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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.

1. 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

Abbreviations: AKT, RAC-alpha serine/threonine-protein kinase; AMPK, AMP-activated kinase; APO, Apolipoprotein; cAMP, Cyclic AMP; CPT-1, Carnitine palmitoyl transferase-1; CREBCyclic, 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

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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].

2. Materials and methods

2.1. 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.*

2.1.1. 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.

2.1.2. 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 ± 0.02 g, 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 weeks), 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 mm^3 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 × *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 μg/ ml). Cells were counted using a hemocytometer based on the trypan blue exclusion method, and only those cultures with > 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 μM *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 μM), Met deficiency (10 mM), adequate Met (100 mM), Met excess (1000 mM), Met