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Characterization of twelve autophagy-related genes from yellow catfish *Pelteobagrus fulvidraco* and their transcriptional responses to waterborne zinc exposure

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ABSTRACT

Autophagy acts as important cytoprotective mechanism in response to adverse environment conditions. The hypothesis of the present study is that autophagy acts as protective responses to waterborne Zn exposure. To this end, the full-length cDNA sequences of 12 key genes related to autophagy in yellow catfish *Pelteobagrus fulvidraco* were cloned, and their mRNA expression profiles and transcriptional responses to waterborne Zn exposure were explored. The 12 genes (SQSTM1, Beclin1, ULK1A, ULK1B, ATG13-1, ATG13-2, ATG101, ATG9A, ATG9B, ATG3, ATG5 and ATG7) mediated the core autophagy machinery, including autophagosome membrane initiation, nucleation, expansion, closure and maturation. All of these members shared similar domain structure to their orthologous genes of other vertebrates. Their mRNAs were widely expressed in various tissues, but at different levels. Zn exposure increased the amount of hepatic autophagic vacuoles, and elevated the mRNA levels of SQSTM1, ULK1A, ULK1B, ATG13, ATG101, ATG9A, ATG9B, ATG3 and ATG7 in a dose- and time-dependent manner, indicating autophagy activation. These results indicated that autophagy acted as an adaptive response to protect from Zn toxicity and confirmed our hypothesis. Moreover, those up-regulated genes may play crucial roles in autophagy response to Zn exposure. For the first time, we characterized the full-length cDNA sequences of twelve autophagy related genes from fish, and determined their transcriptional responses to waterborne Zn exposure, which would contribute to our understanding of the molecular basis of autophagy and Zn toxicity, and also shed new insights on the potential role of autophagy as an adaptive response against metal toxicity in vertebrates.

1. Introduction

Autophagy is a bulk degradation pathway by which cytoplasmic cargo are sequestered inside double membrane vesicle (autophagosome) and delivered to lysosomes for destruction and recycling (Suzuki et al., 2017). Autophagy has been acknowledged to provide a survival strategy that supplies nutrients and maintains energy homeostasis (Mizushima et al., 2008). It has been linked to diverse physiological and pathological processes, such as cellular homeostasis, cell differentiation and growth, neurodegeneration, immunity and lifespan extension (Shintani and Klionsky, 2004). The core autophagy machinery, comprising eighteen autophagy related (ATG) proteins in yeast *Saccharomyces cerevisiae*, plays a predominant role in autophagosome formation. Many of these remain evolutionarily conserved in eukaryotes (Klionsky et al., 2016). They constitute six distinct functional groups: the ATG13-ATG101-ULK1 protein kinase complex, the Beclin1/ATG6 complex, the ATG7-ATG3-ATG5 ubiquitin-like conjugation system, the MAP1LC3 conjugation system, SQSTM1/p62 recruitment and ATG9 vesicle recycling system (Suzuki et al., 2017). Although autophagosome formation plays the crucial role in autophagy, its molecular mechanism remains elusive. Identification of these ATG genes was vital for characterizing the molecular basis and role of autophagy. To date, to our best knowledge, cloning and characterization of genes involved in autophagy have been reported in a very limited number of fish, such as Beclin1 in rare minnow *Gobiocypris rarus* (Gao et al., 2014), olive flounder *Paralichthys olivaceus* (Kong et al., 2011) and common carp *Cyprinus carpio* (Liu et al., 2016), ATG5 in zebrafish *Danio rerio* (Hu et al., 2011) and MAP1LC3 and ATG4 in yellow catfish *Pelteobagrus*

Abbreviations: ATG, autophagy related gene; B2M, beta-2-microglobulin; ELFA, translation elongation factor; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; RPL7, ribosomal protein L7; SEM, Standard Error of Mean; SQSTM1, Sequestosome-1; TBP, TATA-box-binding protein; TUBA, tubulin alpha chain; UBCE, ubiquitin-conjugating enzyme; ULK1, unc-51 like autophagy activating kinase 1

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fulvidraco (Wei et al., 2017). The molecular characterization and tissue distribution of SQSTM1, ULK1A, ULK1B, ATG13, ATG101, ATG9A, ATG9B, ATG3 and ATG7 genes are still unknown in teleosts.

Zinc (Zn) is an essential micromineral that plays important roles as a structural or a catalytic cofactor, and can regulate gene expression and signal transduction (King et al., 2016). However, Zn will be toxic at high concentrations. During the last decades, a dramatic increase in Zn occurs as a consequence of industrial, agricultural and anthropogenic activities, thus fish are often exposed to a significant amount of Zn (Heath, 1987). In some contaminated waters, 0.87 mg Zn/l concentration was reported in the river (Ma et al., 2009). Waterborne Zn toxicity affects Zn homeostasis, oxidative stress, gill morphology and liver damage, which results in higher mortality and reduces growth (Zheng et al., 2011; Huang et al., 2016). As a survival strategy, autophagy may be activated as an adaptive response to adverse environment conditions (Kroemer et al., 2010). Emerging evidence indicate that heavy metals are capable of inducing autophagy (Chatterjee et al., 2014; Wei et al., 2017). For example, lead (Pb) exposure triggers autophagic cell death in rat (Zhang et al., 2012). Cadmium (Cd) leads to autophagic occurrence in skin epidermal cells (Son et al., 2011) and in the kidney of common carp (*Cyprinus carpio*) (Gao et al., 2014). Given the potential role of Zn in autophagy, we hypothesize that autophagy acts as an adaptive response to waterborne Zn exposure toxicity.

Fish are the by far largest group of vertebrates in the world. In spite of this, studies on the autophagic machinery and its function in fish are scarce (Wei et al., 2017). Yellow catfish *Pelteobagrus fulvidraco*, an omnivorous freshwater fish, is a commonly-cultured fish species in many Asian countries and considered as a good model for toxicity research for its small size and easy reproduction. Recently, studies in our laboratory indicated that Zn exposure induced dysregulation of energy metabolism homeostasis, and activated ER stress and the unfolded protein response (UPR) signaling pathway in yellow catfish (Song et al., 2017). In general, autophagy has the role removing intracellular misfolded proteins and damaged organelles, and also has been recognized to be a adaptive catabolic process under adverse environment (Mizushima et al., 2008; Kroemer et al., 2010). Recently, we explored the effect of waterborne Zn exposure on mRNA levels of MAP1LC3 and ATG4 in yellow catfish (Wei et al., 2017). Given the complexity of the autophagic machinery, it is reasonable to hypothesize that waterborne Zn exposure can differentially influence mRNA expression of other ATG genes. To this end, in the present study we identified the full-length cDNA sequences of 12 genes (SQSTM1, Beclin1, ULK1A, ULK1B, ATG13-1, ATG13-2, ATG101, ATG9A, ATG9B, ATG3, ATG5 and ATG7) involved in autophagosome membrane initiation, nucleation, expansion, closure and maturation, and explored their tissue expression profiles in *P. fulvidraco*. Then, we characterised the transcriptional responses of those genes to waterborne Zn. The present study extends our understanding of the molecular basis of autophagy and Zn toxicity, and provides new insights into the adaptive protective mechanism of autophagic responses to metal toxicity in vertebrates.

2. Materials and methods

Two experiments were performed. Exp. 1 was performed to clone 12 autophagy-related genes and explore their mRNA tissue distribution. Exp. 2 determined their transcriptional responses and hepatic ultrastructural alterations to waterborne Zn exposure. The experimental protocol was approved by the Committee of Huazhong Agricultural University on the Ethics of Laboratory Animal Experiments.

2.1. Exp. 1: cloning and tissue distribution of 12 genes

2.1.1. Cloning of the full-length cDNA sequences

The experimental procedures for cDNA cloning were similar to those in our pervious studies (Wu et al., 2016; Wei et al., 2017). Briefly, gill, liver, mesenteric fat, brain, ovary, spleen, muscle, kidney, heart and intestine were removed from yellow catfish (22.5 ± 3.2 g/catfish, mean ± SEM), quickly frozen in liquid N_2 and stored at −80 °C for subsequent analysis. Degenerate primers were used to clone partial cDNA sequences (Supplementary Table 1). 5′ and 3′ RACE PCR were used to obtain their full-length cDNA sequences. The EDITSEQ (DNAstar) was used to find the ORF (open reading frame) of cDNA sequences and translate into amino acid sequences. The Clustal-W multiple alignment algorithm was used to assess sequence alignments of amino acid conservation. The online CDD tool at NCBI was used to analyze the domains. The phylogenetic trees were obtained by the neighbor-joining (NJ) method using MEGA 5.0 (Tamura et al., 2011) based on the JTT + G model (Jones et al., 1992).

2.2. Exp. 2: waterborne Zn exposure

The experimental protocols for waterborne Zn exposure have been described in Song et al. (2017). Briefly, 216 fish (8.54 ± 0.36 g/catfish, mean ± SEM) were randomly stocked in 9 tanks (300-L water volume), 24 fish in each tank. The fish were exposed to one of three nominal Zn concentrations: zero (control, without extra Zn addition), 0.25, and 0.50 mg Zn/L (corresponding to 2.5 and 5% of the 96 h 50% lethal concentration [LC50] of Zn for *P. fulvidraco*, respectively) (Zheng et al., 2013, 2015). Final Zn concentrations were determined to be 0.007 ± 0.001, 0.253 ± 0.004, 0.514 ± 0.070 mg Zn/L (mean ± SEM, n = 16), respectively, for the three treatments using inductively coupled plasma atomic emission spectrometry (ICP-AES). The detection limits are 0.002 mg Zn/L. Quality control blanks and standards were run every 20 samples. The accuracy of the method was evaluated by calibration vs. an international standard (B40094, Yuanye Biology Co., Shanghai, China) was used for the standard. Recovery of metals ranged from 94% to 104%. These analyses were conducted in duplicate.

During the experiment, all fish were fed a commercial pellet diet (dietary Zn contents: 17.45 mg Zn kg^{-1}) to apparent satiation twice daily. The ration level was about 4.0%-4.5% of fish body weight. The experiment continued for 56 days and sampling occurred on days 28 and day 56.

2.3. Sample analysis

2.3.1. Ultrastructural observation

The methods for ultrastructural analyses have been described in our studies (Liu et al., 2011; Song et al., 2016, 2017). Briefly, samples were prefixed in glutaraldehyde solution, followed by rinses with phosphate buffer solution (PBS). Post-fixation occurred in aqueous osmium tetroxide. After rinsing with PBS again, the specimens were dehydrated in a graded ethanol and then embedded in Epon prepared for electron microscope. For analyzing ultrastructural alterations, sample number is 3 (replicates of 3 fish). Ultrastructures were randomly examined with microscope fields for 10 sub-samples, and the results from three independent experiments were then combined for the evaluation of this experiment.

2.3.2. Quantitative RT-PCR (qPCR)

The mRNA expression was assayed by the real-time qPCR method described in Chen et al. (2015) and Wei et al. (2017). The primers used in this analysis are given in Supplementary Table 2. A set of ten housekeeping genes (RPL7, 18S rRNA, β-actin, HPRT, TUBA, B2M, TBP, GAPDH, ELFA and UBCE) were selected in order to test their transcriptional stability. B2M and RPL7 (M = 0.7215), B2M and RPL7 (M = 0.4837) showed the most stable level of expression in both tissues distribution analysis experiment and in waterborne Zn exposure experiments. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), normalizing to the geometric mean of B2M and RPL7 expression levels.

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