Aquatic Toxicology 203 (2018) 69–79

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

CREB element is essential for unfolded protein response (UPR) mediating the Cu-induced changes of hepatic lipogenic metabolism in Chinese yellow catfish (*Pelteobagrus fulvidraco*)

Yu-Feng Song[a], Yi-Huan Xu[a], Mei-Qing Zhuo[a], Kun Wu[a], Zhi Luo[a,b,*]

[a] Key Laboratory of Freshwater Animal Breeding, Ministry of Agriculture, Fishery College, Huazhong Agricultural University, Wuhan 430070, China
[b] Collaborative Innovation Center for Efficient and Health Production of Fisheries in Hunan Province, Changde 415000, China

ARTICLE INFO

Keywords:
Copper
Unfolded protein response
Functional analysis of promoters
CREB
Lipogenic metabolism

ABSTRACT

The present study was conducted to explore the underlying mechanism of unfolded protein response (UPR) mediating the Cu-induced changes of hepatic lipogenic metabolism in a low vertebrate, freshwater teleost yellow catfish *Pelteobagrus fulvidraco*. To this end, three experiments were conducted. In Exp. 1, we cloned the regions of *grp78*, *perk*, *ire-1α* and *atf-6α* promoters, and found that multiple cAMP-response element binding protein (CREB) binding sites were identified in their promoter regions. Furthermore, these CREB binding sites played crucial role in transcriptional regulation of UPR. In Exp. 2, the involvement of *perk*, *ire-1α* and *atf-6α* in Cu-induced changes of hepatic lipid metabolism was confirmed by specific miRNA. In Exp. 3, the regulatory mechanism of CREB underlying UPR mediating Cu-induced hepatic lipogenic metabolism were investigated. Cu induced UPR via the activation of CREB binding sites in the promoter regions of *grp78*, *perk*, *ire-1α* and *atf-6α*. In addition, the inhibition of CREB markedly attenuated the Cu-induced up-regulation of hepatic lipogenic metabolism in hepatocytes. This conclusion was further supported by the results from the trial of CREB over-expression. Taken together, the present study indicated that CREB was essential for UPR mediating Cu-induced lipogenic metabolism, supporting a mechanistic link among CREB, UPR and Cu-induced changes of lipid metabolism.

1. Introduction

Disruption in endoplasmic reticulum (ER) homeostasis creates a state defined as ER stress, which initiates a complex signaling network called as unfolded protein response (UPR) (Hetz, 2012). UPR is an integrated intracellular signaling pathway that induces translational inhibition followed by the up-regulation of ER-resident chaperone (GRP78/BiP) (Hetz, 2012). UPR is characterized by downregulation of protein translation through three ER-transmembrane transducers: PKR-like ER kinase (PERK), inositol requiring enzyme (IRE)-1α and activating transcription factor (ATF)-6α (Volmer et al., 2013). Many environmental insults can disturb ER homeostasis and activate UPR (Han and Kaufman, 2016). In fish, our recent studies indicated that dietary and waterborne copper (Cu) exposure could induce UPR (Song et al., 2015, 2016, 2017); however, at present, the regulatory mechanism of Cu-induced UPR at the transcriptional levels remains to be explored in fish.

In addition, the ER membrane is the site of triglyceride (TG) synthesis and nascent lipid droplet formation (Sturley and Hussain, 2012). In recent years, increasing evidences indicated that UPR played crucial roles in hepatic lipogenic metabolism by mediating the transcriptional regulation of lipogenesis in mammals (Basseri and Austin, 2011; Zhang et al., 2012). In fish, although the involvement of UPR in hepatic lipid metabolism have been confirmed in our earlier studies (Song et al., 2015, 2016, 2017), information involved in the regulatory mechanism of UPR mediating hepatic lipogenic metabolism was very scarce at the transcriptional level.

Cu is a cofactor of many enzymes and plays an important role in fish metabolism; however, it can exert toxicological effects when present in excess amounts (Watanabe et al., 1997). Our recent studies indicated

Abbreviations: ACC, Acetyl-CoA carboxylase; CREB, cAMP-response element binding protein; ATF6, Activating transcription factor 6; ER, endoplasmic reticulum; ERSE, ER stress response element; FAS, Fatty acid synthase; GRP78/BiP, 78-kDa glucose-regulated protein; IRE1, Inositol-requiring enzyme 1; LXR, Liver X receptor; SCD, Stearoyl-coA desaturase; SREBP-1, Sterol-regulator element-binding protein-1; TSS, transcription start site; PERK, Protein kinase RNA-activated-like ER kinase; PPAR, Peroxisome proliferators-activated receptor; UPR, Unfolded protein response

* Corresponding author at: Key Laboratory of Freshwater Animal Breeding, Ministry of Agriculture, Fishery College, Huazhong Agricultural University, Wuhan 430070, China.
E-mail addresses: luozhi99@mail.hzau.edu.cn, luozhi99@aliyun.com (Z. Luo).

https://doi.org/10.1016/j.aquatox.2018.08.002
Received 21 June 2018; Received in revised form 31 July 2018; Accepted 1 August 2018
Available online 01 August 2018
0166-445X/

that both dietary and waterborne Cu influenced hepatic lipogenic metabolism via UPR activation in yellow catfish *Pelteobagrus fulvidraco*, an omnivorous freshwater fish and widely distributed in China and other Asian countries (Song et al., 2015, 2016). Moreover, our studies also suggested UPR mediating Cu-affecting hepatic metabolism in time-, concentration-and tissue-dependent manners in *P. fulvidraco* (Song et al., 2015, 2016). In fact, even different Cu-exposed routes (dietary vs. waterborne) will have differential effects (Song et al., 2015, 2016). Thus, the mechanisms of UPR mediating Cu-induced changes of hepatic lipogenic metabolism in *P. fulvidraco* might be multifactorial and complex. Therefore, it seems necessary and meaningful to investigate the mechanisms of UPR mediating Cu-induced changes of hepatic lipogenic metabolism.

Generally speaking, expression of eukaryotic genes is controlled at the level of transcriptional initiation. Promoters contain cis-acting sequences which serve as binding sites for a wide variety of regulatory factors that control the expression of individual gene (Lenhard et al., 2012). Thus, as a part of our project into the mechanisms of UPR mediating the Cu-induced changes of hepatic lipogenic metabolism in fish, at first, this present study cloned and characterized the promoter sequences of four vital UPR genes (*grp78*, *perk*, *ire-1α* and *atf-6α*) from *P. fulvidraco*, and the crucial role of CREB binding sites in mediating UPR were confirmed by using deletion analysis and site-directed mutagenesis assay. Then, given the close association between CREB and hepatic lipogenic metabolism (Herzig et al., 2003; Qiang et al., 2011), we explored the transcriptional regulation of CREB in Cu-induced UPR and Cu-induced changes of hepatic lipogenic metabolism. Taken together, the present study clearly indicated CREB was essential for UPR mediating the Cu-induced changes of hepatic lipogenic metabolism in *P. fulvidraco*. The results will be beneficial for us to elucidate the regulatory mechanism of UPR mediating the Cu-induced changes of hepatic lipogenic metabolism at the transcription levels, and provides new insights into physiological functions of Cu in fish and probably in other vertebrates.

2. Materials and methods

2.1. Drug treatment

The stock solution of $CuSO_4{\cdot}5H_2O$ was prepared to a concentration of 1 M with sterile double-distilled water. Dulbecco's Modified Eagles Medium (DMEM), 0.25% trypsin-EDTA and fetal bovine serum (FBS) were obtained from Gibco/Invitrogen, USA. 666-15 (specific inhibitor of CREB-mediated gene transcription, (Xie et al., 2017)), dimethyl sulphoxide (DMSO), penicillin, streptomycin, trypan blue and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The concentrations of Cu (10 μM) and 666-15 (an inhibitor for CREB; 15 μM) in the *in vitro* experiment were used based on our preliminary experiments and other studies in mammals (Song et al., 2016; Xie et al., 2017).

2.2. Experimental treatment

Three experiments were carried out. In Exp. 1, the promoter sequences of *grp78*, *perk*, *ire-1α* and *atf-6α* from *P. fulvidraco* were cloned and characterized, and the roles of CREB binding sites in promoter regions of *perk*, *ire-1α* and *atf-6α* were also investigated by using deletion and site-directed mutagenesis assay. In Exp. 2, the involvement of *perk*, *ire-1α* and *atf-6α* in Cu influencing hepatic lipogenic metabolism was demonstrated by specific miRNA. In Exp. 3, the transcriptional regulation of CREB in UPR mediating the Cu-induced changes of hepatic lipogenic metabolism were also investigated.

In the present study, primary hepatocytes were isolated from *P. fulvidraco* liver; HepG2 cell lines were obtained from the Cell Resource Center in the Fishery College of Huazhong Agricultural University. Studies have suggested that primary hepatocyte from *P. fulvidraco* and HepG2 cell lines were good model for studying the relationship between lipid metabolism and UPR (Tenore et al., 2014; Song et al., 2015). We ensured that the experiments were performed in accordance with the experimental protocols of Huazhong Agricultural University (HZAU) and approved by the ethics committee of HZAU.

2.2.1. Exp. 1: cloning and functional analysis of grp78, perk, ire-1α and atf-6α promoters, and investigating the role of CREB binding sites in their promoter regions on transcriptional regulation of UPR

The 5′-cDNA sequences of *grp78*, *perk*, *ire-1α* and *atf-6α* from yellow catfish were obtained according to our previous study (Song et al., 2015). Genomic DNA was extracted from yellow catfish tail fins using a commercial kit (Omega, Norcross, GA, USA). A series of primers (Supplementary Table S1) were designed to determine the position of the first intron of *grp78*, *perk*, *ire-1α* and *atf-6α*. The sequences of *grp78*, *perk*, *ire-1α* and *atf-6α* promoters were cloned using the hiTAIL-PCR method according to our recent studies (You et al., 2017; Zhuo et al., 2018). The specific primers with overlapping sequence were listed in Supplementary Table S1.

For the generation of the luciferase reporter constructs, the PCR products and pGl3-Basic vectors (Promega, Madison, WI, USA) were purified and digested using corresponding endonucleases and then products were ligated using ClonExpress™ II One Step Cloning Kit (Vazyme, Piscataway, NJ, USA) according to our recent studies (You et al., 2017; Zhuo et al., 2018). The plasmids were named as pGl3-1896/+68 of *grp78* vector, pGl3-1780/+57 of *perk* vector, pGl3-1808/+70 of *ire-1α* vector and pGl3-1936/+48 of *atf-6α* vector, respectively, according to the distance from their transcription start sites. Plasmids pGl3-95/+68, pGl3-154/+68, pGl3-449/+68, pGl3-890/+68, pGl3-975/+68, pGl3-1135/+68, pGl3-1225/+68, pGl3-1543/+68, pGl3-1896/+68 of *grp78* vector, which contained unidirectional deletions of the promoters, were generated with the Erase-a-Base system (Promega) using templates of -1896/+68 of *grp78* vector. Similarly, plasmids pGl3 of *perk* vectors, plasmids pGl3 of *ire-1α* vectors and plasmids pGl3 of *atf-6α* vectors were generated with the Erase-a-Base system (Promega) using pGl3-1780/+57 of *perk* vector, pGl3-1808/+70 of *ire-1α* vector and pGl3-1936/+48 of *atf-6α* vector as templates, respectively. All plasmids were sequenced for verification in a commercial company (Tsingke, Wuhan, China). The primer sequences used for plasmid construction are shown in Supplementary Table S2.

To identify CREB binding sites on the promoter regions of *grp78*, *perk*, *ire-1α* and *atf-6α* in yellow catfish, site-directed mutagenesis was performed using Quick Change II Site-Directed Mutagenesis Kit (Vazyme, USA), following the method described in our recent studies (You et al., 2017; Zhuo et al., 2018). For mutation analysis, pGl3-1896/+68 of *grp78* vector, pGl3-1780/+57 of *perk* vector, pGl3-1808/+70 of *ire-1α* vector and pGl3-1936/+48 of *atf-6α* vectors were used as templates, respectively. The mutations were conducted at the positions of -195/-192 and -1040/-1037 for *grp78* promoter, -347/-344 and -1094/-1091 for *perk* promoter, -198/-196 and -962/-959 for *ire-1α* promoter, and -715/-712 and -1467/-1465 for *atf-6α* promoter, respectively in Mutation 1 and Mutation 2. The mutagenesis primers were listed in Supplementary Table S3. Amplification was performed based on the following conditions: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 15 s, annealing at 57 °C for 30 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Then the site-mutated promoter fragments were sub-cloned to pGL3-basic vector using restriction sites Sal I and Hind III. All the plasmids were screened by restriction digestion and the mutations were confirmed by DNA sequencing. Then the plasmid and pRL-TK were co-transfected into HepG2 cells lines using the same method mentioned above. After 4 h, the transfection medium was replaced by 10% FBS-DMEM. After 24 h incubation, cells were harvested to assay the luciferase activity according to the procedure above.