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Upstream regulators of apoptosis mediates methionine-induced changes of lipid metabolism

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Abstract

Although the role of methionine (Met), as precursor for L-carnitine synthesis, in the regulation of lipid metabolism has been explored. Met seems to have tissue- and species-specific regulatory effect on lipid metabolism, implying that the mechanisms in Met regulation of lipid metabolism is complex and may involve the upstream regulatory pathway of lipid metabolism. The present study was performed to determine the mechanism of apoptosis signaling pathways mediating Met-induced changes of hepatic lipid deposition and metabolism in fish, and compare the differences of the mechanisms between the fish and mammals. By iTRAQ-based quantitative proteome analyses, we found that both dietary Met deficiency and excess evoked apoptosis signaling pathways, increased hepatic lipid deposition and caused aberrant hepatic lipid metabolism of yellow catfish *Pelteobagrus fulvidraco*. Using primary hepatocytes from *P. fulvidraco*, inhibition of caspase by Z-VAD-FMK blocked the apoptotic signaling pathways with a concomitant reversal of Met deficiency- and excess-induced increase of lipid deposition, indicating that apoptosis involved the Met-mediated changes of hepatic lipid metabolism. Moreover, we explored the roles of three upstream apoptotic signaling pathways (PI3K/AKT-TOR pathway, cAMP/PKA/CREB pathway and LKB1/AMPK-FOXO pathway) influencing hepatic lipid metabolism of *P. fulvidraco*. The three upstream pathways participated in apoptosis mediating Met-induced changes of lipid metabolism in *P. fulvidraco*. At last, HepG2 cell line was used to compare the similarities of mechanisms in apoptosis mediating Met-induced changes of lipid metabolism between fish and mammals. Although several slight differences existed, apoptosis mediated the Met-induced changes of lipid metabolism between fish and mammals. The present study reveals novel

apoptosis-relevant signal transduction axis which mediates the Met-induced changes of lipid metabolism, which will help understand the mechanistic link between apoptosis and lipid metabolism, and highlight the importance of the evolutionary conservative apoptosis signaling axis in regulating Met-induced changes of hepatic lipid metabolism.

Keywords: Methionine; Apoptosis; Lipid deposition; Lipid metabolism; Signaling pathway

Abbreviations:

AKT, RAC-alpha serine/threonine-protein kinase; AMPK, AMP-activated kinase; APO, Apolipoprotein; cAMP, Cyclic AMP; CPT-1, Carnitine palmitoyl transferase-1; CREB, Cyclic AMP-responsive element-binding protein; FAS, Fatty acid synthase; FBW, Final mean body weight; FCR, Feed conversion rate; iTRAQ, isobaric tags for relative or absolute quantitation; FI, Feed intake; FOXO, Forkhead transcription factors; HSL, Hormone sensitive lipase; LKB1, Liver kinase B1; Met, Methionine; TOR, mammalian target of rapamycin; PI3K, Phosphatidylinositol-3 kinases; PKA, Protein kinase A; SGR, Specific growth rate; TG, Triglycerides; WG, Weight gain

Introduction

Methionine (Met), as a principal donor of the methyl group, is required for protein synthesis and plays important roles in numerous biological processes [1]. Recently, several studies have indicated the important role of Met in lipid deposition and metabolism [2,3]. For example, Met restriction (MR) reduced lipid accumulation, and increased de novo lipogenesis, lipolysis and fatty acid oxidation [4]. Met deficiency and excess also resulted in deregulation in lipid metabolic pathways [5]. Moreover, studies suggested that Met-mediated changes in lipid metabolism were tissues- [6,7], and species-dependent[4,6,8]. Thus, the mechanisms of Met regulating lipid metabolism may be complex and the upstream regulatory pathway of lipid metabolism may be involved in.

As a homeostatic mechanism to maintain cell populations in tissues, apoptosis is a highly organized and genetically controlled form of cell death. Several studies indicated that imbalanced Met could induce apoptosis both *in vivo* and *in vitro* [9,10]. Apoptosis is executed by two fundamental

pathways: the extrinsic pathway is mediated by death receptors on the cellular surface and the intrinsic pathway is organelle-based [11]. The extrinsic pathway is initiated by death receptors, including Fas, TNF receptor (R)1 and TNF-related apoptosis-inducing ligand (TRAIL) receptors. When engaged by their natural ligands, these receptors trigger intracellular cascades that activate death-inducing proteolytic enzymes, especially caspases [12]. In the intrinsic pathway, apoptosis can be initiated by several intracellular organelles. In hepatocytes, mitochondrial dysfunction plays a critical role by amplifying the apoptotic signals and integrating both pathways into the final common pathway which executes the apoptotic changes [13]. The mitochondrial events are regulated by the members of Bcl-2 family which include anti-apoptotic (Bcl-2, Bcl-xL) and pro-apoptotic (Bak, Bid, Bad and Bim) members [14]. Another important organelle in which proapoptotic signals may originate is the endoplasmic reticulum (ER) [15]. In addition, studies indicate that apoptosis is regulated by several upstream regulators, including PI3K/Akt-TOR pathway, cAMP/PKA/CREB pathway and LKB1/AMPK-FOXO pathway [16-18]. Accordingly, it was reasonable to conclude that Met induces the change of lipid metabolism via upstream regulators of apoptosis. However, at present, to our knowledge, although the potential link between apoptosis and lipid metabolism has been postulated [19], the mechanism of apoptosis mediating hepatic lipid metabolism still remains unknown. Our studies might provide the basis to clarify the regulatory mechanism of Met for lipid metabolism.

Lipids act as a major energy source and support various physiological, developmental and reproductive processes in animals, including fish [20]. In general, lipid homeostasis is maintained by the regulation of lipogenesis, lipolysis, β -oxidation and lipid transport, and many crucial enzymes and signaling pathways are involved in these processes [21]. Yellow catfish *Pelteobagrus fulvidraco*, an omnivorous freshwater fish, is widely cultured in the inland freshwater waters in several Asian countries including China for its delicious meat and high market value. However, under intensive aquaculture, excess lipid deposition in yellow catfish become more and more widespread [22], which greatly reduces its taste and economic value. Thus, it is very important to investigate the characteristics of lipid metabolism and explore the pathway for reducing lipid deposition in yellow catfish. Here, given Met is the first-limiting amino acids in some feedstuffs (such as soybean meal) for

fish [23], it is very meaningful to explore the role and mechanism of Met influencing hepatic lipid deposition and metabolism. In addition, taking into account the potential link between apoptosis and lipid metabolism, we hypothesized that upstream regulators of apoptosis mediated the Met-induced changes of lipid metabolism. To that end, as a part of our project into the mechanisms of lipid metabolism and its regulatory pathways, at first, the present study was performed to determine the mechanism of upstream regulators of apoptosis mediating Met-induced changes of hepatic lipid metabolism in fish. On the other hand, in mammals, Met-mediated lipid metabolism showed species-specific response. Thus, it is very meaningful to compare the similarities for Met-induced changes of lipid metabolism between the fish and mammals. Thus, in the present study, we also compared the results between *P. fulvidraco* and HepG2 cell line, a model for studying lipid metabolism in mammals [24].

Materials and methods

Experimental treatments

Four experiments were performed. In Exp. 1, *P. fulvidraco* were fed diets containing three dietary Met levels for 10 weeks, and hepatic proteomic analysis was conducted to obtain the comprehensive understanding on Met-induced changes of physiological function of yellow catfish. In Exp. 2, using primary hepatocytes of yellow catfish and Z-VAD-FMK (specific inhibitor of caspase), the mechanism by which apoptosis signals mediated Met-induced changes of hepatic lipid metabolism was investigated. In Exp. 3, LY294002 (inhibitor of PI3K pathway), H89 (inhibitor against PKA pathway) and Compound C (inhibitor against AMPK pathway) were used to explore the upstream signaling pathways of apoptosis influencing hepatic lipid metabolism in *P. fulvidraco*. In Exp. 4, using HepG2 cell line, we also compared the similarities in the mechanism of Met influencing lipid deposition and metabolism between fish and mammals. We assured that the present experiments followed the guidelines of Institutional Animal Care and Use Committee (IACUC) of Huazhong Agricultural University, Wuhan, China.

Exp. 1: Investigating the effect of dietary Met levels on upstream regulators of apoptosis and lipid metabolism in liver of P. fulvidraco

Diet preparation. Three experimental diets were formulated with L-methionine (Sigma-Aldrich, St. Louis, MO, USA) supplemented at levels of 0, 4 and 6.9 g kg⁻¹ diet at the expense of cellulose (Supplementary Table 1). Different Met contents were added to the diets, based on recent study in *P. fulvidraco* [25], in order to produce three dietary Met groups (Met deficiency, adequate Met and Met excess, respectively). The formulation of the experimental diets was according to Wei et al. [22]. Final Met concentrations in the experimental diets were analyzed in triplicate using an automatic amino acid analyser (Hitachi L-8900, Tokyo, Japan), and the contents were 10.2 (Met deficiency), 13.1 (adequate Met), and 16.3 (Met excess) mg Met kg⁻¹ diet, respectively.

Experimental procedures. Yellow catfish were cultured in a semi-static aquarium system according to our published protocol [22]. Briefly, 270 uniform-sized fish (mean weight: 1.79 ± 0.02g, mean ± SEM) were stocked in 9 fiberglass tanks (300-l in water volume), 30 fish per tank. Each diet was distributed randomly to triplicate tanks. At the termination of the feeding experiment (10 wks), hepatic proteome profiles of *P. fulvidraco* and other relevant indicators were determined.

Exp. 2: Determining the involvement of upstream regulators of apoptosis in Met-induced change of hepatic lipid metabolism in P. fulvidraco in vitro

Primary hepatocytes were isolated from *P. fulvidraco* liver according to our recent studies [22,26]. Firstly, *P. fulvidraco* was cleared of blood by cutting off the branchial arch, and disinfected with 75% alcohol. After all the blood had been cleared, the liver was carefully excised from the abdominal cavity, transferred onto a plastic petri dish, and rinsed twice with PBS supplemented with amphotericin-B (25 µg/ml), streptomycin (100 µg/ml) and penicillin (100 IU/ml). Then the liver was aseptically minced into 1 mm³ pieces with scalpel and scissors, and the tissue was digested by 0.25% sterile trypsin at room temperature on a shaker for 30 min, neutralized with M199 medium containing 10% FBS every 5 min. The cell suspension was gathered. Then, the isolated hepatocytes were filtered through nylon sieves. Hepatocytes were collected in 15-ml sterilized centrifuge tubes, centrifuged at

low-speed ($100 \times g$, 5 min), and washed twice with PBS for debris removal. Finally, the purified hepatocytes were resuspended with M199 medium containing 1 mmol/l L-glutamine, 5% (v/v) FBS, penicillin (100 IU/ml) and streptomycin (100 μ g/ml). Cells were counted using a hemocytometer based on the trypan blue exclusion method, and only those cultures with more than 95% cell viability were used for the subsequent experiments. For each cell culture, a pool of cells from three fish was used.

To explore the role of upstream regulators of apoptosis in Met-induced changes of lipid metabolism, primary hepatocytes were treated with 50 μ M Z-VAD-FMK in RPMI 1640 medium containing different Met levels. For this experiment, eight groups were designed: control (containing 0.1% DMSO), Z-VAD-FMK (50 μ M), Met deficiency (10 mM), adequate Met (100 mM), Met excess (1000 mM), Met deficiency (10 mM) + Z-VAD-FMK (50 μ M), adequate Met (100 mM) + Z-VAD-FMK (50 μ M), and Met excess (1000 mM) + Z-VAD-FMK (50 μ M). Each treatment was performed in triplicate. The inhibitors were added 2 h before the addition of Met. The diagram for *in vitro* experiment was shown in Supplementary Fig. 1. The concentrations of Met and the specific inhibitors were selected according to previous experiments [27], our pilot trials and serum Met concentration in *P. fulvidraco* (91.24 ± 3.41 mM, unpublished data). Total Met concentration in the medium is of physiological relevance compared with that in *P. fulvidraco*. Sampling occurred at 48-h incubation.

Exp. 3: Exploring the upstream regulators of apoptosis participating in Met-induced changes of hepatic lipid metabolism in P. fulvidraco in vitro

In Exp. 3, specific inhibitors LY294002 (1 μ M), H89 (30 μ M) and Compound C (2 μ M) were used to explore the roles of PI3K/AKT-TOR, CAMP/PKA/CREB and LKB1/AMPK-FOXO pathways in apoptosis influencing hepatic lipid metabolism. For PI3K/AKT-TOR pathway, eight groups were designed: control (containing 0.1% DMSO), LY294002 (1 μ M), Met deficiency (10 mM), adequate Met (100 mM), Met excess (1000 mM), Met deficiency (10 mM) + LY294002 (1 μ M), adequate Met (100 mM) + LY294002 (1 μ M), and Met excess (1000 mM) + LY294002 (1 μ M). Each treatment was

performed in triplicate. For CAMP/PKA/CREB and LKB1/AMPK-FOXO pathways, the treatments were similar with PI3K/AKT-TOR pathway. Each treatment was performed in triplicate. The diagram for *in vitro* experiment was shown in Supplementary Fig. 1. The concentrations of the specific inhibitors were selected according to our recent studies and preliminary experiment in *P. fulvidraco* [22,26,28]. The cells were collected for analysis at the 48-h incubation.

Exp. 4: Comparing the similarities in the mechanism of upstream regulators of apoptosis mediating Met-induced changes of hepatic lipid metabolism between fish and mammals

Using HepG2 cells line, specific inhibitors (Z-VAD-FMK, LY294002, H89 and Compound C) were used to compare the similarities in the process of upstream regulators of apoptosis mediating Met-induced changes of hepatic lipid metabolism between fish and mammals, in RPMI 1640 medium containing different levels of Met (Met deficiency, 5 mM; adequate Met, 50 mM; Met excess, 500 mM). The experiment protocols were similar with those in the *in vitro* experiment. For example, in Z-VAD-FMK experiment, there were 8 treatment groups : control (containing 0.1% DMSO), Z-VAD-FMK (50 μ M), Met deficiency (5 mM), adequate Met (50 mM), Met excess (500 mM), Met deficiency (5 mM) + Z-VAD-FMK (50 μ M), adequate Met (50 mM) + Z-VAD-FMK (50 μ M), and Met excess (500 mM) + Z-VAD-FMK (50 μ M). Each treatment was performed in triplicate. The diagram for *in vitro* experiment was shown in Supplementary Fig. 1. The Met concentration was selected according to normal human serum Met concentration [29]. The concentrations of specific inhibitors were based on our previous experiments [22,26,28] and reports on mammals *in vitro* [30]. Sampling occurred at 48-h treatment.

Sample analysis

iTRAQ sample preparation, LC-MS/MS and data analysis

iTRAQ sample preparation

iTRAQ sample preparation was performed according to the methods described previously [31]. Briefly, three frozen liver tissues from each of the nine subgroups were homogenized on ice, with 100

g of homogenate from each used for proteomic screening. Protein lysates were obtained using lysis buffer (7 M urea, 2 M thiourea, and 1% cocktail proteinase inhibitor) followed by centrifugation at 12,000 rpm for 60 min at 4 °C to remove cellular debris. Protein concentrations were established using a Bradford assay, with protein samples (250 µg) digested with trypsin solution overnight at 37 °C and labeled with iTRAQ reagents (10-plex; AB SCIEX, Massachusetts, USA) as follows: Met deficiency-1 (115N tag), Met deficiency-2 (115C tag), Met deficiency-3 (116N tag), adequate Met-1 (116C tag), adequate Met-2 (117N tag), adequate Met-3 (117C tag), Met excess-1 (118N tag), Met excess-2 (118C tag), Met excess-3 (119 tag) according to the manufacturer's instructions. Then, typically peptides labeled with 10-plex iTRAQ tags were pooled and dried.

The peptides were separated on a Shimadzu LC-20AB HPLC Pump system coupled with a high pH RP column. The peptides were reconstituted with buffer A (5% ACN, 95% H₂O, pH 9.8) to 2 ml and loaded into a column containing 5-µm particles (Phenomenex). The peptides were separated at a flow rate of 1 mL/min with a gradient of 5% buffer B (5% H₂O, 95% ACN, pH 9.8) for 10 min, 5-35% buffer B for 40min, 35-95% buffer B for 1 min. The system was then maintained in 95% buffer B for 3 min and decreased to 5% within 1 min before equilibrating with 5% buffer B for 10 min. Elution was monitored by measuring absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled as 20 fractions and vacuum-dried.

LC-MS/MS and data analysis

The mass spectrometry analysis was performed as described in Michalski et al [32], using Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA), coupled with LC-20AD (Shimadzu). The parameters for MS analysis were listed below: electrospray voltage: 1.6 kV; precursor scan range: 300–1600 m/z at a resolution of 35,000 in Orbi trap; MS/MS fragment scan range: >100 m/z at a resolution of 70,000 in HCD mode; normalized collision energy setting: 30%; dynamic Exclusion time: 15 s; Automatic gain control (AGC) for full MS target and MS2 target: 3e6 and 1e5, respectively; The number of MS/MS scans following one MS scan: 20 most abundant precursor ions above a threshold ion count of 20000. Each of the 20 high pH RP-HPLC fractions was

re-suspended in buffer A (2% ACN and 0.1% FA in water) and centrifuged at 20,000 g for 10 min. The supernatant was loaded into a C18 trap column 5 μ L/min for 8min using a LC-20AD nano-HPLC instrument (Shimadzu, Kyoto, Japan) by the autosampler. Then, the peptides were eluted from trap column and separated by an analytical C18 column (inner diameter 75 μ m) packed in-house. The gradient was run at 300 nL/min starting from 8% to 35% of buffer B (2% H₂O and 0.1% FA in ACN) in 35 minutes, then going up to 60% in 5 minutes and to 80% of buffer B for 5 minutes, and finally return to 5% in 0.1 min and equilibrated for 10min.

The software IQuant was used to quantify the labeled peptides with isobaric tags. To assess the confidence of peptides, the PSMs were pre-filtered at a PSM-level false discovery rate (FDR) of 1%. Then, based on the "simple principle" (the parsimony principle), identified sequences of peptide were assembled into a set of confident proteins. In order to control the rate of false-positive at protein level, a protein FDR at 1%, which was based on Picke protein FDR strategy, was estimated after protein inference (Protein-level FDR $\leq$ 0.01). The process for protein quantification included the following steps: Protein identification, Tag impurity correction, Data normalization, Missing value imputation, Protein ratio calculation, Statistical analysis, Results presentation. The transcriptome data of liver from yellow catfish was used for the peptide-protein matching.

Three biological replicates were set for better coverage of the target proteome with reliable statistical consistency. The Paragon database search algorithm and an integrated FDR analysis were implemented in the ProteinPilot software for peptide identification. A 95% confidence interval (CI) was set as the significant threshold for protein identification. Quality control (QC) was performed to determine if a re-analysis step was needed. All the proteins with a FDR less than 1% proceeded with downstream analysis including GO, COG and Pathway. Furthermore, we also performed in depth analysis based on differentially expressed proteins, including Gene Ontology (GO) enrichment analysis, KEGG pathway enrichment analysis and cluster analysis. The protein lists from the 20 LC-MS/MS runs, obtained by fractionation of the iTRAQ pooled sample, were merged. The confidence level of each differentially expressed protein was calculated as a p-value using ProteinPilot,

allowing the results to be evaluated, not only based on the magnitude of the change but also on the confidence level of the change. GO was performed using the bioinformatics analysis tool DAVID (<http://david.abcc.ncifcrf.gov>) to determine the functional classifications of the iTRAQ-identified proteins. The diagram for iTRAQ sample preparation, LC-MS/MS and data analysis was shown in Supplementary Fig. 2.

Histological, histochemical and ultrastructural analyses

Histological, histochemical and ultrastructural analyses were performed according to the method described in our studies [33,34]. Briefly, for histological observation, samples were dehydrated in graded ethanol concentrations and embedded in paraffin wax. Sagittal sections of 6–8 μm thickness were stained with hematoxylin–eosin (H&E) and then prepared for light microscopy. For histochemical observation, specimens were sectioned (8 μm) on a cryostat microtome. Sections were fixed in cold 10% buffered formalin for 10 min, stained with oil-red O and then prepared for light microscopy. For statistics of relative areas for hepatic vacuoles in H&E, lipid droplets in oil-red O staining, we randomly examined 10 microscope fields for each sample and the results from individual observation were then combined for the overall results. All the images were evaluated by double-blind method. For ultrastructural observation, specimens were prefixed in glutaraldehyde solution, followed by rinses with phosphate buffer. Post-fixation occurred in aqueous osmium tetroxide. After rinsing with phosphate buffer again, the specimens were dehydrated in a graded ethanol and then embedded in Epon 812 prepared for electron microscope. Ultrastructural preparations were randomly examined with 10 microscope fields for each sub-samples, and the results from each observation were then combined for the overall results.

Determination of TG, APO-A1 and APO-B contents, and lipometabolic enzymatic activities

TG was determined by glycerol-3-phosphate oxidase p-aminophenol (GPO-PAP) methods following Song et al. [34], using a commercial kit from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. The TG content was expressed as mmol TG per mg cellular protein.

Apo-A1 and APO-B levels were determined with a standard commercial immunoturbidimetric

assay following the method of Mount et al. [35].

HSL activity was determined as described by Reynisdottir et al. [36], using 1(3)-[3H]-oleoyl-2-O-oleylglycerol as substrate. All samples were incubated in triplicate at 25 °C for 30 min on one occasion. The substrate for HSL had only one hydrolyzable ester bond at the 1(3) position and was therefore not a substrate for MGL. Furthermore, under the present incubation conditions (pH 7.0 and no apolipoprotein CII present), lipoprotein lipase activity is negligible. In addition, HSL hydrolyzes tri- and diacylglycerol at the relative rate of 1:10. Therefore, the sensitivity of the assay is enhanced by the use of a diacylglycerol analog as substrate. One unit of enzyme activity equals 1 mmol fatty acid produced/min at 25 °C.

For the determination of FAS activity, liver or hepatocytes samples were homogenized in ice-cold buffer (0.02 M Tris-HCl, 0.25 M sucrose, 2 mM EDTA, 0.1 M sodium fluoride, 0.5 mM phenyl methyl sulphonyl fluoride, 0.01 Mβ-mercapto-ethanol, pH 7.4), and centrifuged at $20,000 \times g$ at 4 °C for 30 min. The supernatant was collected and immediately used for the enzyme analysis. FAS activity was determined according to the method of Chakrabarty and Leveille [37]. One unit of enzyme activity (IU), defined as the amount of enzyme that converted 1μmol of substrate to product per min at 25 °C, was expressed as units per milligram (mg) of soluble protein.

For CPT-1 activity assay, mitochondria were isolated from liver or hepatocytes according to Morash et al. [38]. CPT-1 activity was determined using the method of Bieber and Fiol [39], based on measurement of the initial CoA-SH formation by the 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) reaction from palmitoyl-CoA by mitochondrial samples with L-carnitine at 412 nm. One unit (IU) of CPT-1 activity was defined as 1μmol of product formed per min per mg of mitochondrial protein at 25 °C.

Soluble protein concentration of homogenates was determined by the method of Bradford [40] using bovine serum albumin (BSA) as standard. These analyses were conducted in triplicate.

Determination of caspases-3 and -9 activities

Cytosolic caspases-3 and -9 activities were determined spectrofluorometrically at 460 nm using 380-nm excitation wavelength at 37°C for 150 seconds for caspase-3, and 20 minutes for caspase-9, as described by Khurana et al. [41].

Assay of cAMP, PKA and pAMPK levels

cAMP and PKA levels were performed according to the methods described previously [42], using the direct cAMP and PKA ELISA kit (Enzo Life Sciences). Concentrations of phospho-AMPK (cat. number KHO0651) were determined by ELISA (Invitrogen, Camarillo, CA, USA) according to the manufacturer's instructions [43].

Annexin V and PI staining method

The appearance of phosphatidyl-serine on the extracellular side of the cell membrane was quantified by Annexin V/PI staining following the method of Sheng et al. [44]. Cells were stained with Annexin V-FITC and PI at room temperature as recommended by the manufacturer. Cells were subjected to fluorescence-activated cell sorting (FACS) analysis using a flow cytometer with apoptotic cells being Annexin V-positive/PI-negative.

Terminal Transferase-Mediated dUTP Nick End Labeling (TUNEL) assay

TUNEL staining was conducted using *in vitro* Cell Death Detection Kit (TdT-FragEL™ DNA Fragmentation Detection Kit, Cat. No.: QIA33, Calbiochem, Merck, USA), used either fluorescein or alkaline phosphatase (AP) as recommended by the supplier according to manufacturer's procedures [45].

Flow cytometric analysis

For flow cytometric analysis, a FACSort (Becton Dickinson, Sunnyvale, CA) equipped with a single Argon ion laser was used according to the methods described previously [46]. Excitation was done at 488 nm and the emission filters used were 515-545 BP (green; FITC) and 600 LP (red; PI). A minimum of 5000 cells per sample were analysed. Electronic compensation was used to eliminate

bleed through of fluorescence. Data analysis was performed with the standard Lysis and Cellfit software (Becton Dickinson).

mRNA abundance determination by real-time quantitative PCR (Q-PCR)

Analyses on gene transcript levels were conducted by Q-PCR method described in our studies [34]. For Q-PCR analysis, three replicate tanks and three fish for each tank were sampled for gene expression. Q-PCR was performed in a 20 µl reaction mixture including 10 µl SYBR Premix Ex Taq™ II, 0.4 µl each primer, 1µl diluted first-strand cDNA product and 8.2 µl ddH₂O. Reactions were based on a three-step method as followed: at 95 °C for 30 s, 40 cycles of 5 s at 95 °C, 10 s at 57 °C and 30 s at 72 °C. All reactions were performed in duplicate and each reaction was verified to contain a single product of the correct size by agarose gel electrophoresis. A non-template control and dissociation curve were performed to ensure that only one PCR product was amplified and that stock solutions were not contaminated. Standard curves were constructed for each gene using serial dilutions of stock cDNA. The amplification efficiencies of all genes were approximately equal and ranged from 98 to 103 %. A set of eight common housekeeping genes (β-actin, 18s-rRNA, GAPDH, RPL7, HPRT, B2M, UBCE and TUBA) were selected from our transcriptome database for *P. fulvidraco*, and also selected from GenBank for HepG2 cell in order to test their transcription stability. Our pilot experiment indicated that β-actin and TUBA (β-actin and GAPDH, M=0.27) showed the most stable level of expression across the experimental conditions as suggested by geNorm [47]. Thus, the relative expression levels were normalized to the geometric mean of the combination of two genes and calculated using the $2^{-\Delta\Delta C_t}$ method [48].

Western Blotting

For Western blotting analysis, cells were plated in 6-well plates, incubated overnight, and subsequently treated with PBS, GCRV, or poly (I:C). At 48-h posttreatment, cells were washed with PBS and lysed in lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride, serine/threonine phosphatase inhibitor, tyrosine phosphatase inhibitor, and protease inhibitor PMSF. After clarification by centrifugation at 12,000 rpm for 15 min, 30 µg of supernatant proteins was

separated by 8-12% SDS-PAGE. The separated polypeptides were electroblotted onto nitrocellulose (NC) filter membranes (Millipore) using a trans-blot SD semidry electrophoretic transfer cell (Jim-X, Dalian, China), and then the blotted membranes were incubated with blocking TBST buffer (0.5 M Tris-Cl, 150 mM NaCl, 0.5% Tween 20, and 1% bovine serum albumin) at room temperature for 2 h. Subsequently, the membranes were incubated with appropriate primary antibodies for 2 h at room temperature in blocking TBST buffer, washed thrice with TBST buffer, and then incubated with secondary antibody for 1 h at room temperature. After again washing thrice with TBST buffer, the NC membranes were scanned and imaged by an Odyssey® CLx Imaging System (LI-COR). For hybridization, the anti-p-Akt and anti-GAPDH antisera were diluted 1:1,000, commercial primary antibodies 1:5,000, and secondary antibodies 1:10,000.

Statistical analysis

All data were expressed as mean $\pm$ standard error of means (SEM). Prior to statistical analysis, an arcsine transformation was used before processing percentage data. All data were tested for normality of distribution using the Kolmogorov–Smirnov test. The homogeneity of variances among the different treatments was tested using the Bartlett’s test. Then, data were subjected to one-way ANOVA and Tukey’s multiple range test using SPSS 19.0 software, and the minimum significant level was set at $P < 0.05$. Differences between adequate Met groups and imbalanced Met groups in the *in vivo* study were analyzed by Student’s T-test for independent samples.

Results

Growth performance and feed utilization

Survival and feed intake (FI) showed no significant differences among three treatments. Compared to adequate Met, dietary Met deficiency and excess significantly decreased final mean body weight (FBW), weight gain (WG) and specific growth rate (SGR), and significantly increased feed conversion rate (FCR) (Supplementary Table 2).

Overview of Quantitative Proteomics Analysis in liver of *P. fulvidraco*

Overall, 22,217 unique peptides corresponding to 4,554 proteins were identified (Supplementary Fig. 3A). In terms of protein mass distribution and peptide length distribution, good coverage (more than 90% of the total proteins) was obtained for proteins larger than 10 kDa (Supplementary Fig. 3B). MS raw files have been submitted to ProteomeXchange (accession number: PXD007696).

For COG annotations, proteins were functionally classified into 24 COG categories. Among these categories, most enriched terms were “General function of prediction only” (Supplementary Fig. 3F). The GO annotations of biological processes revealed several proteins implicated in metabolic process and in the regulation of cellular processes (Supplementary Fig. 3G). The information of differentially expressed protein in all groups were shown in Supplementary Fig. 4.

Dietary Met deficiency or excess activated upstream regulators of apoptosis and caused aberrant lipid metabolism in liver of *P. fulvidraco*

A global understanding of the effect of dietary Met deficiency or excess on signaling pathways and hepatic lipid metabolism

A 1.2-fold ($P < 0.05$) cut-off was used to implicate significant changes in the abundance of differentially expressed proteins (DEPs) among three Met groups. There were a total of 796 proteins identified as DEPs, 412 of which were increased and 384 were decreased in Met deficiency vs adequate Met groups (Fig. 1A and B); 842 proteins identified as DEPs, and 442 proteins were increased and 400 were decreased in Met excess vs adequate Met groups (Fig. 1D and E).

Statistics of pathway enrichment indicated that dietary Met deficiency or excess posed significant effects on biological pathways, such as signal transduction, apoptosis and lipid metabolism (Fig. 1C and F). Among signal transduction pathways, three important pathways, including PI3K/AKT-TOR pathway, cAMP/PKA/CREB pathway and LKB1/AMPK-FOXO pathway, were enriched in both Met deficiency and Met excess groups (Supplementary Table 3). The majority of proteins involved in apoptosis were increased following dietary Met deficiency or excess. These DEPs participated in lipid

metabolism, including lipogenesis, lipolysis, β -oxidation and lipid transport. Several crucial proteins in lipid metabolism were differentially regulated by dietary Met deficiency or excess (Supplementary Table 3).

Effect of dietary Met levels on upstream regulators of apoptosis in liver of P. fulvidraco

As shown in Fig. 2B, dietary Met deficiency up-regulated the mRNA abundance of *lkb1*, *ampka1*, *ampkb1* and *foxo3a* (LKB1/AMPK-FOXO pathway); dietary Met excess up-regulated the mRNA abundance of genes involved in cAMP/PKA/CREB pathway, and down-regulated the mRNA abundance of genes involved in PI3K/AKT-TOR pathway.

Compared to dietary adequate Met, both dietary Met deficiency and excess up-regulated the mRNA abundance of genes in death receptor pathway, ER stress pathway and mitochondrial pathway (Fig. 2C), and also increased the activities of caspase-3 and -9, indicating the occurrence of apoptosis (Fig. 2D). Additionally, as shown in Fig. 2A, dietary Met deficiency and excess caused chromatin margination and nuclear convolution in hepatocytes of *P. fulvidraco*.

Effect of dietary Met deficiency or excess on lipid metabolism in liver of P. fulvidraco

First, the amount of vacuoles in H&E and lipid droplets in oil-red O increased in imbalanced dietary Met groups (Fig. 3A). The relative areas for hepatic vacuoles in H&E staining and for lipid droplets in oil-red O staining, and the TG concentrations further supported the results (Fig. 3B and C). However, dietary Met deficiency and excess had different effects on lipogenesis, lipolysis, oxidation and lipid transport: dietary Met deficiency down-regulated the mRNA abundance of *hsl2*, *atgl*, *fatp4*, *apoa-1*, *apob* and *lpl*, and HSL activity, and contents of APO-A1 and APO-B; dietary Met excess down-regulated the mRNA abundance of *cpt-1*, *acadm*, *echs1*, *fatp4*, *apoa-1*, *apob* and *lpl*, and CPT-1 activity, and contents of APO-A1 and APO-B, up-regulated the mRNA abundance of *fas*, *scd1*, *acca*, and FAS activity (Fig. 3D, E, F and G). The results of genes transcription involving in lipid metabolism were further confirmed by cluster analysis (Supplementary Fig. 6).

Upstream regulators of apoptosis mediated the Met-induced changes of hepatic lipid metabolism in

P. fulvidraco

Compared to single Met deficiency or excess treatment, pretreatment of Z-VAD.FMK markedly attenuated the Met deficiency- and excess-induced increase of the FITC+/PI+ positive rate, apoptosis index, and rates of apoptosis (Fig. 4A, B, C and D). At the transcriptional level, the increase in mRNA abundance of proapoptotic genes and the reduction in mRNA abundance for antiapoptotic genes induced by Met deficiency or excess were alleviated by Z-VAD.FMK incubation (Fig. 4E). In addition, Z-VAD.FMK incubation also reduced the activities of caspase-3 and -9 (Fig. 4F). These results indicated that Met deficiency or excess-induced apoptosis was significantly attenuated by Z-VAD.FMK incubation.

Compared to the single Met deficiency group, Z-VAD.FMK pretreatment markedly alleviated the Met deficiency-induced reduction of lipolysis and lipid transport (Fig. 4). Met excess-induced elevation of lipogenesis, and reduction of oxidation and lipid transport were also significantly attenuated by Z-VAD.FMK incubation. Furthermore, along with apoptosis attenuation, Z-VAD.FMK also significantly attenuated the Met deficiency- and excess-induced elevation of TG contents (Fig. 4J).

Three distinct upstream regulators of apoptosis were involved in Met-induced changes of hepatic lipid metabolism in P. fulvidraco

As shown in Supplementary Fig. 7, about PI3K/AKT-TOR pathway, compared to the single Met excess group, LY294002 pretreatment and then excess Met incubation reduced the mRNA abundance of *pi3kca*, *akt*, *torc1* and *sgk-1*, and the protein levels of p-Akt, and enhanced the mRNA abundance of *casp3*, *casp9*, *chop* and *bax*, apoptosis index, and activities of caspase-3 and -9. Meanwhile, LY294002 markedly up-regulated the Met excess-induced increase of TG content. However, interestingly, we also found that wortmannin, another inhibitor of PI3K/AKT, had opposite effect on apoptosis and Met-induced changes of hepatic lipid metabolism, which implying that wortmannin treatment may reduce apoptotic rate or caspase activity independent of PI3Kinase (Supplementary Fig. 7, 9, 12, 14). About cAMP/PKA/CREB pathway, pretreatments with H89 showed similar effect with

Wortmannin incubation. Pretreatments with H89 significantly influenced these parameters from Met excess groups, but had no significant effect on Met deficiency groups (Supplementary Fig. 8). Regarding the LKB1/AMPK-FOXO pathway (Fig. 5), pre-incubation with Compound C followed by Met incubation alleviated the Met deficiency-induced increase of apoptosis rates, activities of caspase-3 and -9, and the mRNA abundance of *casp3*, *casp9*, *chop* and *bax*. Compound C also significantly influenced the Met deficiency-induced reduction of lipolysis and lipid transport. However, although Compound C shown suppressive effect on Met excess-induced increase of apoptosis rates, activities of caspase-3 and -9, there are no statistically significant difference between Met excess and Met excess plus Compound C groups.

Similarities existed in apoptosis mediating the Met-induced changes of hepatic lipid metabolism between fish and mammals

At first, the roles of upstream regulators of apoptosis participating in the Met-induced changes of hepatic lipid metabolism were explored in HepG2 cells line (Fig. 6). Both Met deficiency and excess up-regulated apoptosis as evidenced by the FITC+/PI+ positive rate, apoptosis index, rates of apoptosis, activities of caspase-3 and -9 and the mRNA abundance of marker genes involved in apoptosis. The Met deficiency- and excess-evoked apoptosis was significantly attenuated by Z-VAD.FMK pretreatment. Furthermore, Met deficiency had significant effect on lipolysis, oxidation and lipid transport; Met excess showed significant effect on lipogenesis, lipolysis, oxidation and lipid transport. Additionally, Met deficiency and excess caused TG accumulation, which was also markedly alleviated by Z-VAD.FMK in HepG2 cells line.

As shown in Supplementary Fig. 12, 13, LY294002 pretreatment markedly up-regulated Met deficiency- and excess- induced apoptosis by inhibiting PI3K/AKT-TOR pathway. LY294002 down-regulated the levels of p-Akt and up-regulated activities of caspase-3 and -9, and TG content. Regarding the cAMP/PKA/CREB pathway, pretreatments with H89 had significant effect on these parameters only from the Met deficiency group, but not these caused by Met excess. In addition, H89 pretreatment had similar effect to LY294002 pretreatments in HepG2 cells line with Met treatment.

Discussion

Recently, studies about the potential role of Met in lipid metabolism at transcription levels were reported [4]. Compared with genome and transcriptome, proteome provided a more realistic picture about the underlying mechanism of Met mediating lipid metabolism. In this study, pathway enrichment analysis indicated that Met deficiency- and excess- posed significant effects on biological pathways, such as signal transduction, apoptosis and lipid metabolism. Hence, we proposed that upstream regulators of apoptosis were responsible for disturbances in hepatic lipid metabolism caused by imbalance Met, and dietary Met deficiency or excess disrupted hepatic lipid metabolism by influencing processes of lipogenesis, lipolysis, oxidation and lipid transport, which in turn caused lipid accumulation in the liver of *P. fulvidraco*, in agreement with other studies [5]. Similarly, Zhou et al. [4] pointed out that Met restriction (MR) increased de novo lipogenesis, lipolysis and fatty acid oxidation, with a reduction of fat accumulation. Interestingly, the present study suggested that dietary Met deficiency and excess had differential effects on the hepatic lipid metabolism in *P. fulvidraco*: (i) dietary Met deficiency reduced lipolysis and lipid transport; and (ii) dietary Met excess reduced oxidation and lipid transport pathway, and increased lipogenesis, in partial agreement with other studies [49]. On the other hand, our study showed that both dietary Met deficiency and excess activated upstream regulators of apoptosis in liver of *P. fulvidraco*, in agreement with reports in mammals [9,10]. Apoptosis will occur when the cells lack a specific nutrient [10]. In the present study, proteome analyses showed that both dietary Met deficiency and excess led to significant changes in the expression of proteins involved in upstream regulators of apoptosis. Meantime, at the transcriptional level, both dietary Met deficiency and excess had significant effect on mRNA transcription of all the tested apoptosis marker genes. The present study also indicated that both dietary Met deficiency and excess activated the activities of caspase-3 and -9 both *in vivo* and *in vitro*, which would in turn catalyze the specific cleavage of cellular apoptotic proteins [50], and caused the occurrence of apoptosis [51]. Similarly, previous studies indicated Met deficient diets could change

the levels of lipid accumulation in rodents by influencing the activities of caspase [52,53]. In addition, the potential link between apoptosis and lipid metabolism has been postulated in other study [19]. Thus, we speculate the apoptotic mediator (caspases) played a bridge role for apoptosis mediating lipid metabolism. In addition, it is worthy to point out that in the present study, although other factors (such as stress) may mediate the Met deficiency and excess-induced occurrence of apoptosis, the majority of proteins involved in apoptosis were increased following dietary Met deficiency or excess. Taken together, these results suggested that both dietary Met deficiency and excess activated upstream regulators of apoptosis and caused aberrant hepatic lipid metabolism in *P. fulvidraco*.

One of objectives of the current study was to explore the mechanism by which upstream regulators of apoptosis mediated the Met-induced changes in lipid metabolism. To this end, specific caspase inhibitors Z-VAD-FMK were used. Our current study found that Z-VAD-FMK markedly alleviated the Met deficiency- and excess-activated apoptosis pathways in *P. fulvidraco*, similar to those in mammalian cells [54]. Importantly, the current study also showed that upstream regulators of apoptosis mediated the Met deficiency- and excess-induced changes in hepatic lipid metabolism since Z-VAD-FMK markedly attenuated the Met deficiency- and excess-induced effects on gene transcription and enzymatic activity involved in hepatic lipid metabolism. To our knowledge, very limited information was available on the role of apoptosis in hepatic lipid metabolism, and involved in FAS and CPT-1 [55,56]. Additionally, Huang et al [19] pointed out that bioactive lipid molecules promoted apoptosis via the intrinsic pathway by modulating mitochondrial membrane permeability and activating different enzymes including caspases. Thus, the present study demonstrated that upstream regulators of apoptosis were involved in the Met deficiency- and excess-induced dysregulation of hepatic lipid metabolism in fish.

The mechanisms by Met deficiency- and excess-activating apoptosis may be multifactorial and regulated by multiple signal transduction pathways. In mammals, studies indicated that PI3K/AKT-TOR inhibition [57], the activation of cAMP/PKA/CREB and LKB1/AMPK-FOXO [58,59], would contribute to the occurrence of apoptosis, in agreement with the present *in vitro* experiments. Thus, the present study indicated that three signaling pathways mediated Met deficiency-

and excess-induced apoptosis in yellow catfish. Moreover, the PI3K/AKT-TOR inhibition, and cAMP/PKA/CREB and LKB1/AMPK-FOXO activation were markedly alleviated by LY294002, H89 and Compound C, respectively; the dysregulation of hepatic lipid metabolism induced by Met deficiency or Met excess were also significantly attenuated. These observations indicated that the PI3K/AKT-TOR, cAMP/PKA/CREB and LKB1/AMPK-FOXO pathways mediated the Met deficiency- and excess-induced changes in hepatic lipid metabolism. Similarly, our recent studies also suggested that these pathways were involved in lipid metabolism in *P. fulvidraco* [22,26,28]. Here, considering the involvement of apoptosis in Met deficiency- and excess-induced changes of hepatic lipid metabolism, the present study indicated that the three pathways mediated Met deficiency- and excess-induced apoptosis, providing a mechanism for the aberrant lipid metabolism induced by imbalanced Met in yellow catfish. Interestingly, we also revealed that Met deficiency or excess modulated hepatic lipid metabolism via different upstream pathways: (i) Met deficiency via LKB1/AMPK-FOXO pathway, and (ii) Met excess via PI3K/AKT-TOR and cAMP/PKA/CREB pathways. Similarly, Yen et al. [10] showed that different nutrient status could differentially induce apoptosis in different upstream signals. Meanwhile, the present study also indicated that another inhibitor of PI3K/AKT, wortmannin, did not inhibit but PI3K/AKT-TOR pathway. This result implied that wortmannin may involve Met-induced apoptosis independent of PI3K pathway, similar to these by Wang et al. [60] and Yan et al. [61]. Thus, taken together, for the first time, we found that two distinct signaling axis existed in the effects of dietary Met on hepatic lipid metabolism in *P. fulvidraco*.

Because of whole-genome duplication in teleost fishes [62], one should keep cautious when extrapolating the results in the fish to mammals. We therefore compared the similarities in the process of upstream regulators of apoptosis mediating imbalanced Met-induced changes of hepatic lipid metabolism between yellow catfish and human cells. In the present study, two main similarities existed: (i) Met deficiency and excess activated upstream regulators of apoptosis and caused dysregulation of lipid metabolism both in *P. fulvidraco* primary hepatocytes and in HepG2 cells, (ii) upstream regulators of apoptosis were involved in Met deficiency or excess-induced changes of lipid

metabolism both in *P. fulvidraco* primary hepatocytes and in HepG2 cells. Indeed, the conserved effects of imbalanced Met on apoptosis have been found in various cell and species [63]. Furthermore, these findings confirmed an evolutionary conserved role of upstream regulators of apoptosis in regulation of lipid metabolism, which also supported our hypothesis. However, several distinctions were observed: (i) Met deficiency and excess induced TG accumulation via different processes of lipid metabolism between *P. fulvidraco* and HepG2 cell, and (ii) upstream regulators of apoptosis modulated lipid metabolism via different pathways between *P. fulvidraco* and HepG2 cell. Similarly, Yen et al. [10] suggested that essential nutrient deficiency and excess activated different upstream apoptotic pathways that ultimately converged on a common execution pathway. These findings suggested the mechanisms of apoptosis-related signaling pathways regulating lipid metabolism have species-specific responses.

In conclusion, the present study clearly indicated that both dietary Met deficiency and excess evoked upstream regulators of apoptosis and induced the changes of hepatic lipid metabolism in fish. Upstream regulators of apoptosis mediated the Met deficiency- and excess-induced changes of hepatic lipid metabolism. Moreover, two distinct signaling axis were involved in Met-induced changes of hepatic lipid metabolism. We also observed the similarities of the mechanism involved in the upstream regulators of apoptosis mediating Met-induced changes of lipid metabolism between *P. fulvidraco* and HepG2 cell (Supplementary Fig.10; Fig. 7). These findings highlight the importance of the upstream regulators of apoptosis in the regulation of Met-induced changes of lipid metabolism.

Conflict Of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Authors' contributions

Song Y.F. and Luo Z. designed the experiment. Song Y.F. conducted the experiment and data analysis with the help of Gao Y., Li D.D. and Pan Y.X. Hogstrand C. provided some critical suggestions for data analysis and manuscript writing. Song Y.F. drafted the manuscript. Hogstrand C.

and Luo Z. revised the manuscript. All the authors reviewed and approved the manuscript.

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Figure captions

FIGURE 1. Pathway enrichment analysis of DEPs involved in signal transduction, apoptosis and lipid metabolism in liver of *P. fulvidraco* fed different levels of dietary Met. (A) Volcano plots of DEPs from Met deficiency vs adequate Met. (B) Histogram showing numbers of DEPs from Met deficiency vs adequate Met. DEPs were indicated in *red* (increase) and *green* (reduction). Black meant proteins that were not differentially expressed. (C) Statistics of pathway enrichment of DEPs from Met deficiency vs adequate Met. Rich Factor was the ratio of the number of DEPs annotated in this pathway to all protein number annotated in this pathway. Greater Rich Factor meant greater intensiveness. (D, E and F) From Met excess vs adequate Met, volcano plots of DEPs, histogram showing numbers of DEPs, and statistics of pathway enrichment of DEPs, respectively.

FIGURE 2. Dietary Met deficiency or excess induced the changes of upstream regulators of apoptosis in liver of *P. fulvidraco*. (A) Liver ultrastructure (TEM, original magnification $\times 10000$, bars 1 μm) of *P. fulvidraco* fed different levels of dietary Met. Abbreviation: endoplasmic reticulum (er); hepatocyte nucleus (N); nucleolus (n); chromatin margination (marg); nuclear convolution (con). Liver cells of fish fed adequate dietary Met, showing the typical appearance of hepatocyte nucleus and nucleolus. Hepatocytes in Met deficiency and excess groups showed the chromatin margination and nuclear convolution. (C and D) Effect of dietary Met deficiency or excess on the transcription of genes involved in upstream regulators of apoptosis (PI3K/AKT-TOR, cAMP/PKA/CREB and LKB1/AMPK-FoxO). mRNA transcription values were normalized to β -actin and GAPDH expressed as a ratio of the control on adequate Met (control = 1). (E) Dietary Met deficiency and excess increased the activities of caspase-3 and -9. Values are mean $\pm$ SEM (n = 3 replicate tanks, and for each tank, three fish were sampled for gene transcription and four fish for activities of Caspase-3 and -9). Asterisks indicate significant differences between Met deficiency or excess and adequate Met group ($P < 0.05$).

FIGURE 3. Dietary Met deficiency or excess caused aberrant lipid metabolism in liver of *P. fulvidraco*. (A) Liver histology (H&E) and histochemistry (oil-red O staining) (original magnification $\times 200$, bars $50\mu\text{m}$) of *P. fulvidraco* fed different levels of dietary Met. Abbreviation: hepatocytes (he); vacuoles (va); pyknotic nuclei (pn). Lipid was red-colored and nuclei-blue colored after staining with oil-red O. Dietary Met deficiency and excess increased the amount of vacuoles (H&E) and lipid droplets (oil-red O staining). (B and C) The relative areas, analyzed by Image-Pro Plus 6.0, for hepatic vacuoles (H&E) and lipid droplets (oil-red O staining) of *P. fulvidraco* fed different levels of dietary Met. (D, E and F) Effects of dietary Met deficiency or excess on the activities of FAS, HSL and CPT-1 (D), and the contents of APO-A1 and APO-B (E) and TG (F), in liver of *P. fulvidraco*. (G) Effect of dietary Met deficiency or excess on the transcription of genes involved in lipogenesis, lipolysis, oxidation and lipid transport. mRNA transcription values were normalized to β -actin and GAPDH expressed as a ratio of the control on adequate Met (control = 1). Values are mean $\pm$ SEM (n = 3 replicate tanks, and for each tank, three fish were sampled for gene transcription and four fish for H&E, oil-red O staining, the activities of FAS, HSL and CPT-1 and the contents of APO-A1, APO-B and TG). Different letters indicated significant differences among groups ($P < 0.05$).

FIGURE 4. The involvement of upstream regulators of apoptosis in imbalance Met-induced aberrant lipid metabolism in hepatocyte of *P. fulvidraco* in vitro. (A, B, C and D) Met deficiency or excess-induced increase of the FITC+/PI+ positive rate (B), apoptosis index (C), and rates of apoptosis (D) were markedly alleviated by Z-VAD.FMK in primary hepatocyte of *P. fulvidraco* in vitro. (E) Met deficiency or excess-induced change in the transcription of genes involved in upstream regulators of apoptosis were markedly attenuated by Z-VAD.FMK in primary hepatocyte of *P. fulvidraco* in vitro. mRNA transcription values were normalized to β -actin and GAPDH expressed as a ratio of the control on adequate Met (control = 1). (F) Z-VAD.FMK significantly attenuated the Met deficiency or excess-induced increase of caspase-3 and -9 activities. (G) Met deficiency or excess-induced change in the mRNA abundances of genes involved in lipid metabolism were markedly attenuated by Z-VAD.FMK in primary hepatocyte of *P. fulvidraco*. mRNA transcription values were normalized to β -actin and GAPDH expressed as a ratio of the control on adequate Met (control = 1). (H, I and J)

Effect of Z-VAD.FMK on Met deficiency or excess-induced change in the activities of FAS, HSL and CPT-1, and the contents of APO-A1 and APO-B and TG. Values are mean $\pm$ SEM (n = 3 independent biological experiments). Different letters indicated significant differences among groups ($P < 0.05$).

FIGURE 5. LKB1/AMPK-FOXO pathway which regulates apoptosis is involved in imbalanced Met-induced changes of lipid metabolism in hepatocytes of *P. fulvidraco*. (A) Met excess-induced change in the transcription of genes involved in LKB1/AMPK-FOXO pathway were markedly attenuated by Compound C. mRNA transcription values were normalized to β -actin and GAPDH expressed as a ratio of the control on adequate Met (control = 1). (B) Compound C significantly attenuated the Met excess-induced increase of pAMPK content. (C, D and E) Compound C significantly attenuated the Met excess-induced increase of apoptosis rates (C and D), caspase-3 and -9 activities (E). (F) Met excess-induced change in the transcription of genes involved in apoptosis signaling pathways were markedly attenuated by Compound C. mRNA transcription values were normalized to β -actin and GAPDH expressed as a ratio of the control on adequate Met (control = 1). (G) Compound C significantly attenuated the Met excess-induced increase of TG content. (H) Met excess-induced change in the mRNA abundances of genes involved in lipid metabolism were markedly attenuated by Compound C. mRNA transcription values were normalized to β -actin and GAPDH expressed as a ratio of the control on adequate Met (control = 1). Values are mean $\pm$ SEM (n = 3 independent biological experiments). Different letters indicated significant differences among groups ($P < 0.05$).

FIGURE 6. The involvement of upstream regulators of apoptosis in imbalanced Met-induced changes of lipid metabolism in HepG2 cells line. (A, B, C and D) Met deficiency or excess-induced increase of the FITC+/PI+ positive rate (B), apoptosis index (C), and rates of apoptosis (D) were markedly alleviated by Z-VAD.FMK in HepG2 cells line *in vitro*. (E) Met deficiency or excess-induced change in the transcription of genes involved in upstream regulators of apoptosis were markedly attenuated by Z-VAD.FMK in HepG2 cells line *in vitro*. mRNA transcription values were normalized to β -actin and GAPDH expressed as a ratio of the control on adequate Met (control = 1). (F) Z-VAD.FMK significantly attenuated the Met deficiency or excess-induced increase of caspase-3

and -9 activities. (G) Met deficiency or excess-induced change in the mRNA abundances of genes involved in lipid metabolism were markedly attenuated by Z-VAD.FMK in HepG2 cells line *in vitro*. mRNA transcription values were normalized to β -actin and GAPDH expressed as a ratio of the control on adequate Met (control = 1). (H, I and J) Effect of Z-VAD.FMK on Met deficiency or excess-induced change in the activities of FAS, HSL and CPT-1, and the contents of APO-A1 and APO-B and TG. Values are mean $\pm$ SEM (n = 3 independent biological experiments). Different letters indicated significant differences among groups ($P < 0.05$).

FIGURE 7. Graphical conclusions for the mechanism of upstream regulators of apoptosis mediating methionine deficiency or excess-induced changes of hepatic lipid metabolism in *P. fulvidraco* and HepG2 cells line. The yellow words meant the significant influence by Met deficiency; the blue words meant the significant influence by Met excess; the black word meant significant influences by both Met deficiency and excess. The up and down arrows meant the increase and the reduction, respectively.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

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Supplementary Material

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