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Original Paper

Amino Acids Attenuate Insulin Action on Gluconeogenesis and Promote Fatty Acid Biosynthesis via mTORC1 Signaling Pathway in trout Hepatocytes

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Key Words

Amino acids • mTORC1 • Insulin action • Fatty acid biosynthesis • Gluconeogenesis • Rainbow trout

Abstract

Background/Aims: Carnivores exhibit poor utilization of dietary carbohydrates and glucose intolerant phenotypes, yet it remains unclear what are the causal factors and underlying mechanisms. We aimed to evaluate excessive amino acids (AAs)-induced effects on insulin signaling, fatty acid biosynthesis and glucose metabolism in rainbow trout and determine the potential involvement of mTORC1 and p38 MAPK pathway. **Methods:** We stimulated trout primary hepatocytes with different AA levels and employed acute administration of rapamycin to inhibit mTORC1 activation. **Results:** Increased AA levels enhanced the phosphorylation of ribosomal protein S6 kinase (S6K1), S6, and insulin receptor substrate 1 (IRS-1) on Ser³⁰² but suppressed Akt and p38 phosphorylation; up-regulated the expression of genes related to gluconeogenesis and fatty acid biosynthesis. mTORC1 inhibition not only inhibited the phosphorylation of mTORC1 downstream targets, but also blunted IRS-1 Ser³⁰² phosphorylation and restored excessive AAs-suppressed Akt phosphorylation. Rapamycin also inhibited fatty acid biosynthetic and gluconeogenic gene expression. **Conclusion:** High levels of AAs up-regulate hepatic fatty acid biosynthetic gene expression through an mTORC1-dependent manner, while attenuate insulin-mediated repression of gluconeogenesis through elevating IRS-1 Ser³⁰² phosphorylation, which in turn impairs Akt activation and thereby weakening insulin action. We propose that p38 MAPK probably also involves in these AAs-induced metabolic changes.

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Introduction

The liver is a key organ in the systemic response to insulin, controlling both glucose and lipid metabolism [1]. In hepatocytes, binding of insulin to their receptors leads to recruitment and phosphorylation of the insulin receptor substrate 1 (IRS-1) on tyrosine residues, which in turn recruits phosphoinositide 3-kinase (PI3K) and activates Akt, a critical node of the insulin signaling pathway leading to at least the regulation of glucose uptake, glycogen synthesis, glycolysis and gluconeogenesis [2]. The activation of Akt inhibits glucose production through direct phosphorylation of transcription factors Forkhead-box Class O1 (FoxO1) [3] and peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) [4]. Another important anabolic role of insulin is to activate fatty acid synthesis. Insulin stimulates hepatic lipogenesis by increasing the mRNA and the processed nuclear form of sterol regulatory element-binding protein-1c (SREBP-1c), a transcription factor that activates all the genes needed to produce fatty acids and triglycerides in liver [5]. Insulin-dependent SREBP-1c transcriptional regulation also requires liver X receptor (LXR) activation [6, 7].

Dietary proteins and amino acids (AAs) have emerged as potent modulators of insulin signaling, thereby regulating glucose and lipid metabolism. Under appropriate conditions, AAs (particularly leucine, isoleucine, alanine, and arginine) can enhance pancreatic insulin secretion and repress hepatic gluconeogenesis through the IRS-Akt-Foxo pathway [8, 9]. Furthermore, AAs abundance can be sensed by mechanistic target of rapamycin complex 1 (mTORC1) [10], a crucial signaling node controlling cell growth and proliferation, which promotes *de novo* lipogenesis and glycolysis, through mTORC1-mediated activation of SREBP-1c and hypoxia inducible factor 1 α (HIF1 α), respectively [11, 12]. Moreover, excessive AAs can induce elevated activation of the mTORC1/S6K1 pathway, which elicits a negative feedback signal that hyperphosphorylates IRS-1 at serine residues, leading to reduced IRS-1 function and impaired activation of the PI3K/Akt pathway, thereby diminishing insulin action [13, 14]. Persistent activation of mTORC1 signaling pathway may lead to insulin resistance [14, 15], which is defined as the inability of insulin to promote efficient glucose uptake by peripheral tissues, and commonly associated with obesity, human type 2 diabetes and nonalcoholic fatty liver disease [16, 17].

The p38 mitogen-activated protein kinase (MAPK) is a major kinase in the MAPK family and plays a pivotal role in regulating many essential cellular processes, such as proliferation, inflammation, differentiation, survival and migration [18, 19]. Recent studies show that p38 MAPK also responds to key metabolic factors, thereby controlling glucose and lipid metabolism [20]. Cao et al. [21] demonstrated that activation of p38 by fasting in liver and glucagon in primary hepatocytes stimulates hepatic gluconeogenesis via regulating the phosphorylation of cAMP-response element-binding protein (CREB) and the expression of PGC1 α . Furthermore, p38 plays an inhibitory role on hepatic lipogenesis, probably through the inhibition of the transcription of central lipogenic genes, such as SREBP-1c and PGC-1 β [22]. However, whether nutrients regulate p38 MAPK activation, thereby regulating intermediary metabolism remains largely unknown.

Rainbow trout, like domestic cat and dolphin, exhibits relatively high dietary protein/AAs requirement, poor utilization of dietary carbohydrates and glucose intolerant phenotype, thus it represents a relevant carnivorous model organism for the investigation of protein and glucose metabolism [23, 24]. Furthermore, given that healthy carnivores exhibit metabolic changes after fasting or a carbohydrate rich diet that are similar to those observed in humans with diabetes, trout can be used as a comparative natural model for the study of common pathologies associated with type 2 diabetes [23]. Therefore, understanding how nutrients regulate intermediary metabolism in trout may have dual significance.

Despite the well-studied roles of protein/AAs in modulating insulin action and intermediary metabolism in mammals, relatively little is known in trout. Diets of rainbow trout generally contain more than 40% protein, which has long been suspected as a causal factor for the persistent hyperglycemia and absence of postprandial down-regulation of

gluconeogenesis [25, 26]. In line with this hypothesis, Kirchner et al. [26] observed that low protein intake reduced hepatic gluconeogenic enzyme activities and gene expression (FBPase and G6Pase). Furthermore, in a previous study, we also demonstrated that AAs increased gluconeogenic mRNA levels (G6Pase and mPEPCK) independently of the insulin/TOR signaling pathway [27]. However, whether excessive AAs elevate hepatic gluconeogenesis via affecting insulin signaling has not been investigated in fish thus far. As for the regulation of lipogenesis, previous *in vivo* studies tend to indicate that dietary protein play a potent role in regulating mTOR signaling pathway [28], and lipogenic and glycolytic pathways in fish [28-30]. Moreover, our *in vitro* studies demonstrated that AAs (particularly leucine) together with insulin successfully stimulated the activation of mTOR signaling pathway [27, 31], which was proven as an upstream activator for hepatic lipogenesis in trout liver and hepatocytes [27, 32]. However, whether different AA levels affect mTORC1 activation and thereby regulating hepatic lipogenesis in fish is still unknown.

The aim of this work was to (1) study whether excessive amino acids (AAs) affect the regulation of hepatic metabolism-related gene expression and (2) determine the potential involvement of mTORC1 and p38 MAPK pathways in these regulations.

Material and Methods

Animals

Sexually immature rainbow trout (240 to 300g) 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 two days with a commercial diet (T-3P classic, Trouw, France). The experiments were carried out in accordance with the clear boundaries of EU legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e. Directive 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decree no. 2001-464, 29 May, 2001). The investigators carrying out the experiment had "level 1" or "level 2" certification, bestowed by the Direction Départementale des Services Vétérinaires (French veterinary services) to carry out animal experiments (INRA 2002-36, 14 April, 2002).

Hepatocyte Cell Culture

Isolated liver cells were prepared as previously described [25, 27]. Cells were finally taken up in modified Hanks' medium supplemented with 1.5 mM CaCl₂, 1% defatted BSA, 3 mM glucose, MEM essential amino acids (1X) (Invitrogen Carlsbad, California, USA), MEM non-essential amino acids (1X) (Invitrogen Carlsbad, California, USA) and antibiotic antimycotic solution (1X) (Sigma, St. Louis, MO, USA) as the basic culture medium. 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 3×10⁶ cells/ well and incubated at 18°C. The culture medium was changed 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 [33, 34]. For the first experiment involving different amino acid levels, 48 h-cultured cells were stimulated with the mediums containing 4×10⁻⁹ mol l⁻¹ of bovine insulin (Sigma, St. Louis, MO, USA) corresponding to the post-prandial level of insulin irrespective of dietary carbohydrate level [35], and one fold (I*1AA), two fold (I*2AA), or four fold concentrated MEM essential and non-essential amino acids mixture (I*4AA), respectively, while the control cells maintained the basic culture medium (1AA but no insulin). In the second experiment, 48 h-cultured hepatocytes were pre-incubated for 30 min with or without 100 nM rapamycin (Rapa), a pharmacological TOR inhibitor. The mediums were then replaced by the medium containing 4×10⁻⁹ mol l⁻¹ insulin and four fold concentrated MEM essential and non-essential amino acids mixture (I*4AA). Cells were harvested at 45 min for western blot analysis or resuspended in TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) 24 h after stimulation and stored at -80°C for subsequent mRNA extraction. The time intervals were chosen based on the phosphorylation peaks of TOR signaling pathway or relevant metabolic gene expression peaks, respectively (data not shown).

Protein extraction and Western blotting

At the end of the stimulation period, cells were carefully washed with two times 1ml of cold phosphate buffered saline (PBS). Then 300µl of cell lysis buffer [36] were 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 12,000g for 30 min at 4°C. The resulting supernatant fractions were recovered and stored at -80°C. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with BSA as standard. Lysates (2 µg of total protein per lane for Akt/S6/4EBP1, 7 µg for TOR/S6K1/p38 MAPK and 17 µg for IRS-1, respectively) were subjected to SDS-PAGE and Western blotting using the appropriate antibodies. Anti-phospho-Akt (Ser⁴⁷³) (no. 9271), anti-phospho-Akt (Thr³⁰⁸) (no. 9275), anti-carboxyl terminal Akt (no. 9272), anti-phospho-TOR (Ser²⁴⁴⁸) (no. 2971), anti-TOR (no. 2972), anti-phospho-S6 protein kinase 1 (Thr³⁸⁹) (no. 9205), anti-S6 protein kinase 1 (no. 9202), anti-phospho-S6 (Ser^{235/236}) (no. 4856), anti-S6 (no. 2217), anti-phospho-4E-BP1 (Thr^{37/46}) (no. 9459), anti-4E-BP1 (no. 9452), anti-phospho-IRS-1 (Ser³⁰²) (no. 2384), anti-phospho-p38 MAPK (no. 9211) and anti-β-Tubulin (no. 2146) were purchased from Cell Signaling Technologies (Ozyme, Saint Quentin Yvelines, France). Anti-p38 MAPK (no. sc-535) was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). All of these antibodies (except anti-phospho-IRS-1 (Ser³⁰²), anti-phospho-p38 MAPK and anti-p38 MAPK) were successfully cross-reacted with rainbow trout [32]. For anti-phospho-IRS-1 (Ser³⁰²), anti-phospho-p38 MAPK and anti-p38 MAPK antibody, the molecular weight and amino acid sequences were monitored in the SIGENAE database [37] to check for a good conservation of the antigen sequence. After washing, membranes were incubated with an IRDye Infrared secondary antibody (LI-COR Biosciences, Lincoln, NE, USA). Bands were visualized by Infrared Fluorescence using the Odyssey Imaging System (LI-COR Biosciences) and quantified by Odyssey Infrared imaging system software (Version 3.0, LI-COR Biosciences).

Gene expression analysis

Total RNA samples were extracted from hepatocytes using TRIzol[®] Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations, quantified by spectrophotometry (absorbance at 260 nm) and its integrity was assessed using Agarose Gel Electrophoresis. A 1 µg sample of the resulting total RNA was reverse transcribed into cDNA using the SuperScript[™] III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and random primers (Promega, Charbonnières, France) according to the manufacturers' instructions. Target gene expression abundance was determined by quantitative real-time (q) RT-PCR, using specific primers [38-42]. Primers targeting alanine transaminase 2 (forward: TGGGTGCGTACAGTGCCAGT; reverse: GACGCACCTCACACACAC; Sigenae AU081029.s.om.10), aspartate transaminase 1 (forward: TCAAGAGTGCCAGGAACATCA; reverse: AGCGTCTCTGAAGATGGGTGT; Sigenae CA359859.s.om.10), aspartate transaminase 2 (forward: TCTGTGCCAGTCCCTTCTC; reverse: GGAGGGTTGGACCAGGT; Sigenae CA344854.s.om.10.) and branched-chain α-ketoacid dehydrogenase α subunit (forward: TCGCTGTGTACAACGCTACC; reverse: GCCTCCATCACCATCTTGC) were newly designed using Primer3 software. To confirm specificity, amplicons were purified and sequenced (Beckman Coulter Genomics, UK).

qRT-PCR was performed as previously described [36]. Quantitative RT-PCR analyses were focused on several key enzymes of hepatic metabolism, which were glucokinase (GK; EC 2.7.1.2), 6-phosphofructo-1-kinase (6PF1K; EC 2.7.1.11), pyruvate kinase (PK; EC 2.7.1.40) and transcription factor hypoxia-inducible factor 1-α (HIF-1α) for glycolysis; glucose transporter 2 (GLUT2) for glucose transport; glucose-6-phosphatase (G6Pase; EC 3.1.3.9) for glucose dephosphorylation; fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) and phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) for gluconeogenesis; ATP citrate lyase (ACLY; EC 2.3.3.8) and fatty acid synthase (FAS; EC 2.3.1.85) for lipogenesis; glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) for pentose phosphate shunt; Δ6 fatty acyl desaturase (D6D; EC 1.14.19.3), Stearoyl-CoA desaturase-1 (SCD1 or D9D; EC 1.14.19.1), elongation of very long chain fatty acids like-5 (Elovl5; EC 2.3.1.199) and elongation of very long chain fatty acids like-2 (Elovl2; EC 2.3.1.199) for fatty acid bioconversion, and two relevant transcription factors sterol regulatory element binding protein 1-like (SREBP1c) and liver X receptor (LXR); serine dehydratase (SD; EC:4.3.1.17), alanine transaminase (ALAT; EC 2.6.1.2), aspartate transaminase (ASAT; EC 2.6.1.1), branched-chain α-ketoacid dehydrogenase (BCKD; EC 1.2.4.4) and branched-chain α-ketoacid dehydrogenase kinase (BCKDK; EC 2.7.11.4) for amino acid catabolism. When different isoforms of a gene were known in rainbow trout (as for G6Pase), gene expression analysis was performed on each isoform. Elongation factor-1α (EF1α) was employed as

a non-regulated reference gene, as previously used in rainbow trout and it was stably expressed in our investigations (data not shown).

Statistical analysis

The results of western blot ($N=4$) and gene expression ($N=6$) are expressed as means + s.e.m. and were analyzed using one-way ANOVA, followed by a Tukey test. In cases where data were nonparametric or not homoscedastic, data transformations, such as logarithms, square roots and reciprocals, were used to meet ANOVA criteria. Normality was assessed using the Shapiro–Wilk test, while homoscedasticity was determined using Levene’s test. For all statistical analyses, the level of significance was set at $P<0.05$.

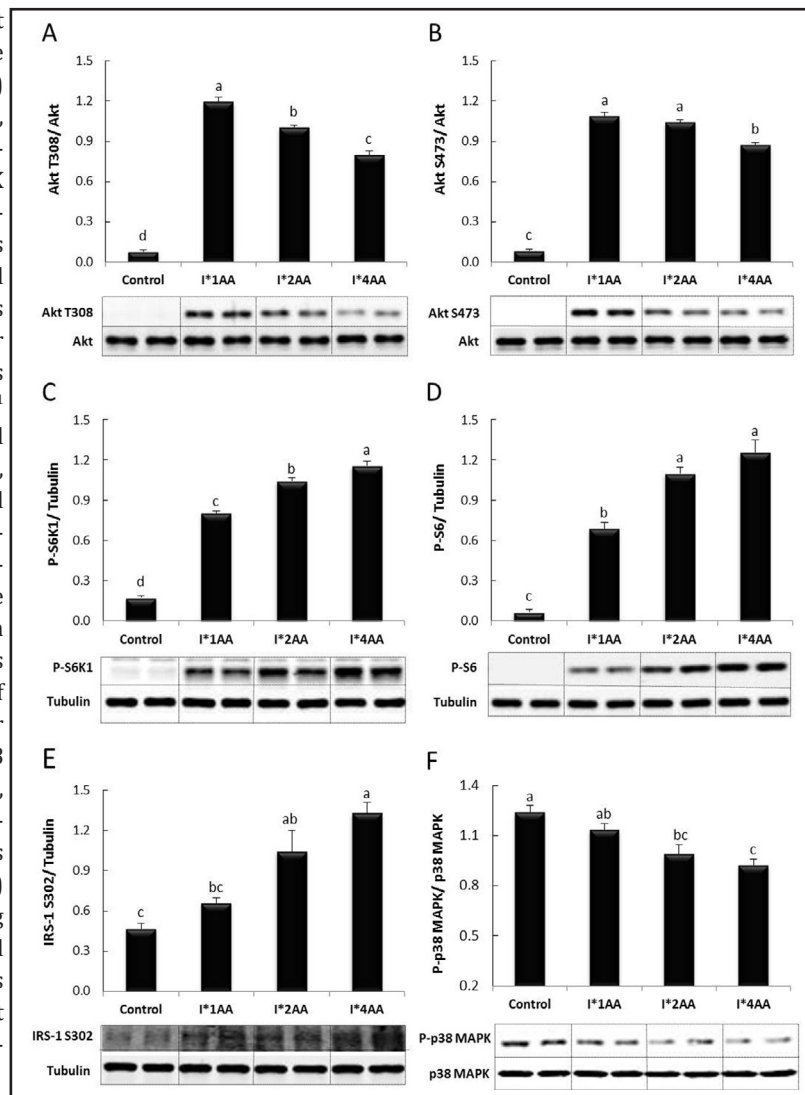
Results

Amino acid levels affected the phosphorylation of Akt/TOR signaling pathway

First, we investigated whether the phosphorylation of Akt/TOR signaling pathway was affected by different amino acid levels (Fig. 1).

As expected, I*1AA treated cells showed higher Akt (on Thr³⁰⁸ and Ser⁴⁷³), S6K1 and S6 phosphorylation levels compared to control cells, indicating that insulin effectively triggered

Fig. 1. Effects of different amino acid levels on the phosphorylation of (A) Akt Ser⁴⁷³, (B) Akt Thr³⁰⁸, (C) S6K1, (D) S6, (E) IRS-1 Ser³⁰² and (F) p38 MAPK protein in trout hepatocytes. The experiment was conducted in 48 h-cultured trout hepatocytes. Cells were then stimulated for 45 min with the mediums containing 4×10^{-9} mol l⁻¹ insulin (I), and one fold (I*1AA), two fold (I*2AA), or four fold concentrated amino acids (I*4AA), respectively, while the control cells maintained the basic culture medium (1AA but no insulin). Gels were loaded with 2 µg of total protein per lane for Akt/S6, 7 µg for S6K1/p38 MAPK and 17 µg for IRS-1, respectively. A representative blot is shown. Results are means + s.e.m. ($N=4$) and were analyzed using one-way ANOVA, followed by a Tukey test. Values with different superscript letters are significantly different at $P<0.05$.



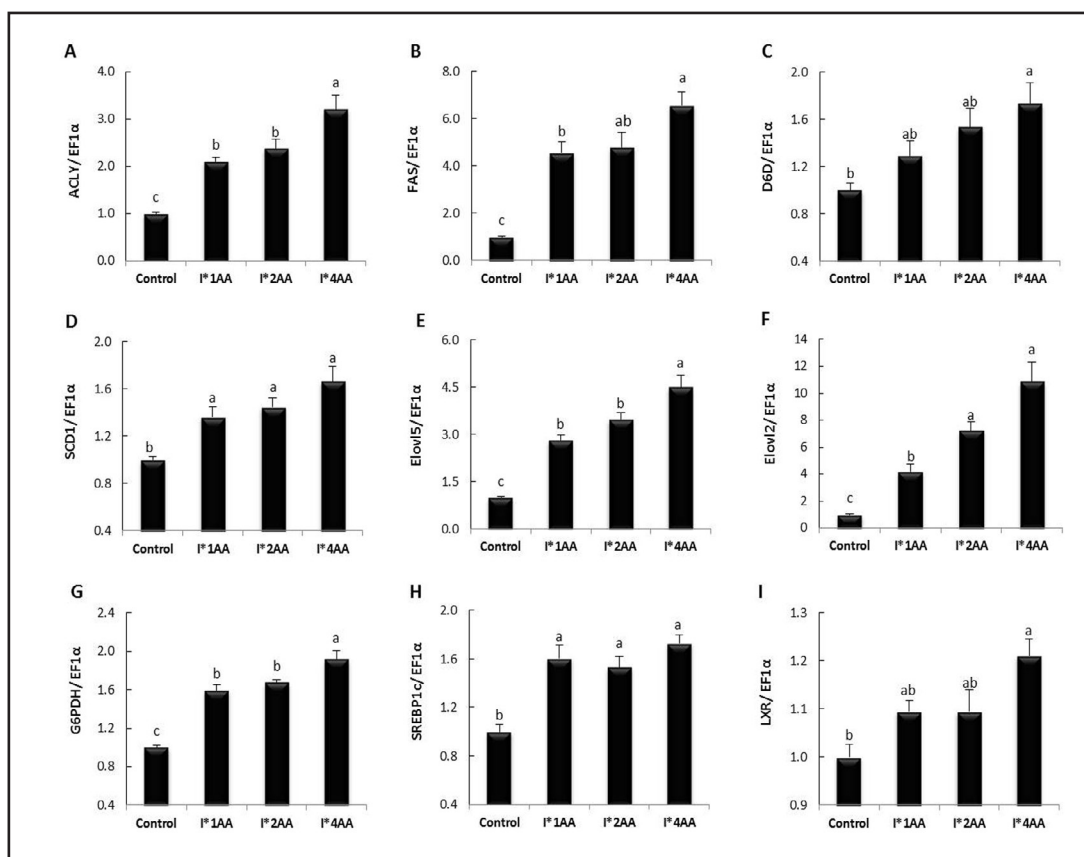


Fig. 2. Effects of different amino acid levels on the regulation of fatty acid biosynthetic gene expression in trout hepatocytes. mRNA levels of genes involved in *de novo* lipogenesis including (A) ACLY and (B) FAS, fatty acid bioconversion including (C) D6D, (D) SCD1, (E) Elovl5 and (F) Elovl2, NADPH production including (G) G6PDH, and relevant transcriptional factors (H) SREBP1c and (I) LXR in the primary hepatocytes of rainbow trout after 24 h stimulation. The experiment was conducted in 48 h-cultured trout hepatocytes. Cells were then stimulated with the mediums containing 4×10^{-9} mol l $^{-1}$ insulin (I), and one fold (I*1AA), two fold (I*2AA), or four fold concentrated amino acids (I*4AA), respectively, while the control cells maintained the basic culture medium (1AA but no insulin). Expression values are first normalized with elongation factor-1 alpha (EF1 α)-expressed transcripts then further normalized to the levels of control hepatocytes. Results are means + s.e.m. (N=6) and were analyzed using one-way ANOVA, followed by a Tukey test. Values with different superscript letters are significantly different at P<0.05.

the activation of Akt/TOR signaling pathway as previously demonstrated [27]. Of note, the phosphorylation of IRS-1 on Ser302 and p38 MAPK was not affected by the addition of insulin in 1AA treated cells.

Increased amino acid levels gradually enhanced S6K1 phosphorylation (Fig. 1C). Furthermore, phosphorylated S6 in I*2AA and I*4AA treated cells was also higher compared to I*1AA treated cells (Fig. 1D). In contrast, increased amino acid levels gradually decreased Akt phosphorylation on Thr³⁰⁸ (Fig. 1A) and the phosphorylation of Akt on Ser⁴⁷³ in I*4AA treated cells was significantly lower than in I*1AA or I*2AA treated cells (Fig. 1B). Because the phosphorylation of IRS-1 on serine residues is known as a potential link between TOR/S6K1 and Akt protein phosphorylation in mammals, we further studied the phosphorylation of IRS-1 on Ser³⁰² and found that phosphorylated IRS-1 on Ser³⁰² in I*4AA treated cells was significantly higher than in I*1AA treated cells (Fig. 1E). Moreover, the phosphorylation of p38 MAPK in I*4AA treated cells was markedly lower compared to I*1AA treated cells (Fig. 1F).

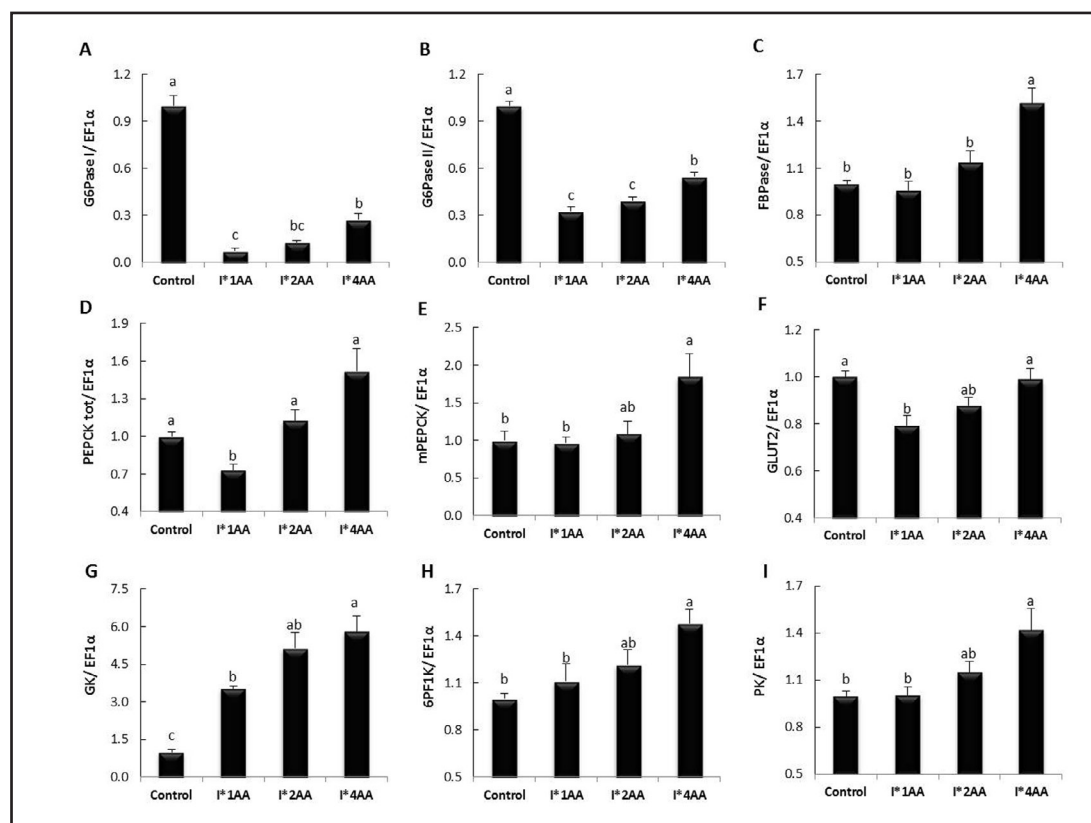


Fig. 3. Effects of different amino acid levels on the regulation of glucose metabolism-related gene expression in trout hepatocytes. mRNA levels of genes involved in gluconeogenesis including (A) G6Pase I, (B) G6Pase II, (C) FBPase, (D) PEPCKtot and (E) mPEPCK, glucose transport including (F) GLUT2, and glycolysis including (G) GK, (H) 6PF1K and (I) PK in the primary hepatocytes of rainbow trout after 24 h stimulation. The experiment was conducted in 48 h-cultured trout hepatocytes. Cells were then stimulated with the mediums containing 4×10^{-9} mol l⁻¹ insulin (I), and one fold (I*1AA), two fold (I*2AA), or four fold concentrated amino acids (I*4AA), respectively, while the control cells maintained the basic culture medium (1AA but no insulin). Expression values are first normalized with elongation factor-1 alpha (EF1α)-expressed transcripts then further normalized to the levels of control hepatocytes. Results are means + s.e.m. (N=6) and were analyzed using one-way ANOVA, followed by a Tukey test. Values with different superscript letters are significantly different at $P < 0.05$.

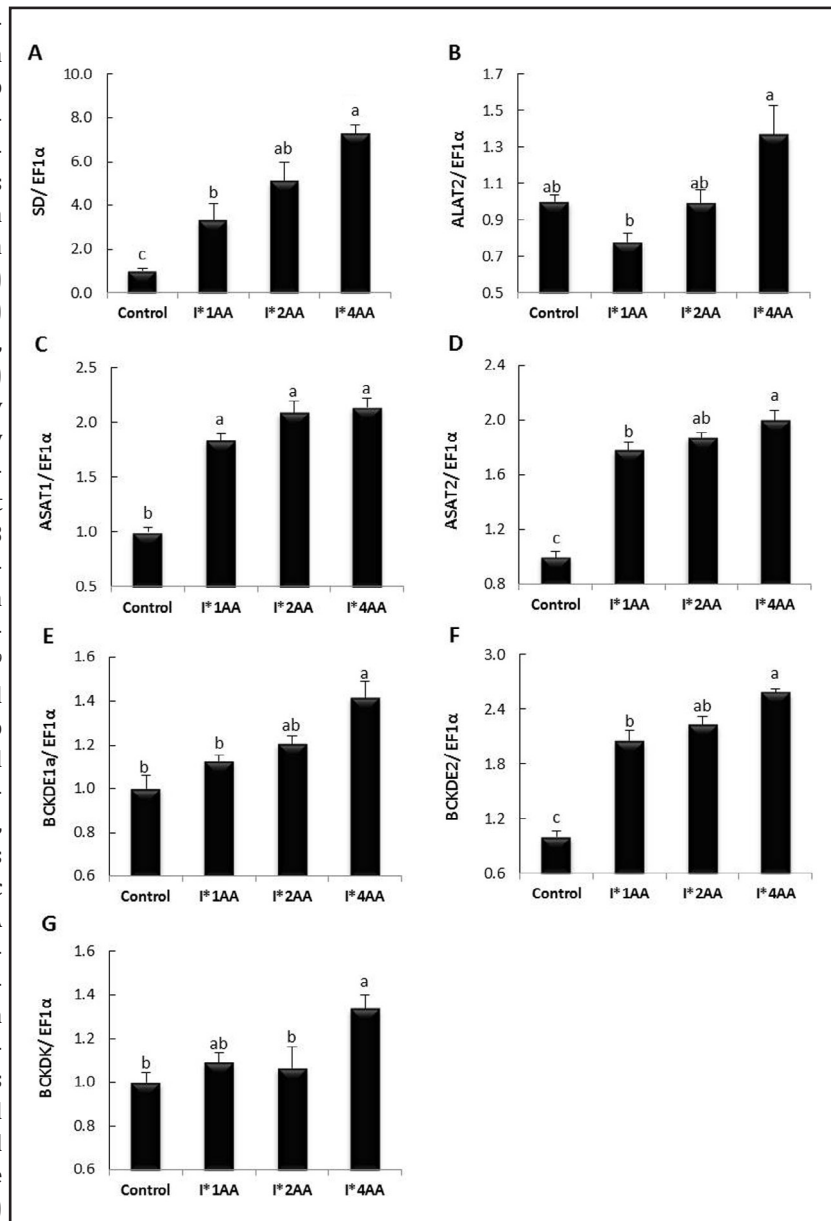
Fatty acid biosynthetic gene expression was up-regulated by high level of AAs

We further investigated the effects of different amino acid levels on the regulation of fatty acid biosynthetic gene expression in trout hepatocytes (Fig. 2).

Except D6D (Fig. 2C) and LXR (Fig. 2I), all the examined mRNA levels of the key enzymes involved in *de novo* lipid synthesis including ACLY, FAS, SCD1, Elovl5, Elovl2 and G6PDH, and SREBP1c, a transcription factor that activates *de novo* lipogenesis, were all significantly up-regulated in I*1AA treated cells compared to control cells, indicating that insulin significantly up-regulated fatty acid biosynthetic gene expression in trout hepatocytes, which is consistent with previous demonstrations in trout [27, 43] and mammals [44].

Moreover, mRNA levels of the key enzymes involved in *de novo* lipogenesis, including ACLY (Fig. 2A) and FAS (Fig. 2B), fatty acid bioconversion including Elovl5 (Fig. 2E) and Elovl2 (Fig. 2F), and NADPH production including G6PDH (Fig. 2G) were all significantly higher in I*4AA treated cells than in I*1AA treated cells. Furthermore, similar enhancements were also observed between I*2AA and I*4AA treated cells for the mRNA levels of ACLY (Fig. 2A), Elovl5 (Fig. 2E) and G6PDH (Fig. 2G). Of note, despite no significant difference was observed

Fig. 4. Effects of different amino acid levels on the regulation of amino acid catabolic gene expression in trout hepatocytes. mRNA levels of genes involved in amino acid catabolism including (A) SD, (B) ALAT2, (C) ASAT1, (D) ASAT2, (E) BCKDE1 α , (F) BCKDE2 and (G) BCKDK in the primary hepatocytes of rainbow trout after 24 h stimulation. The experiment was conducted in 48 h-cultured trout hepatocytes. Cells were then stimulated with the mediums containing 4×10^{-9} mol l $^{-1}$ insulin (I), and one fold (I*1AA), two fold (I*2AA), or four fold concentrated amino acids (I*4AA), respectively, while the control cells maintained the basic culture medium (1AA but no insulin). Expression values are first normalized with elongation factor-1 alpha (EF1 α)-expressed transcripts then further normalized to the levels of control hepatocytes. Results are means + s.e.m. (N=6)



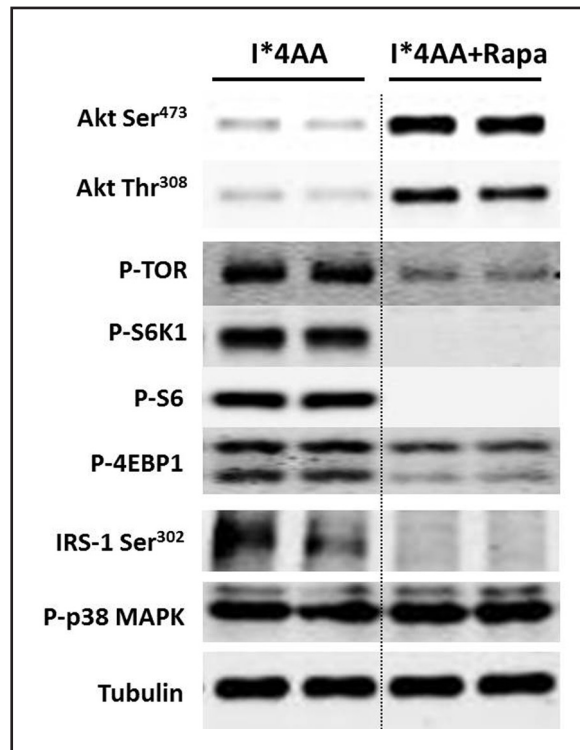
and were analyzed using one-way ANOVA, followed by a Tukey test. Values with different superscript letters are significantly different at $P < 0.05$.

for the mRNA levels of D6D (Fig. 2C), SCD1 (Fig. 2D) or LXR (Fig. 2I), they all exhibited similar patterns as the other examined fatty acid biosynthetic genes, which were positively regulated by increased amino acid levels.

High level of AAs up-regulated gluconeogenic and glycolytic gene expression

We also investigated the effects of different amino acid levels on the regulation of gluconeogenic and glycolytic gene expression in trout hepatocytes (Fig. 3). The addition of insulin significantly decreased the mRNA levels of G6Pase I (Fig. 3A), G6Pase II (Fig. 3B), PEPCKtot (Fig. 3D) and GLUT2 (Fig. 3F), and significantly increased GK mRNA level (Fig. 3G), while no significant variations was observed for the mRNA levels of FBPase (Fig. 3C), mPEPCK (Fig. 3E), 6PF1K (Fig. 3H) and PK (Fig. 3I). Messenger RNA levels of the key enzymes involved in gluconeogenesis (G6Pase I, G6Pase II, FBPase, PEPCKtot and mPEPCK),

Fig. 5. Effects of mTORC1 overactivation (I*4AA) and inhibition (I*4AA+Rapa) on the phosphorylation of Akt Ser⁴⁷³, Akt Thr³⁰⁸, TOR, S6K1, S6, 4E-BP1, IRS-1 Ser³⁰² and p38 MAPK protein in trout hepatocytes. Cells were pre-incubated for 30 min with or without 100 nM rapamycin (Rapa), a TOR inhibitor. The culture medium was then replaced for 45 min by the medium containing 4×10^{-9} mol l⁻¹ insulin (I) and four fold concentrated amino acids (I*4AA). Gels were loaded with 2 µg of total protein per lane for Akt/S6/4EBP1, 7 µg for TOR/S6K1/p38 MAPK and 17 µg for IRS-1, respectively. Western blots were performed on four individual samples and similar results were obtained. This figure includes representative blots.



glucose transporter (GLUT2), and glycolysis (GK, 6PF1K and PK) were all significantly higher in I*4AA treated cells than in I*1AA treated cells, indicating that high level AAs up-regulated gluconeogenic and glycolytic gene expression.

High level of AAs up-regulated AA catabolic gene expression

As shown in Fig.4, the addition of insulin upregulated the mRNA levels of SD (Fig. 4A), ASAT1 (Fig. 4C), ASAT2 (Fig. 4D) and BCKDE2 (Fig. 4F), while no changes for the mRNA levels of ALAT2 (Fig. 4B), BCKDE1α (Fig. 4E) and BCKDK (Fig. 4G). Despite no modulation for ASAT1, messenger RNA levels of SD, ALAT2, ASAT2, BCKDE1α and BCKDE2 were all significantly higher in I*4AA treated cells compared to I*1AA treated cells, and BCKDK increased in I*4AA treated cells compared to I*2AA treated cells, indicating that high level AAs up-regulated AA catabolic gene expression.

Excessive AAs decreased Akt phosphorylation through mTORC1-dependent manner

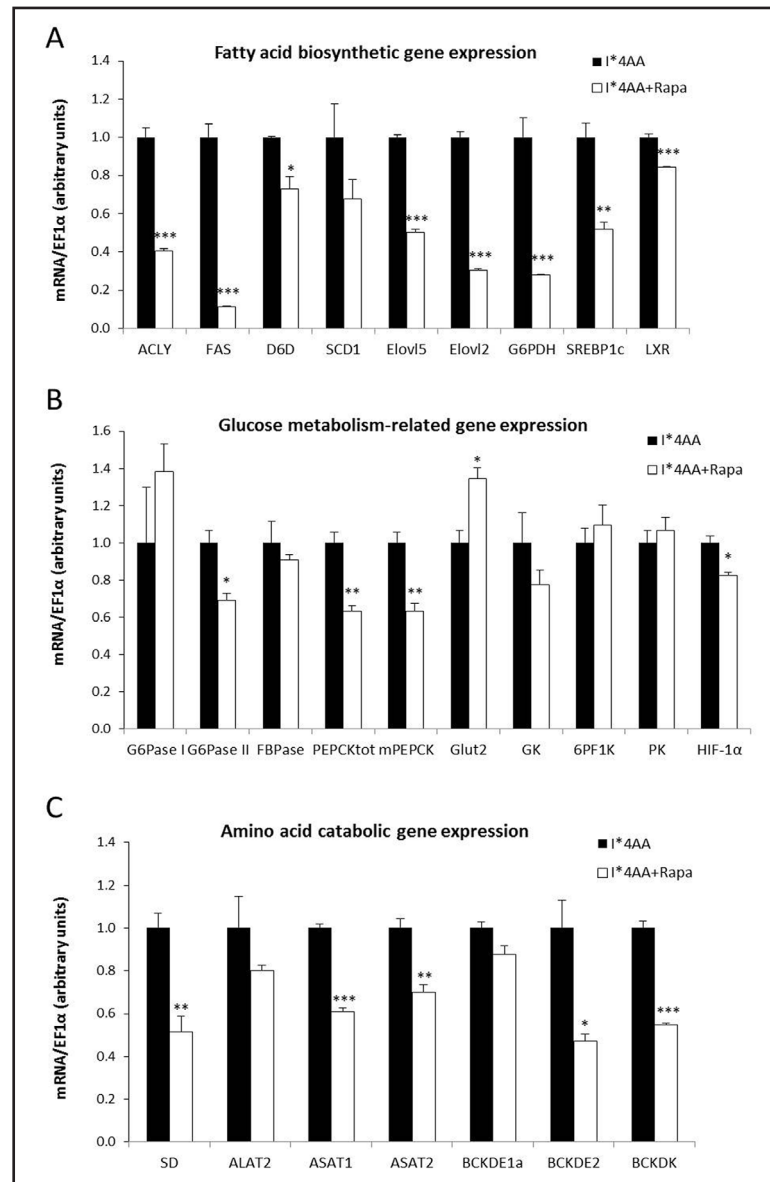
In order to investigate whether excessive AAs reduced Akt phosphorylation through mTORC1/S6K1-induced negative feedback loop involving the phosphorylation of IRS-1 on serine residues, we further treated trout hepatocytes with I*4AA in the presence or absence of rapamycin.

As expected, rapamycin partially inhibited the phosphorylation of TOR and 4E-BP1 and totally abolished the phosphorylation of S6K1 and S6 as previously observed [27, 32]. More importantly, we observed that rapamycin significantly inhibited the phosphorylation of IRS-1 on Ser³⁰² and strongly increased the Akt phosphorylation (on Thr³⁰⁸ and Ser⁴⁷³) (Fig. 5). p38 phosphorylation was not affected by rapamycin, indicating that the phosphorylation of p38 MAPK is mTORC1-independent, which is compatible with previous demonstration [45].

Inhibition of mTORC1 activation resulted in suppressed fatty acid biosynthetic and gluconeogenic gene expression

We further examined the effects of mTORC1 overactivation and inhibition on the regulation of fatty acid biosynthetic, gluconeogenic, glycolytic and AA catabolic gene expression in trout hepatocytes. Messenger RNA levels of the key enzymes involved in *de*

Fig. 6. Effects of mTORC1 overactivation (I*4AA) and inhibition (I*4AA+Rapa) on the regulation of fatty acid biosynthetic, gluconeogenic and glycolytic gene expression in trout hepatocytes. (A) mRNA levels of genes involved in *de novo* lipid synthesis including ACLY, FAS, D6D, SCD1, Elovl5, Elovl2, G6PDH, SREBP1c and LXR; (B) mRNA levels of genes involved in gluconeogenesis including G6Pase I, G6Pase II, FBPase, PEPCKtot and mPEPCK, glucose transport GLUT2, and glycolysis including GK, 6PF1K, PK and HIF1- α ; (C) mRNA levels of genes involved in amino acid catabolism including SD, ALAT2, ASAT1, ASAT2, BCKDE1 α , BCKDE2 and BCKDK, in the primary hepatocytes of rainbow trout after 24 h stimulation. Cells were pre-incubated for 30 min with or without 100 nM rapamycin (Rapa), then stimulated with the medium containing 4×10^{-9} mol l⁻¹ insulin (I) and four fold concentrated amino acids (I*4AA) for 24 h. Expression values are first normalized with elongation factor-1 alpha (EF1 α)-expressed transcripts then further normalized to the levels of I*4AA hepatocytes (N=6). "*" indicates significant difference at P<0.05, "**" indicates P<0.01, "***" indicates P<0.001.



de novo lipid synthesis (ACLY, FAS, D6D, Elovl5, Elovl2 and G6PDH), gluconeogenesis (G6Pase II, PEPCKtot and mPEPCK) and amino acid catabolism (SD, ASAT1, ASAT2, BCKDE2 and BCKDK) were all significantly reduced by rapamycin (Fig. 6A, 6B and 6C). In addition, three transcription factors, SREBP1c and LXR for activating *de novo* lipid synthesis, and HIF-1 α for regulating glycolysis, were significantly reduced by rapamycin (Fig. 6A and 6B). Messenger RNA levels of SCD1, G6Pase I, FBPase, GK, 6PF1K, PK, ALAT2 and BCKDE1 α were not significantly affected by rapamycin.

Discussion

Rainbow trout, like the other carnivorous animals consumes diets with relatively high protein content compared to humans, rodents and other terrestrial domestic animals

[23, 46]. Presumably, ingested protein/AAs not only regulate protein metabolism, but also modulate glucose and lipid metabolism. The current studies were designed to evaluate the effects of different AA levels on the regulation of hepatic fatty acid biosynthetic, glucose metabolism-related and amino acid catabolic gene expression, and also to assess the potential involvements of Akt/mTORC1 and p38 MAPK signaling pathway in these regulation processes using freshly isolated trout hepatocytes. We demonstrated that high levels of AAs up-regulate hepatic fatty acid biosynthetic gene expression through an mTORC1-dependent manner, while attenuating insulin-mediated down-regulation of gluconeogenesis through elevated IRS-1 phosphorylation on Ser³⁰², which in turn impairs the activation of Akt pathway and insulin action.

AAs modulated insulin signaling and gluconeogenesis

Our first effort revealed that high levels of AAs attenuated insulin/Akt signaling through mTORC1/S6K1-induced feedback loop in rainbow trout hepatocytes. Increased AA levels not only elevated the phosphorylation of mTORC1 downstream effectors, S6K1 and S6, but also enhanced the phosphorylation of IRS-1 on Ser³⁰² and decreased the phosphorylation of Akt on both Ser⁴⁷³ and Thr³⁰⁸. Given that IRS-1 Ser³⁰² phosphorylation is known as a direct target of mTORC1 and S6K1 in mammals [14, 47], which disrupts the ability of the phosphotyrosine-binding domain to interact with activated insulin receptor, thereby decreasing insulin signaling [48], we first inferred that high levels of AAs enhanced IRS-1 Ser³⁰² phosphorylation and repressed Akt phosphorylation through mTORC1/S6K1-induced feedback loop. By using rapamycin, we further demonstrated that mTORC1 inhibition blunted IRS-1 Ser³⁰² phosphorylation and restored Akt phosphorylation in excessive (four fold concentrated) AAs-treated hepatocytes, confirming the existence of mTORC1/S6K1-operated negative feedback loop decreasing insulin sensitivity under AAs excess conditions in trout. Therefore, we indeed demonstrated that increased AA levels promoted mTORC1/S6K1 activation, which in turn operated a negative feedback loop toward Akt signaling pathway through, at least in part, enhanced IRS-1 phosphorylation on Ser³⁰². This demonstration is in agreement with previous *in vitro* [49-51] and *in vivo* [52, 53] mammalian investigations.

An intriguing finding of the present study is that high levels of AAs elevated hepatic gluconeogenic gene expression through attenuating insulin signaling. Consistent with previous demonstrations in trout [25, 27], insulin effectively suppressed the gene expression of two key gluconeogenic enzymes, G6Pase and PEPCK. However, increased AA levels attenuated these suppressions and increased the gene expression of FBPase, suggesting that insulin-mediated down-regulation of hepatic gluconeogenesis was attenuated by increased AA sufficiency. This finding is in agreement with previous *in vivo* findings in trout and sea bass, which reported that higher hepatic gluconeogenic enzyme activities and/or mRNA levels were associated with high dietary protein diet/intake compared to low protein diet/intake [26, 54-58]. Furthermore, the negative relation between Akt phosphorylation and gene expression of G6Pase and PEPCK was consistently observed in all the insulin-, different AA levels- and rapamycin-mediated modulations, indicating that hepatic gluconeogenesis was negatively controlled by Akt phosphorylation in trout hepatocytes, as in mammals [2, 59]. Therefore, we concluded that high levels of AAs attenuated insulin-mediated down-regulation of hepatic gluconeogenic gene expression through mTORC1/S6K1-induced feedback loop toward Akt pathway. Similar inhibitory effects on insulin action as a consequence of increased AA supply have also been reported in mammalian hepatocytes [50, 60], muscle cells [49] and adipocytes [51, 61, 62], as well as *in vivo* studies [53, 63, 64]. The present work together with previous study [27] provides strong evidences that increased AA sufficiency up-regulates hepatic gluconeogenesis through insulin/TOR-independent manner(s) and as well as impairing insulin action. However further studies are needed to test whether AAs (particularly glutamine) also promote glucose production through stimulating hexosamine biosynthetic pathway/O-GlcNAc signaling [65]. Furthermore, considering that persistent activation of mTORC1 and S6K1 may promote insulin resistance and glucose intolerance [13, 15], our finding, that high levels of AAs up-regulated hepatic

gluconeogenic gene expression through attenuating insulin signaling, indeed highlighted the potential role of AAs as an inducer for insulin resistance and impaired glucose homeostasis in carnivorous species such as salmonids. However, it is noteworthy that AAs elevation *in vivo* can also stimulate higher insulin secretion [66, 67], which may counteract the effects of AAs on glucose production [53]. Thus, whether high protein diet/intake modulates insulin action and glucose production *in vivo* still needs to be investigated in fish.

AAs regulated fatty acid biosynthesis and glycolysis

Impaired insulin signaling by high levels of AAs partially lost its ability to inhibit hepatic gluconeogenesis, yet it retained its ability to promote fatty acid biosynthesis. mTORC1 inhibition by rapamycin markedly suppressed the expression of genes involved in *de novo* lipogenesis (ACLY and FAS), fatty acid bioconversion (D6D, Elovl5 and Elovl2), NADPH production (G6PDH), and transcriptional factors SREBP1c and LXR, confirming that mTORC1 signaling is essential to stimulate specific metabolic pathways, including *de novo* lipogenesis, fatty acid bioconversion and the oxidative arm of the pentose phosphate pathway (PPP), which is consistent with previous demonstration in mammals [68]. Furthermore, we observed that enhanced gene expression of ACLY, FAS, Elovl5, Elovl2 and G6PDH by high levels of AAs, was also associated with enhanced mTORC1 activation. Therefore, we concluded that increased AA levels up-regulated fatty acid biosynthetic gene expression through an mTORC1-dependent manner. This conclusion is consistent with previous findings in trout [27, 32, 39], which showed that mTOR activation is positively related to elevated lipogenic mRNA levels and/or enzyme activities. The results are also consistent with the findings in mammals [69, 70] and in fish [28-30], showing that high protein diets induced higher fatty acid synthetic gene expression and/or enzyme activities compared to low protein diets. In contrast, all of these results differ from the *in vitro* findings of Allee et al. [71] and Rosebrough et al. [72], who observed that lipogenesis and malic enzyme activity were inversely related to dietary protein levels. Part of these discrepancies may be ascribed to species/tissue specificity or varied protein levels. mTORC1 activation regulates fatty acid biosynthetic gene expression probably through the transcription factors, SREBP1c and LXR [6]. Whereas insulin and amino acids effectively enhanced the expression of SREBP1c and LXR, we failed to demonstrate their AAs-induced changes at the transcriptional level. Given that both SREBP1c and LXR can be regulated transcriptionally and posttranscriptionally [73, 74], we presume that in trout hepatocytes high levels of AAs contribute to the posttranscriptional regulation of SREBP1c and LXR, probably through posttranslational modifications including phosphorylation, ubiquitinylation, and acetylation.

Increased AA availability also elevated glycolytic gene expression. GK, also known as hexokinase IV, plays a pivotal role in blood glucose homeostasis by catalyzing the phosphorylation of glucose, providing metabolites for glycolysis, glycogenesis and pentose phosphate pathway in hepatocytes [75]. We observed that insulin significantly up-regulated GK mRNA level in trout hepatocytes, which is consistent with the previous demonstrations in fish [25, 27, 76] and mammals [77-79] but differs from other studies based on intraperitoneal administration of insulin in trout [80, 81]. GK and the other two glycolytic genes, 6PF1K and PK, were all up-regulated by increased AA levels. This result is in line with the foresaid elevated fatty acids biosynthetic gene expression, as elevated glycolysis can render more available substrates to support the enhanced fatty acid biosynthesis and increased NADPH-demanding. Given that glycolysis is known to be positively regulated by the transcription factors HIF-1 α and SREBP1c [68, 82], and both HIF-1 α and SREBP1c mRNA levels were inhibited by rapamycin in the current study, we thus suggest that high levels of AAs up-regulated hepatic glycolysis through an mTORC1-dependent manner as well. This deduction is consistent with the observation of Lansard et al. [27], who observed that insulin combined with AAs activated TOR signaling pathway and up-regulated the expression of GK, 6PF1K and PK. As for GLUT2 gene expression, despite it was significantly decreased by insulin and up-regulated by rapamycin and elevated AAs, all the changes were quite minor, confirming that GLUT2, which has low affinity for glucose, is poorly regulated by metabolic factors [83].

AAs modulated AA catabolism and p38 MAPK phosphorylation

AAs are not only signaling molecules, but are also glucose and fatty acids precursors, which can supply carbon skeleton for hepatic gluconeogenesis and lipogenesis via AA catabolism [84]. As expected, increased AA levels markedly elevated the expression of genes related to AA catabolism (SD, ALAT2, ASAT2, BCKDE1 α , BCKDE2 and BCKDK), indicating that elevated AA levels promoted hepatic fatty acid biosynthesis and glucose metabolism not only through modulating relevant signaling pathways, but also serving as gluconeogenic and lipogenic substrates by promoting AA catabolism. The present study also demonstrated that several AA catabolic genes were controlled by the mTOR pathway. Further studies are needed to explore the mechanisms linking these signaling pathways to AA catabolic gene expression.

p38 MAPK phosphorylation was not affected by insulin in hepatocytes, which is consistent with previous demonstrations showing that insulin-mediated p38 MAPK activation mainly occur in adipocytes and skeletal muscle [85]. p38 phosphorylation was insensitive to rapamycin, indicating that high levels of AAs decreased p38 phosphorylation through an mTORC1-independent manner. The AA-induced decrease of p38 phosphorylation was associated with the up-regulation of hepatic lipogenic, G6PDH and gluconeogenic gene expression. These results are compatible with previous reports indicating that p38 MAPK negatively regulates hepatic lipogenic and G6PDH mRNA levels [86, 87]. However, they contrast with the other studies reporting that p38 plays a stimulatory role in hepatic gluconeogenic gene expression [88, 89]. Nevertheless, we uncovered a new player involved in AA signaling, which confirmed previous deductions by Casas-Terradellas et al. [90] and may provide new insights into the understanding of nutrients-mediated intermediary metabolism.

Conclusions and prospects

Taken together, we demonstrated that, in primary trout hepatocytes, increased AA levels could (i) up-regulate fatty acid biosynthetic gene expression through an mTORC1-dependent manner, (ii) attenuate insulin-mediated repression on hepatic gluconeogenesis through elevated IRS-1 phosphorylation on Ser³⁰², which in turn impairs the activation of Akt pathway and weakens insulin action, (iii) elevate AA catabolic gene expression, and (iiii) partly suppress p38 MAPK phosphorylation. The results that, under excessive AAs conditions, insulin partially lost its ability to suppress gluconeogenesis but continued to promote fatty acid biosynthetic gene expression, resemble the pathological features observed in the insulin-resistant state [91, 92], which further highlights the potential of carnivorous fish to be used as a natural model for the study of common mammalian pathologies. Future studies are needed to determine which AA truly contributes to mTORC1 activation, and gene expression regulation, and how dietary AA profiles may influence these regulations.

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

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