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Research paper

Effect of tributyltin chloride (TBT-Cl) exposure on expression of *HSP90β1* in the river pufferfish (*Takifugu obscurus*): Evidences for its immunologic function involving in exploring process

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ABSTRACT

HSP90β1 (known as glyco-protein 96, GP96) is a vital endoplasmic reticulum (ER) depended chaperonin among the HSPs (heat shock proteins) family. Furthermore, it always processes and presents antigen of the tumor and keeps balance for the intracellular environment. In the present study, we explored the effect of tributyltin chloride (TBT-Cl) exposure on *HSP90β1* expression in river pufferfish, *Takifugu obscurus*. The full length of *To-HSP90β1* was gained with 2775 bp in length, with an ORF (open reading frame) encoding an 803 aa polypeptide. A phylogenetic tree was constructed and showed the close relationship to other fish species. The *HSP90β1* mRNA transcript was expressed in all tissues investigated with higher level in the gill and liver. After the acute and chronic exposure of TBT-Cl, the *To-HSP90β1* mRNA transcript significantly was up-regulated in gills. Moreover, the histology study indicated the different injury degree of TBT-Cl in liver and gill. Immunohistochemistry (IHC) staining results implied the cytoplasm reorganization after TBT-Cl stress and the function of immunoregulation for To-HSP90β1 to TBT-Cl exposure. All the results indicated that *HSP90β1* may be involved in the resistance to the invasion of TBT-Cl for keeping autoimmune homeostasis.

1. Introduction

Heat shock proteins (HSPs) are a series of special proteins which could generate and be activated in the environment of heat stress and other biological stresses (Ritossa, 1962). HSPs are also called stress proteins (SPs) due to taking part in regulating other stress responses such as oxidative stress, heat, infection, toxicosis and so on (Erlejman et al., 2014a; Sørensen et al., 2003). It has been proved that HSPs have imperative roles in inhibiting protein aggregation, helping in folding the nascent proteins, and are considered to protect cells against oxidative stress (Fu et al., 2011; Jiang et al., 2012; Parsell and Lindquist, 1993). HSPs are a cluster of highly conserved molecular chaperones which were ubiquitously expressed in tissues. They are segmented into distinct multigenic families, like HSP110, HSP90, HSP70, HSP60, HSP40 and other small HSPs. Among them, HSP90 is often found in a constitutive dimmer, which participates in controlling multiple regulatory pathways such as stress defense and apoptosis (Rajeshkumar et al., 2013). In *Crassostrea hongkongensis*, HSP90 plays a vital role in response to both osmotic stress and bacterial invasion (Fu et al., 2011). For many fish species, the HSP90 have been connected to cytoprotection and cell survival (Csermely et al., 1998; Smith et al., 2015), performing a protective and inducible role (Xu et al., 2014; Zhang et al., 2015). HSP90 in the pufferfish liver was also found induced by ammonia stress, indicating that this kind of protein hammered at protecting body from oxidative stress and apoptosis (Cheng et al., 2015). It's worth noting that *HSP90β1* (GP96), a subtype of HSP90 members, associated with major histocompatibility complex (MHC) class I molecule, which indicated it might be involved in immune response (Suto and Srivastava, 1995). The mRNA level of *HSP90β1* preferentially expressed in hepatocellular carcinoma and significantly increased in hepatoma cell line. Its expression had a down-regulation when the oncocytes differentiation was inducted by sodium butyrate. This indicated

Abbreviations: HSPs, heat shock proteins; *To-HSP90β1*, *Takifugu obscures* heat shock protein 90β1; TBT-Cl, tributyltin chloride; IHC, immunohistochemistry; SPs, stress proteins; GP96, glyco-protein 96; ORF, open reading frame; bp, base pair; aa, amino acid; mRNA, messenger ribonucleic acid; cDNA, complementary DNA; ER, endoplasmic reticulum; MHC, histocompatibility complex; qPCR, quantitative real-time PCR; LC_{50}, medial lethal concentration; MS-222, tricaine methanesulfonate; NCBI, National Center for Biotechnology Information; PBS, phosphate-buffered saline; H & E, hematoxylin-eosin; SD, standard deviation; UTR, untranslated region; kDa, kilo-Dalton; pI, isoelectric point; BLAST, Basic Local Alignment Search Tool; ATP, adenosine triphosphate; NEFs, nucleotide exchange factors; BiP, heavy-chain binding protein; GRP, glucose-regulated protein; UPR, unfolded protein response; IgG, immunoglobulin G

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that *HSP90β1* had correlations with occurrence and development of cancer and cell differentiation (Cai et al., 1993; Heike et al., 2000b). Studies had shown that some stress factors made increases of expression level for GP96, in the meanwhile, its immunogenicity was also aggrandized and rose with the expression level increasing (Dai et al., 2003). In the process of autoimmunity, the cell surface expression level of an endoplasmic reticulum-dependent GP96 initiated systemic autoimmune diseases in the body (Liu et al., 2003).

Tributyltin chloride (TBT-Cl) is one of the most representative chemical compounds of Tributyltin (TBT). In view of its fatal toxicity to hydrobios, TBT-Cl was severed as a threat to water security (Antizar-Ladislao, 2008; Organization, 2001). TBT residual in water from various channels had become a noticeable problem, which made TBT contamination for aquatic ecosystems (Antizar-Ladislao, 2008; Tessier et al., 2007). TBT induced imposex in mollusks and fishes, which suggested that this toxic substance exerted a force on aquatic animal gonad function (Matthiessen, 2008; Mcallister and Kime, 2003; Nakayama et al., 2004; Shimasaki et al., 2003). Furthermore, TBT was found to be an inducer in the accumulation course of adipose and altered fatty acid levels in *Marisa cornuarietis* (Inadera and Shimomura, 2005; Janer et al., 2007; Meador et al., 2011). In zebra fish, TBT indeed altered multiple and complex activities of mRNA level in lipid metabolism and cell damage, which implied the underlying molecular mechanism of TBT on hepatic steatosis (Zhang et al., 2016).

Takifugu obscures, commonly known as river pufferfish, is an anadromous fish and an economic species. Studies on pufferfish aquaculture and its ecological environment have been regarded as a hot topic in the meanwhile (Kai et al., 2005; Van, 2004; Yamanoue et al., 2009). The pufferfish are important and scarce sources at the lower reach of the Yangtze River and the river mouth area in China. *T. obscures* was always chosen as a model to explore its adaptive and resisting mechanisms when exposed to different kinds of environmental stress factors (Kato et al., 2005; Kim et al., 2010b). However, the physiological function of the pufferfish under the TBT-Cl exposure keeps unclear. It's attractive to us that studying the mechanism of TBT-Cl exposure in *T. obscures* may have a profound meaning.

In this regard, *HSP90β1* gene in *T. obscures* from transcriptome sequencing databases was characterized by bioinformatic analysis, and the phylogenetic tree was constructed based on HSP90 sequences of other species. Tissues expressions were detected by quantitative real-time PCR (qPCR) method. After exposing to different concentrations of TBT-Cl in the acute and chronic experiment, the *HSP90β1* mRNA level was checked through qPCR. The histochemistry and IHC test were performed to verify the damaging effect of TBT-Cl to the pufferfish. This study may supply a deep understanding for the unique function of *HSP90β1* in the course of fighting with the adverse effect of TBT-Cl and explain the conceivable mechanism in immunoreaction.

2. Materials and methods

2.1. Animals

T. obscurus (two months old, average length = 10 ± 1.5 cm with average weight = 25.1 ± 2.23 g) were obtained from the aquaculture base in Freshwater Fisheries Research Center (FFRC, Wuxi, China). The pufferfish were kept in 100 L cylindrical opaque polypropylene aquaria and supplied with commercial feed twice a day at regular intervals. After 7-day acclimation, robust animals were chosen until 24 h feeding before the experimental treatment. The water was exposed to air for a week to remove chlorine. During the experiment, the temperature was kept at 26 ± 2 °C, the dissolved oxygen and pH were maintained at 7.93 ± 0.45 mg/L and 7.83 ± 0.12, respectively. The blood, liver, gill, heart, muscle, stomach, intestine, kidney, spleen and brain were sampled for the follow-up study. All operations to the pufferfish were carried out in strict accordance with the recommendation in the criterion for the care and use of laboratory animals.

2.2. TBT-Cl exposure and sampling

Healthy fish were randomly chosen and divided into four groups. On the basis of 96 h acute toxicity experiment (96 h-LC_{50} = 19.62 μg/L), the pufferfish were exposed to three kind of concentrations of TBT-Cl (10% 96 h-LC_{50}, 20% 96 h-LC_{50} and 50% 96 h-LC_{50}) and the DMSO (dimethyl sulphoxide) solution (V (DMSO): V (water) = 1‰). Six individuals were put into a group randomly. After the exposure, at the time point of 96 h, fish were collected (n = 6) and anesthetized in diluted tricaine methanesulfonate (MS-222, Sigma, USA) at the concentration of 100 mg/L. The fish were put on the ice and sampled with liver and gill.

For the chronic toxicity experiment, the treatment group (900 ng/L of TBT-Cl) and a control group (DMSO group) were set. 24 fish were randomly put in each container. The experimental period was 30 days, and sampling was performed every 10 days. Six animals were collected at each timepoint (n = 6). The fish were collected in each group randomly. The liver and gill were sampled for later use.

After 30 d exposure experiment, the 30 days recovery test was followed. The water in all groups was changed to aerated tap-water. Fish were collected every 15 days. Six animals were gained in each group randomly (n = 6). After normal saline wash, the fish put on ice were rapidly sampled and its liver and gill were sampled. All the serum and tissues were snap-frozen in liquid nitrogen after labeled and stored at −80 °C for later assay.

2.3. Total RNA extraction and cDNA preparation

Total RNA was isolated from the harvested pufferfish tissue using Trizol reagent (Invitrogen, USA) according to the kit's instruction, then dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at −80 °C. The cDNA template was prepared containing 2 μg total RNA by reverse transcription reaction by a PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan) following the protocol. The concentration and quality of RNA and DNA products were measured by spectrophotometry and agarose gel electrophoresis, respectively.

2.4. The full-length cloning and phylogenetic analysis

Target sequences of cDNA encoding *HSP90β1* was obtained from the library of transcriptome sequencing (unpublished data). The specific primer for *HSP90β1* was designed using Primer Premier 5.0 (*To-HSP90β1*-S: TGGTGGGAGCGGTGGCTTGTCAGTCCTCTTGT; *To-HSP90β1*-A: AGAACCACAGTGGAGCTGGAACTCTCAGAC). The full-length template for cloning *To-HSP90β1* was verified by PCR amplification. Its product was determined by agarose gel electrophoresis. The biological sequence obtained was analyzed by BLAST (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi) in NCBI (National Center for Biotechnology Information) (Mcginnis and Madden, 2004). The program ClustalW2 was used to perform multiple sequence alignment (Chenna et al., 2003; Thompson et al., 2002). The phylogenic tree was constructed by MEGA 6.0 through a neighbor-joining (NJ) algorithm based on the deduced amino acid sequence of HSP90 from some other species (Kelly et al., 2006; Yu et al., 2015).

2.5. qPCR detection of tissues expression patterns

The cDNA samples (n = 6) collected from blood, liver, stomach, intestine, gill, heart, muscle, kidney, spleen and brain above were all performed to determine their expressions by qPCR. The primer of *To-HSP90β*1 was designed (RT-*HSP90β1*-F: CCCTGGAGAAGGACTTTG AGC, RT-*HSP90β1*-R: GGGGTGTTTGGGGTTGATTT). *β-Actin* (RT-*β-actin*-F: AGAGGGAAATCGTGCGTGAC, RT-*β-actin*-R: CAAGGAAGGA TGGCTGGAAG) in *T. obscurus* (GeneBank accession number: EU871643) was measured as the internal control to normalize the level of qPCR results. Before beginning the qPCR program, the specificity and