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Induction of liver GST transcriptions by *tert*-butylhydroquinone reduced microcystin-LR accumulation in Nile tilapia (*Oreochromis niloticus*)

Shan He, Xu-Fang Liang *, Jian Sun, Dan Shen

Key Laboratory of Freshwater Animal Breeding, Ministry of Agriculture, College of Fisheries, Huazhong Agricultural University, Wuhan 430070, China

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ABSTRACT

The cyanobacterial toxin, MC-LR, is predominantly presented during toxic cyanobacterial blooms and is consumed by phytoplanktivorous fish and zooplanktivorous fish directly. Detoxification of MC-LR in liver was believed to begin with conjugate formation with GSH, catalyzed by GSTs. MC-LR GSH conjugates display increased solubility and are subjected to accelerated biliary excretion. In this study, we showed that the mRNA transcriptions of GSTA, GPX and UCP2 were increased within 8 h following MC-LR exposure in isolated hepatocytes of Nile tilapia, confirming the roles of phase II enzymes, especially GSTs, in MC-LR detoxification in tilapia. The widely used food-additive, synthetic antioxidant, *tert*-butylhydroquinone (tBHQ) has been shown to induce phase II enzymes including GSTs, via the antioxidant responsive elements (ARE) locate in the regulatory regions of these genes. Our results also showed that the transcription of various GSTs, including GSTA, GSTR2 and GSTT were significantly induced by tBHQ in Nile tilapia. In consistence, fish fed on tBHQ-containing diet (0.01 percent tBHQ) showed significantly reduced MC-LR accumulation in liver tissues 48 h after an oral administration of a single dose of 250 μg MC-LR/kg body weight (bwt). The findings in this research suggested that tBHQ could reduce MC-LR accumulations in liver, likely through the induction of phase II metabolizing enzymes such as GSTs. Subacute effects of tBHQ and its potential applications in fishery need to be further investigated.

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1. Introduction

The occurrence of toxic cyanobacterial blooms in eutrophic fresh waters has been a worldwide problem. Despite of the various aquatic organisms that can be affected, phytoplanktivorous fish and zooplanktivorous fish that consume cyanobacteria are exposed directly to cyanotoxins, which can be transferred through food web subsequently. Effects of cyanobacterial toxins usually involved changes in liver enzymology, liver damage and ionic imbalance, although reduced reproduction and abnormities in embryonic development were also observed (Oberemm et al., 1999; Liu et al., 2002). Mass mortalities of fish and bird have also been reported in rare cases (Ibelings and Havens, 2008). However, due to multiple factors that co-occurred during cyanobacterial blooms, the contributions of cyanotoxins were difficult to evaluate.

Among the many cyanotoxins, microcystins (MCs), especially MC-LR, are prominently presented and are highly toxic (Martins and Vasconcelos, 2009). MCs are microcyclic heptepeptides and are known to be potential hepatotoxins (Codd, 1995; Dawson, 1998). Intraperitoneal injection of MC in fish usually resulted in mortality following liver failure while oral exposure of the same toxin seemed to cause only mild symptoms. Upon ingestion, MC-LR is actively degraded. The limited amount of toxin that is taken up into blood and organs is detoxified and excreted (Ibrahem et al., 2012). However, accumulations of MC-LR are commonly found in the gut and liver tissues across fish species, as well as in fish feces. As consequences, MC-LR is found in both higher trophic levels and organisms of the benthic community such as in macroinvertebrates, in which accumulation of toxins may prolong the negative impacts on the ecosystem. The detection of MC-LR in fish muscles (Magalhaes et al., 2001) and the risk of drinking MC-LR contaminated water raised general concerns on the potential threats to human health if these fish would be consumed, especially when the amount of the toxin in muscle tissues exceeded the tolerable daily intake (0.04 mg/kg/d), as recommended by the World Health Organization.

Direct hepato-cytotoxicity of MC-LR is due to the inhibition of protein phosphatases, which results in hyperphosphorylation of cellular proteins (Rymuszka et al., 2010). Detoxification of MC-LR may involve both phase I and phase II metabolizing enzymes, which catalyze bioactivation into electrophilic species and conjugate formation, respectively. The detailed detoxification pathways seemed to vary in different fish that exposed to MC-LR via

* Corresponding author. Fax: +86 27 8728 2114.
E-mail address: xufang_liang@yahoo.com (X.-F. Liang).

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different routes (He et al., 2010; Ziková et al., 2010). However, amongst others, formation of MC-LR-GSH conjugates, catalyzed by phase II detoxification enzymes glutathione *S*-transferases (GSTs), has been shown in most organisms and results in reduced toxicity and accelerated excretion of MC-LR (Kondo et al., 1996; Pflugmacher et al., 1998; Takenaka, 2001). Covalent binding to GSH also depletes the cellular GSH pool, which subsequently causes increased oxidative stress due to the formation of reactive oxygen species (ROS) (Ishisaki et al., 2001; Murphy et al., 1991). ROS induce a wide range of cellular responses including lipid peroxidation, which is one of the indirect toxic effects of MC-LR. In the meantime, oxidative stresses also activate protein kinase transcription factors such as nuclear factor erythroid 2-related factor 2 (Nrf2), which in turn induces phase II enzymes via a *cis*-acting regulatory sequence known as the antioxidant responsive element (ARE) (Tony-Kong et al., 2001). Given the complexity in ecosystem during cyanobacteria blooms, factors such as the existance of synergistic compounds in the ecosystem, temperature, as well as the status and the feeding histories of the exposed animals, can all affect the metabolism and the toxity of MC-LR.

tert-butylhydroquinone (tBHQ) is an phenolic antioxidant that is widely used as a food-additive. Dietary intake of tBHQ was shown to alter the activities of the endogenous antioxidant enzymes through activation of Nrf2 in both mammals and fish (Kobayashi et al., 2002; Nouhi et al., 2011). Induction of a wide range of antioxidative potentials such as elevation of the glutathione levels and GSTs, via the activation of AREs found in the 5′-flanking regions of several phase II enzyme genes, was believed to be responsible for the protective effect of tBHQ (Favreau et al., 1991; Rushmore and Pickett, 1990).

In this study, the influence of tBHQ on the accumulation and metabolism of MC-LR was investigated in a phytoplanktivorous fish, Nile tilapia. The transcriptions of both phase I and phase-II detoxification enzyme, including cytochrome P450 1A1 (Cyp1A1), glutathione peroxidase (Gpx), uncoupling protein 2 (Ucp2), Glutathione *S*-transferases (GSTs), as well as heat stock protein 70 (Hsp70), were analyzed in isolated hepatocytes exposed to MC-LR. Transcription of GSTA was significantly induced by MC-LR exposure. The expression of GSTR, a rho class GST, which found no homologue in mammals and was suggested to play important roles in reducing the harmful effects of xenobiotic exposure (Martinez-Lara et al., 2002; Konishi et al., 2005), was not affected by MC-LR exposure. Due to the implicated roles for GSTs in detoxification, several subclasses of GSTs were cloned from the liver of Nile tilapia and were subsequently characterized by phylogenetic analyses in this study. The transcriptions of these GSTs in respond to MC-LR exposure and the effects of tBHQ on the transcription of these GSTs, following MC-LR exposure, were further investigated in the liver tissues in vivo using real-time RT-PCR. In line with the increased transcriptions of GSTs, our results also showed that oral administration of tBHQ significantly accelerated MC-LR metabolism and less MC-LR was detected in the liver tissues of Nile tilapia treated with tBHQ.

2. Material and methods

2.1. Materials

Cyanobacterial toxin MC-LR and *tert*-butylhydroquinone (tBHQ) were purchased from ALEXIS (USA) and SIGMA (USA), respectively. All the primers used in this research were synthesized by Sangon (Shanghai, China).

2.2. Hepatocytes isolation and MC-LR exposure

Nile tilapias (340 ± 21.2 g) were obtained from Guangdong Freshwater Fish Farm (Panyu, Guangdong Province, China). During a three-week acclimatization, fish were kept in 96 l of fresh water with continuous system of water filtration and aeration at constant temperature (24 ± 2 °C). Fish were fed with commercial feed at a rate of 4 percent of body weight two times (9:00 and 15:00) a day.

Fish were then killed by a sharp blow on the head and livers were collected individually. After extensive wash to eliminate as much blood as possible, hepatocytes were isolated by 0.25 percent trypsin digestion at 28 °C for 40 min. Cells were suspended in Hanks buffer and washed with the same medium for three times. Isolated hepatocytes were maintained in 6-well petri dishes in DMEM containing 100 IU/ml penicillin, 100 μg/ml streptomycin and 5 percent fetal bovine serum and cultured at 28 °C, supplied with 5 percent CO_2. The cells were then exposed to 10 μg/l MC-LR or the above described media only ('control' treatment) for various periods as indicated in results. Every treatment was conducted in sextuplicate. The number of cells was counted and cell viability was determined for each sample using Trypan blue exclusion at each time point before quantification of gene expression.

2.3. Rearing of Nile tilapia and exposure scenario

Nile tilapia of mean body weight of 89.34 ± 4.12 g, were obtained from Guangdong National Tilapia Farm (Guangdong Province, China). During a three-week acclimatization, fish were kept in aquariums with continuous system of water filtration and aeration at constant temperature (27 ± 2 °C). Fish were fed with commercial fish diet at a rate of 4 percent of body weight two times (9:00 and 15:00) a day.

Fish were maintained in two separate tanks and were fasted for 24 h before oral gavaging of 4 percent of body weight commercial fish diet containing 0.01 percent tBHQ and 250 μg/kg body weights (bwt) of MC-LR, representing the environmentally relevant daily maximum dose of MC-LR, while the control fish were fed with the same diet but tBHQ free. These fish were then maintained in the same aquariums. At each time point, six fish from each group were sacrificed and the liver tissues were sampled at 7 h, 24 h and 72 h post treatment. The amount of MC-LR and relevant gene transcription in the liver tissues were determined.

2.4. Total RNA isolation from hepatocytes and liver tissues

Total RNA was isolated from cultured hepatocytes or liver tissues using SV Total RNA Isolation System (Promega, USA), as described in the manufacturer's instruction. The extracted RNA was resuspended in 50 μl RNase-free water and quantified using Eppendorf Biophotometer (Germany). Samples were stored at −80 °C before use.

2.5. Cloning of partial cDNAs of Nile tilapia GSTR2, GSTM, GSTT

Reverse transcription was performed with oligo $(dT)_{20}$ primer using First Strand cDNA Synthesis Kit (Toyobo, Japan). Three pairs of degenerate primers GSTR01F and GSTR02R, GSTM01F and GSTM02R, GSTT01F and GSTT02R (Table 1) were used to amplify Nile tilapia GSTR2, GSTM and GSTT partial cDNAs with Taq polymerase (Takara, Japan). The PCR parameters were 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 40 °C for 1 min and 72 °C for 1 min. Final extention was performed at 72 °C for 5 min for each reaction. PCR products of the expected lengths were purified from 2.0 percent agarose gel and subsequently cloned into the pMD19-T Vector (Takara, Japan). The constructs were verified by sequencing using an ABI Prism™ 377 (Perkin Elmer, USA).

2.6. Cloning and analysis of full-length cDNA sequences of Nile tilapia GSTR2

Gene-specific primers for 5′-RACE and 3′-RACE were designed according to the sequenced PCR fragments of Nile tilapia GSTR2 cDNA. 5′-RACE was performed using Smart RACE cDNA Amplification Kit (Clontech, Palo Alto, California). One-microgram total RNA was reversely transcribed with the 5′-RACE CDS primer and Smart II A oligonucleotide (provided in the kit). In the first PCR, the cDNA was amplified with GSTR25'01R (5′-TGATGACATTGTCTCCATG-3′) and Universal Primer A Mix (UPM, provided in the kit). The second PCR was performed using GSTR25'02R (5′-CTTGTTGGGACTTGTGTT-3′) and Nested Universal Primer A (NUP, provided in the kit). The 5′-RACE PCR products were purified from agarose gel and cloned into the pMD-19 vector for sequencing.

The 3′-RACE reaction was performed using a 3′-Full RACE Core Set (TaKaRa, Japan). Total liver RNA was reverse transcribed to cDNA with oligo (dT)-3′site adaptor primer (provided in the kit) using a sequential program of 30 °C for 1 min, 50 °C for 30 min, and 95 °C for 5 min. The cDNA was first amplified with primer GSTR23'01F (5′-CCAACAGTTGGCACTCTCTT-3′) and 3′site adaptor primer (provided in the kit). The secondary PCR was performed using GSTR23′02F (5′-GGAGAGTATCACAATCTGC-3′) primer and 3′site adaptor primer. The 3′-RACE PCR products were purified from agarose gel and cloned into the pMD-19 vector for sequencing.

Phylogenetic comparison of full length protein sequences was performed using Mega 3.0 software (http://www.megasoftware.net/) and the phylogenetic tree was constructed using the Neighbor-joining (NJ) method.

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