003). Rainbow trout, in fact, exhibit an absence of post-prandial inhibition of glucogenic gene expression [especially glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (mPEPCK)] that can be restored by reducing dietary protein levels (Kirchner et al. 2003). We examined insulin and TORC1 signalling and mRNA expression of metabolism-related genes under stimulation by insulin, amino acids and a combination of both. Attention was focused on asparagine synthase (AS), which is responsible for the biosynthesis of asparagine from aspartate and glutamate, and genes related to gluconeogenesis (G6Pase, fructose-1,6-bisphosphatase FBPase and mPEPCK), glycolysis (glucokinase GK, 6-phospho-fructo-1-kinase 6PF1K and pyruvate kinase PK), lipogenesis (fatty acid synthase FAS, ATP-citrate lyase ACLY) and amino acid degradation (serine dehydratase SD). Transcript accumulation of transcription factor SREBP1 was also evaluated.

Methods

Animals

Sexually immature rainbow trout (350–400 g) were obtained from the INRA experimental fish farm facilities (INRA, Donzacq, France). Fish were maintained in tanks kept in open circuits at 18°C with well-aerated water under natural photoperiod conditions. Trout were fed to satiety every 2 days 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 (authority: Prefecture of Pyrénées Atlantiques, 64000 Pau, for INRA St Pée-sur-Nivelle). Animal experiments were in accordance with national law (Decret No. 2001-464, 29 May 2001) as applicable in INRA according to INRA No. 2002-36, 4 April 2002.

Hepatocyte cell culture

Isolated liver cells were prepared from 3-day fasted rainbow trout. Trout were left unfed for 3 days in order to empty the digestive tract and then facilitate the liver in situ perfusion. At the time of experiments, fish were anaesthetized by placing them in water containing 60 mg l^{-1} aminobenzoic acid, and hepatocytes were isolated by the in situ perfusion method described by Mommsen et al. (1994). Livers were excised and minced with a razor blade in modified Hanks' medium (136.9 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 5 mM $NaHCO_3$ and 10 mM HEPES) supplemented with 1 mM EGTA. After filtration and centrifugation (120*g*, 2 min), the resulting cell pellet was resuspended three successive times in modified Hanks' medium [1.5 mM $CaCl_2$ and 1.5% defatted bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA)]. Cells were finally taken up in modified Hanks' medium supplemented with 1.5 mM $CaCl_2$, 1% defatted BSA, 3 mM glucose, MEM essential amino acids (1×) (Invitrogen, Carlsbad, CA, USA), MEM non-essential amino acids (1×) (Invitrogen, Carlsbad, CA, USA) and antibiotic antimycotic solution (1×) (Sigma, St. Louis, MO, USA). Cell viability (>98%) was assessed using the trypan blue exclusion method (0.04% in 0.15 M NaCl) and cells were counted using a haemocytometer. The hepatocyte cell

suspension was plated in a six-well Primaria culture dish (BD, USA) at a density of 3×10^6 cells/well and incubated at 18°C. The incubation medium was replaced every 24 h over the 48 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 1998). Cell viability and cytotoxicity were monitored throughout culture using CellTiter 96® aqueous one solution cell proliferation assay (Promega, Madison, WI, USA) and Cyto Tox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA), respectively, according to the manufacturer's recommendations. Osmotic pressures of all cell culture media were verified before experiments and calibrated to 300 mosmol l^{-1} using a K7400 osmometer (Knauer, Berlin, Germany). For experiments, 48-h cultured hepatocytes were subjected to amino acid deprivation in amino acid-free incubation medium for 2 h. Then, according to each experimental design, cells were incubated in either amino acids-free medium (control), in medium containing 4×10^{-9} M of bovine insulin (Sigma, St. Louis, MO, USA) corresponding to the post-prandial level of insulin irrespective of dietary carbohydrate level (del sol Novoa et al. 2004), in medium containing onefold (1×) or fourfold (4×) concentrated amino acids, or in medium containing insulin plus amino acids as specified in the figure legends. In experiments involving rapamycin (specific TOR inhibitor), the inhibitor was added 30 min prior to stimulation with insulin and/or amino acids. At the end of the stimulation period, cells were prepared for western blot analysis or resuspended in TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and stored at −80°C for subsequent mRNA extraction.

Gene expression analysis

Total RNA samples were extracted from hepatocytes using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. One microgram of the resulting total RNA was reverse transcribed into cDNA using the SuperScript III RNaseH-Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and oligo dT primers (Promega, Charbonnières, France) according to the manufacturers' instructions. Target gene expression levels were determined by quantitative RT-PCR. Primers were designed to overlap an intron if possible (Primer3 software) using known sequences in trout nucleotide databases (Genbank and INRA-Sigenae) as previously described (Plagnes-Juan et al. 2008; Polakof et al. 2009; Skiba-Cassy et al. 2009), except for AS in the liver (forward primer : CTGCACACGGTCTGGAGCTG; reverse primer: GGATCTCGTCTGGGATCAGGTT; tcay 0019b.e.21_3.1.s.om.8). The different PCR products were initially checked by sequencing to confirm the nature of the amplicon.

qRT-PCR was carried out on an iCycler iQ™ real-time PCR detection system (BIO-RAD, Hercules, CA, USA) using iQ TM SYBR® Green Supermix. qRT-PCR analyses were focused on several key enzymes of hepatic metabolism (Plagnes-Juan et al. 2008): (a) glucose-6-phosphatase (G6Pase), fructose-1,6-bisphosphatase FBPase and the mitochondrial isoform of phosphoenolpyruvate carboxykinase (mPEPCK) involved in gluconeogenesis, (b) for glycolysis, glucokinase (GK) that catalyses the phosphorylation of glucose, 6-phospho-fructo-1-kinase (6PF1K) that catalyses the transfer of a phosphate group from ATP to fructose-6-phosphate to yield fructose-1,6-bisphosphate, and pyruvate kinase (PK) that catalyses the conversion of phosphoenolpyruvate to pyruvate (Towle et al. 1997), (c) two enzymes involved in fatty acid metabolism, ATP-citrate lyase (ACLY) and fatty acid synthase (FAS), that successively allow the synthesis of fatty acids, primarily palmitate, by catalysing the production of cytosolic acetyl-CoA and oxaloacetate from citrate then promoting the condensation of malonyl-CoA and acetyl-CoA (Smith et al. 2003), (d) serine dehydratase (SD), which catalyses the pyridoxal 50-phosphate-dependent deamination of serine and threonine in the liver to produce pyruvate and 2-oxobutyrate, respectively (Ishikawa et al. 1965), and (e) transcript accumulation of the transcription factor SREBP1.

Elongation factor 1α (EF1α) was employed as a non-regulated reference gene, as previously used in rainbow trout. No changes in EF1α gene expression were observed in our investigations (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 programme (20 s at 95°C followed by 30 s at specific primer hybridisation 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 PCR amplification, respectively) and negative controls (reverse transcriptase- and RNA-free samples, respectively).

Relative quantification of target gene expression was performed using the mathematical model described by 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. PCR efficiency (E) was measured by the

slope of a standard curve using serial dilutions of cDNA. PCR efficiency values ranged between 1.90 and 2.

Protein extraction and Western blotting

Following incubation, cell pellets were washed with 1 ml of phosphate buffered saline. Then 300 µl of cell lysis buffer [150 mM NaCl, 10 mM Tris, 1 mM EGTA, 1 mM EDTA (pH 7.4), 100 mM sodium fluoride, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1% Triton X-100, 0.5% NP-40-IGEPAL and a protease inhibitor cocktail (Roche, Basel, Switzerland)] was added before being stored the culture dish at −80°C overnight. Then cells were scraped, collected, and lysed on ice for 30 min. Lysates were centrifuged at 12,000*g* for 30 min at 4°C. The resulting supernatants were aliquoted and stored at −80°C. Protein concentrations were determined using the Bio-Rad protein assay kit (BIO-RAD, Hercules, CA, USA). Cell lysates (20 µg of protein) were subjected to SDS-PAGE and Western blotting using the appropriate antibody. All antibodies used were obtained from Cell Signaling Technologies (Ozyme, Saint Quentin Yvelines, France). Anti-phospho Akt (Ser^{473}), anti-carboxyl terminal Akt, anti-phopho-FoxO1 (Ser^{256}), anti-FoxO1, anti-phospho-mTOR (Ser^{2448}), anti-mTOR, anti-phospho-S6K1 (Thr^{389}), anti-S6K1, anti-phosphor-S6 ($Ser^{235/236}$) and anti-S6 were purchased from Cell Signaling Technologies (Ozyme, Saint Quentin Yvelines, France). These antibodies have been shown to cross-react successfully with rainbow trout proteins of interest (Seiliez et al. 2008). After washing, membranes were incubated with an IRDye infrared secondary antibody (LI-COR Inc. Biotechnology, Lincoln, NE, USA). Bands were visualised by Infrared Fluorescence using the Odyssey® Imaging System (LI-COR Inc. Biotechnology, Lincoln, NE, USA).

Statistical analysis

The results of gene expression analysis were expressed as mean ± SD ($n = 6$) and analysed by one-way ANOVA followed by Student–Newman–Keuls test. For all statistical analyses, the level of significance was set at $P < 0.05$.

Results

Specific and combined effects of insulin and amino acids on Akt-TOR signalling

The effects of insulin and/or amino acids on Akt/TOR signalling pathways were investigated in rainbow trout hepatocytes using western blot analyses. As illustrated in Fig. 1, proteins of the Akt/TOR signalling pathways,

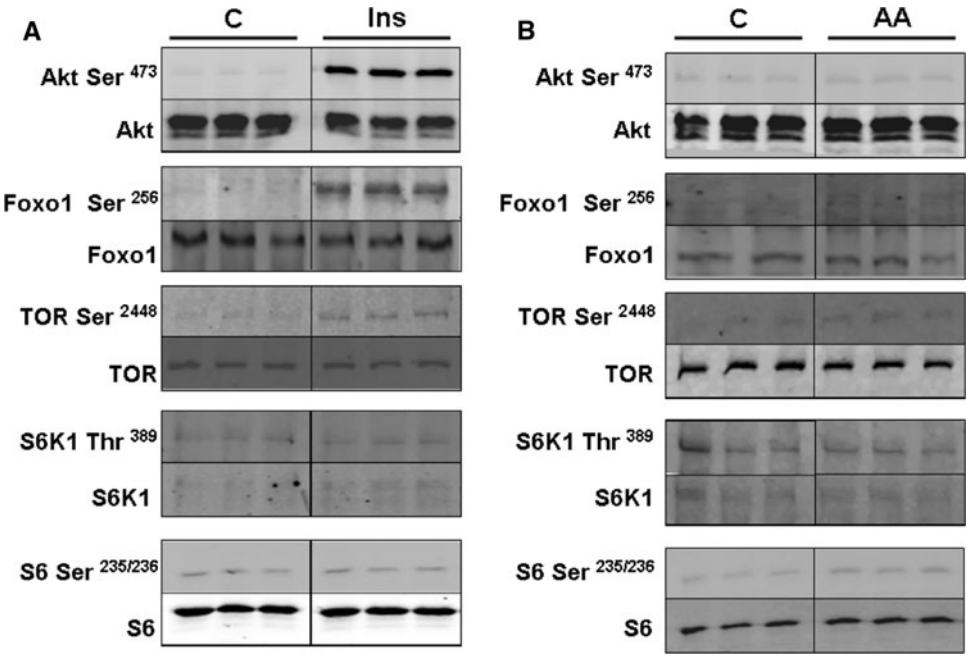

Fig. 1 Specific effect of insulin (**a**), amino acids (**b**) on the phosphorylation of Akt, FoxO1, TOR, S6K1 and S6 protein in trout hepatocytes. Cells were subjected to amino acid deprivation for 1 h 30 min and then were preincubated for 30 min with or without 100 nM rapamycin, a TOR inhibitor. The culture medium was then replaced for 15 min with an amino acid-free medium (control *C*), an amino acid-free medium containing 4×10^{-9} mol l^{-1} insulin (*Ins*) or a medium containing 1× amino acids (*AA*). The gel was loaded with 20 µg of total protein per lane. Western blots were performed on six individual samples and similar results were obtained. This figure includes representative *blots*

including Akt, FoxO1, TOR, S6K1 and the ribosomal protein S6, were identified. The levels of these total proteins were not modified by with insulin or amino acids treatment (Fig. 1). As expected, insulin (4×10^{-9} mol l^{-1}) improved the phosphorylation of Akt (Ser^{473}) and FoxO1 (Ser^{256}), and slightly enhanced the phosphorylation of TOR (Ser^{2448}), but had no effect on S6K1 (Thr^{389}) or S6 ($Ser^{235/236}$) protein phosphorylation (Fig. 1a). On the other hand, we found that amino acids had no effect on the phosphorylation state of any of the proteins tested (Fig. 1b). Figure 2 shows that combined insulin and amino acid stimulation had no further effect on the insulin-induced phosphorylation of Akt (Ser^{473}) or FoxO1 (Ser^{256}) but caused even greater stimulation than insulin alone with regard to TOR phosphorylation, and induced the phosphorylation of S6K1 (Thr^{389}) and S6 ($Ser^{235/236}$) protein. Finally, as expected, we observed no effect of addition of rapamycin on the phosphorylation of Akt and FoxO1, whereas the activating effect of the combination of insulin and amino acids on TOR (Ser^{2448}), S6K1 (Thr^{389}) and S6 ($Ser^{235/236}$) phosphorylation was totally abolished by the rapamycin treatment (Fig. 2). The phosphorylation of tested proteins was not affected by rapamycin alone (data not shown).

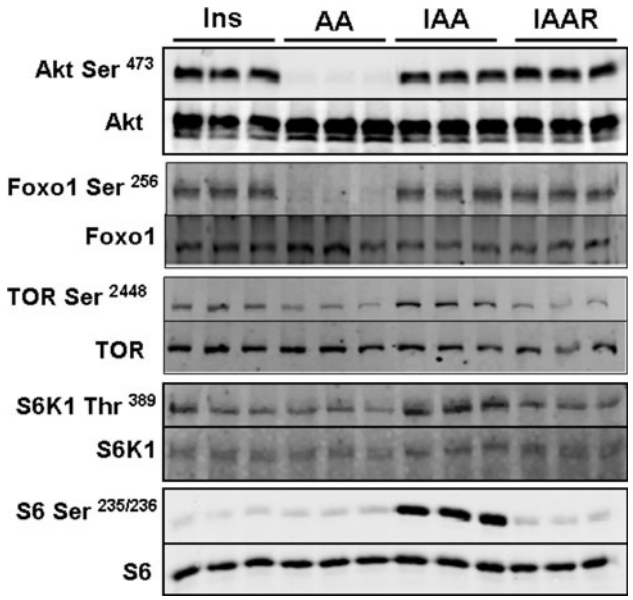

Fig. 2 Combined effects of insulin and amino acids on the phosphorylation of Akt, FoxO1, TOR, S6K1 and S6 protein in trout hepatocytes. Cells were subjected to amino acid deprivation for 1 h 30 min and then were preincubated for 30 min with or without 100 nM rapamycin, a TOR inhibitor. The culture medium was then replaced for 15 min by an amino acid-free medium containing 4×10^{-9} mol l^{-1} insulin (*Ins*), a medium containing 1× amino acids (*AA*), or a 1× amino acid medium containing 4×10^{-9} mol l^{-1} insulin (*AAI* and *AAIR*). The gel was loaded with 20 µg of total protein per lane. Western blots were performed on six individual samples and similar results were obtained. This figure includes representative *blots*

Specific effects of amino acids on metabolism-related gene expression

In order to test the effects of amino acids on gene expression in rainbow trout hepatocytes, cells were amino acid deprived for 2 h then stimulated for 24 h with a pool of amino acids. The specific effects of amino acids on AS, G6Pase, FBPase, mPEPCK, GK, 6PF1K PK, FAS, ACLY, SREBP1 and SD gene expression are presented in Fig. 3. AS is a marker gene that is known to be induced by amino acid starvation in mammals. As expected, AS was down-regulated after 24 h of stimulation with amino acids in a dose-dependent manner (Fig. 3). Amino acids up-regulated the gene expression of G6Pase, mPEPCK and 6PF1K in dose-dependent manner. PK and SD gene expression was increased by amino acids but only with the 4× concentration. Expression of the other target genes (FBPase, GK, FAS, ACLY and SREBP1) was not affected by amino acids.

Combined effects of insulin and amino acids on gene expression

In order to investigate the combined effect of amino acids and insulin on gene expression and to determine the potential involvement of the TOR pathway in such regulation, cells were stimulated with insulin, amino acids or a combination of insulin and amino acids in the presence or absence of the TOR inhibitor rapamycin (Fig. 4). To exclude a particular effect of rapamycin on gene expression, cells were also treated with rapamycin alone. We found that rapamycin had no effect on the mRNA levels of FBPase, mPEPCK, GK, 6PF1K, FAS, ACLY, SREBP1 and SD compared to the control situation, but weakly down- and up-regulated G6Pase and PK, respectively. Our results regarding gluconeogenic genes showed that insulin and amino acids had opposite effects on the regulation of G6Pase mRNA levels. Insulin decreased G6Pase gene expression twofold while amino acids increased G6Pase mRNA accumulation fourfold. However, the combination of insulin and amino acids dramatically (sixfold) decreased mRNA levels of G6Pase, compared to amino acids alone and reached level significantly lower than that measured in control cells. This decrease was totally abolished when TOR was inhibited by rapamycin. We found that using insulin and amino acids separately had no significant effect on FBPase mRNA levels, but together increased FBPase gene expression compared to control cells. Addition of rapamycin did not change this effect. Moreover, whereas insulin decreased (about twofold) the expression of mPEPCK, amino acids increased mPEPCK expression about twofold. Combination of insulin and amino acids 1.5-fold increased mPEPCK gene expression but this increase was significantly lower compared to that obtained

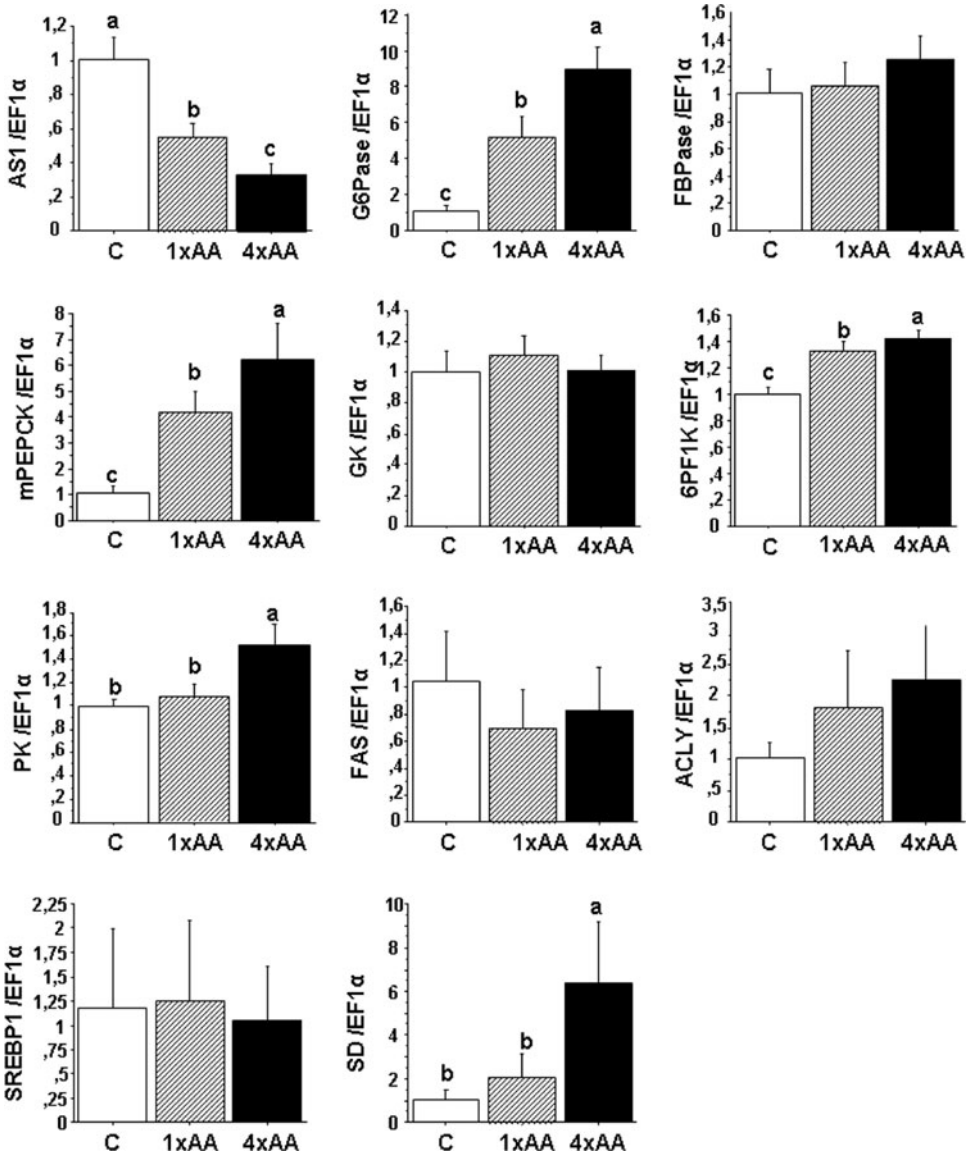

Fig. 3 Dose effect of amino acids on the expression levels of mRNA encoding hepatic genes in 24-h stimulated rainbow trout hepatocytes. Cells were subjected to amino acid deprivation for 2 h. The culture medium was then replaced for 24 h by an amino acid-free medium (control *C*), or onefold (*AA*) or fourfold (4×AA) concentrated amino acid medium. Aspargine synthase (*AS*), glucose 6-phosphatase (*G6Pase*), fructose 1,6-bisphosphatase (*FBPase*), mitochondrial phosphoenolpyruvate carboxykinase (*mPEPCK*), glucokinase (GK), 6-phospho-fructo-1-kinase (6PF1K), pyruvate kinase PK, fatty acid synthase (*FAS*), ATP-citrate lyase (*ACLY*), sterol responsive element binding protein (*SREBP1*) and serine dehydratase (*SD*) mRNA levels were estimated using real-time RT-PCR. For each treatment, six replicates were performed. Similar results were obtained from three separate primary hepatocyte cell cultures. Expression values were normalised with elongation factor 1α (*EF1α*)-expressed transcripts. Results are expressed as mean ± SD ($n = 6$) and were analysed by one-way ANOVA followed by Student–Newman–Keuls test for multiple comparison ($P < 0.05$). Different letters represent significantly different values

with amino acids alone. Addition of rapamycin also slightly decreased this combined effect on mPEPCK gene expression. Expression of the GK gene was 2.5-fold reduced by insulin but not modulated by amino acids alone. Insulin plus amino acids resulted in significant up-regulation (1.6-fold) of GK gene expression, and this effect was suppressed by addition of rapamycin. Amino acids induced three- and fivefold increase in 6PF1K mRNA levels in the absence and presence of insulin, respectively, whereas insulin alone was unable to modulate 6PF1K gene expression. This combined effect of insulin and amino acids on 6PF1K was counteracted by rapamycin. Expression of the PK gene was slightly but significantly increased (1.3-fold) by insulin, not modified by amino acids, and increased twofold by the combination of both. Once again, this combined effect was abolished by rapamycin. On the

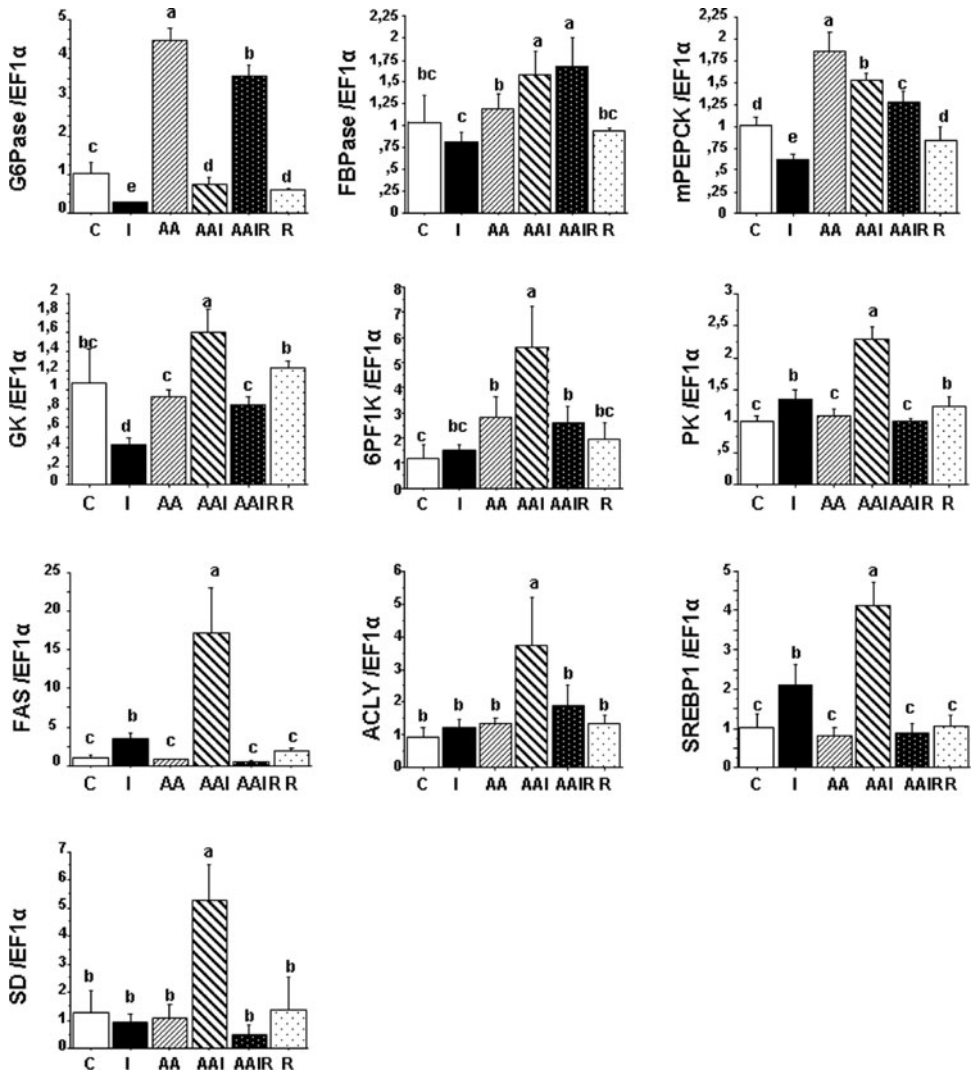

Fig. 4 Combined effects of insulin and amino acids on the expression levels of mRNA encoding hepatic genes in 24-h stimulated rainbow trout hepatocytes. Cells were subjected to amino acid deprivation for 1 h 30 min and then were preincubated for 30 min with or without 100 nM rapamycin, a TOR inhibitor. The culture medium was then replaced for 24 h with an amino acid-free medium (control group *C* and rapamycin group *R*), an amino acid-free medium containing 4×10^{-9} mol l^{-1} insulin (*Ins*), a medium containing 1× amino acids (AA), or a 1× amino acid medium containing 4×10^{-9} mol l^{-1} insulin (*AAI* and *AAIR*). Glucose 6-phosphatase (*G6Pase*), fructose 1,6-bisphosphatase (*FBPase*), phosphoenolpyruvate carboxykinase (*mPEPCK*), glucokinase (*GK*), 6-phosphofructo-1-kinase (*6PF1K*), pyruvate kinase PK, fatty acid synthase (*FAS*), ATP-citrate lyase (*ACLY*), sterol responsive element binding protein (*SREBP1*) and serine dehydratase (*SD*) mRNA levels were estimated using real-time RT-PCR. For each treatment, six replicates were performed. Similar results were obtained from three separate primary hepatocyte cell cultures. Expression values were normalised with elongation factor 1α (*EF1α*)-expressed transcripts. Results are expressed as mean ± SD ($n = 6$) and were analysed by one-way ANOVA followed by Student–Newman–Keuls test for multiple comparison ($P < 0.05$). Different letters represent significantly different values

other hand, we found that FAS and SREBP-1 gene expression was regulated in the same way: insulin increased the expression of FAS and SREBP-1 (3.5- and 2-fold, respectively) and the insulin stimulatory effect was dramatically enhanced (five- and twofold, respectively) in the presence of amino acids. This increase was totally inhibited in the presence of rapamycin. Finally, mRNA levels of ACLY and SD were not affected by insulin or by amino acids but strongly increased when cells were treated simultaneously with insulin and amino acids (3.5-fold and 5-fold, respectively). This effect was totally suppressed by addition of rapamycin.

Discussion

There is growing evidence that amino acids have an important role in controlling gene expression (Jousse et al.

2004). Using rainbow trout hepatocytes, the purpose of the study reported here was to establish the role of amino acids in the regulation of hepatic intermediary metabolism-related expression of genes involved in gluconeogenesis, glycolysis, lipogenesis and amino acid catabolism. The aim was also to demonstrate the interaction between amino acids and insulin and the possible involvement of the TOR signalling pathway in such regulation.

Particular effects of amino acids on metabolic gene expression

Our results demonstrate for the first time that amino acids are not only able to regulate the expression of genes involved in the metabolism of amino acids but also affect the expression of a wider range of hepatic metabolism-related genes involved in gluconeogenesis and glycolysis. In fact, we demonstrated that amino acids decreased AS gene expression while they up-regulated mRNA levels of G6Pase, mPEPCK, PK and SD enzymes. To our knowledge, and except for AS (Gong et al. 1991; Guerrini et al. 1993), no findings have been published to date on the effects of amino acids on the regulation of expression of these intermediary metabolism-related genes. Whereas AS gene transcription is known to be controlled by amino acid starvation through the activation of the GCN2 transduction pathway (Barbosa-Tessmann et al. 2000), the molecular pathway involved in the regulation of G6Pase, mPEPCK, PK and SD by addition of amino acids remains to be determined. In our study, cells stimulated with 1×AA, which corresponded to the concentration of amino acids of a standard cell culture medium, had enough amino acids to permit proteosynthesis (Mommsen et al. 1994; Plagnes-Juan et al. 2008) compared cells deprived of amino acids. The dose-dependent action of amino acids (1×AA vs. 4×AA) thus suggested that the control of G6Pase, mPEPCK, PK and SD mRNA levels by amino acids was not linked to altered protein synthesis. Some amino acids are known to induce cell swelling because of increased intracellular osmolarity following Na+-dependent, concentrative, amino acid transport across the plasma membrane (Meijer 2003). It is also well known that cell swelling leads to phosphorylation of the ribosomal protein S6 (Meijer 2003) and decreased levels of gluconeogenic rate-limiting enzymes (Goswami et al. 2004). Since opposite effects were observed in the present study (i.e. absence of phosphorylation of S6 with amino acids alone and increased expression of gluconeogenic genes), we can exclude the involvement of amino acid-induced cell swelling in the regulation of gene expression. We therefore concluded that amino acids have specific effects on the mRNA levels of G6Pase, mPEPCK, PK and SD. These effects seem to be independent of the activation of the TOR pathway since our results clearly indicated that amino acids were unable to induce the phosphorylation of TOR, S6K1 and S6 without insulin. Further investigations will be necessary to elucidate the molecular mechanism distinct from the TOR pathway mediating the particular effects of amino acids on such intermediary metabolism-related genes expression.

Combined effects of amino acids and insulin on metabolic gene expression

Recent findings have suggested that the PI3K-Akt-TOR pathway regulates lipid biosynthesis in an orchestrated manner. Indeed, Porstmann et al. (2008) demonstrated that mTORC1 positively regulates SREBP1, a transcription factor that controls the expression of genes encoding proteins involved in lipid metabolism (ACLY and FAS). In this context, our aim was to elucidate how hepatocytes integrate signals generated by insulin and amino acids to regulate not only lipogenic genes but also genes related to other types of metabolism and the role played by the TOR signalling pathway in these interactions between insulin and amino acids. For this purpose, we stimulated primary cell cultures of hepatocytes with a combination of insulin and amino acids in order to fully activate the TOR pathway. To distinguish the involvement of the TOR pathway, rapamycin was used to inhibit TOR activation. As previously demonstrated in mammals (Porstmann et al. 2008), we confirmed in this cellular model that the stimulation of FAS, ACLY and SREBP1 gene expression was associated with the activation of TOR, since rapamycin abolished the combined stimulatory effect of insulin and amino acids. Similar regulation was also observed for SD. SD catalyses the pyridoxal phosphate (PLP)-dependent deamination of serine to produce pyruvate. In mammals, SD is involved in the regulation of liver gluconeogenesis from serine in different dietary, hormonal and developmental states by providing pyruvate (Snell 1984). In rainbow trout hepatocytes, pyruvate originating from SD activity might instead be directed to lipid synthesis and contribute to enhanced lipogenesis, as previously proposed for a line of rainbow trout genetically selected for high muscle fat content (Skiba-Cassy et al. 2009). We thus confirmed in this study using a different model from mammals that lipogenesis is probably under the control of the TOR pathway in fish.

The lipogenic metabolism was not the only type of metabolism subjected to combined insulin and amino acid regulation. Compared to amino acid-treated cells, expression of GK, 6PF1K and PK was also up-regulated by insulin combined with amino acids. Insulin is considered to be a positive regulator of glycolysis. PK gene transcription in mammals is induced by insulin-stimulated glucose metabolism (Alam and Saggerson 1998; Towle 2005). We

demonstrated here that the ability of insulin to stimulate glycolytic gene expression was enhanced in the presence of amino acids. In excessive nutrient situations in mammals, amino acids are able to trigger negative feedback on the early events of the insulin signalling pathway and generate insulin resistance (Tremblay et al. 2007a; Tremblay and Marette 2001). In our study, where hepatocytes were not placed in excessive nutrient conditions, the positive combined effects of insulin and amino acids on the expression of several genes and the persistence of insulin-induced Akt and FoxO1 phosphorylation in cells stimulated by both insulin and amino acids (compared to insulin alone) suggested that in our conditions amino acids did not impair the insulin signalling pathways. In fact, it was quite the reverse, since rapamycin inhibited TOR activation and totally abolished the molecular effects of the insulin/amino acids combination on GK, 6PF1K and PK mRNA levels. We therefore hypothesize that insulin and amino acids both contribute to the regulation of glycolysis through activation of the TOR pathway.

In mammals, as in fish, gluconeogenic genes are mainly under insulin control (Barthel and Schmoll 2003; Plagnes-Juan et al. 2008). Insulin inhibits expression of mPEPCK and G6Pase enzymes at the transcription level through the activation of Akt (Liao et al. 1998; Schmoll 2000). We demonstrated here for the first time that amino acids may also contribute to the regulation of gluconeogenesis by activating the TOR signalling pathway in concert with insulin. In fact, insulin inhibited G6Pase gene expression in both the presence and absence of amino acids, but such inhibition was completely abolished by rapamycin only in the presence of amino acids. This suggests that insulin regulates G6Pase gene expression by activating several pathways, including at least the Akt-FoxO1 pathway and the TOR pathway, together with amino acids. Such regulation did not apply to all enzymes of gluconeogenesis. For example, rather than a decrease, FBPase was subjected to an increase in gene expression under stimulation with insulin and amino acids compared to untreated cells, insulin alone, and amino acid-treated cells. Nevertheless, such regulation that is totally independent of the activation of TOR is in agreement with previous studies in our laboratory showing a stimulatory effect of insulin on FBPase gene expression in rainbow trout hepatocytes (Plagnes-Juan et al. 2008). The pattern of regulation was again different for mPEPCK, with a dramatic up-regulation of mPEPCK mRNA levels under amino acid conditions. In agreement with previous published findings in mammals and fish (Barthel and Schmoll 2003; Plagnes-Juan et al. 2008), insulin resulted in a weak but significant decrease in mPEPCK mRNA levels in the presence or absence of amino acids. Surprisingly, blocking the TOR pathway slightly strengthened the inhibitory action of insulin, suggesting that the TOR pathway may be involved in molecular mechanisms regulating PEPCK gene expression.

In conclusion, this study demonstrated that hepatic metabolism is regulated by a combination of several hormonal-nutrient pathways, and showed the essential role of amino acids in the regulation of hepatic metabolism-related gene expression. The ways by which amino acids regulate gene expression are still not fully understood but may at least include the TOR pathway, the activation of which clearly required simultaneous induction by insulin.

Acknowledgments We thank M. Larquier and L. Gruyère for technical assistance, and F. Terrier, Y. Hontang and F. Sandres for fish rearing in the INRA experimental farm (Donzacq, France). This study was supported by the European Union 6th Framework project (Contract No. 016249-2, Sustainable aquafeeds to maximise the health benefits of farmed fish for consumers—AQUAMAX), French national research agency (ANR-08-JCJC-0025, Low utilisation of dietary carbohydrates in carnivorous rainbow trout: role of amino acids, glucose and insulin interactions?) and the Aquitaine Region (No. CCRRDT-20051303004AB). The AQUAMAX European project (Contract No. 016249-2) also provided M.L. with a fellowship.

Conflict of interest statement None.

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