Comparative Biochemistry and Physiology, Part C 152 (2010) 473–479

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Effect of cadmium exposure on expression of antioxidant gene transcripts in the river pufferfish, *Takifugu obscurus* (Tetraodontiformes)

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ARTICLE INFO

Article history:
Received 30 December 2009
Received in revised form 2 August 2010
Accepted 2 August 2010
Available online 7 August 2010

Keywords:
Takifugu obscurus
Cadmium chloride
Antioxidants
CAT
GR
GPx
Mn-SOD
Cu/Zn-SOD

ABSTRACT

Cadmium (Cd) is a non-essential toxic heavy metal with the potential to induce oxidative stress. Cd toxicity and its capacity for accumulation in aquatic habitats have earned its recognition as a pollutant of immediate and widespread concern. To obtain a better understanding of oxidative stress-associated gene expression in different tissues, six antioxidant genes such as catalase (*CAT*), glutathione reductase (*GR*), glutathione peroxidase 1a (*GPx1a*), glutathione peroxidase 1b (*GPx1b*), Cu/Zn superoxide dismutase (*Cu/Zn-SOD*), and Mn superoxide dismutase (*Mn-SOD*) were cloned and fully sequenced in the river pufferfish, *Takifugu obscurus*. On tissue specific mRNA expression, the liver showed the highest expression when compared to other tissues, even though each antioxidant gene showed different modes of expression patterns in the examined tissues. Of the various antioxidant genes, *GR* was the most highly expressed in the liver, followed by *CAT*, *GPx1*, and *Cu/Zn-SOD*. For the time-course experiment, all the antioxidant genes were significantly induced over time except for *Cu/Zn-SOD* in the liver, and there was a 5-fold induction in hepatic *GR*, *CAT*, and *Mn-SOD* mRNA compared to the control. These findings indicate that the liver of *T. obscurus* has a robust antioxidant system. In addition, these results suggest that Cd exposure modulates the expression of antioxidant genes, and would indicate that the antioxidant genes would be a relevant biomarker of trace metal pollution such as Cd exposure in *T. obscurus*.

1. Introduction

Environmental contamination of cadmium (Cd) in aquatic habitats is an important global problem due to its persistence and capacity for bioaccumulation (Satarug et al., 2003; Ruangsomboon and Wongrat, 2006). In polluted marine environments, fish are continuously exposed to ambient Cd through both water and food. Several mechanisms of Cd-induced toxicity have been suggested in both in vivo and in vitro situations (Stacey and Klaassen, 1981; Goering and Klassen, 1984; Klaassen and Liu, 1998). In fact, previous studies have shown that Cd disturbs the ion-regulatory system in several organisms (McGeer et al., 2000; Radi and Matkovics, 1988; Waisberg et al., 2003), ultimately leading up to the interference of the cellular signaling network and gene regulation at different levels (Wang et al., 2004).

Regarding the oxidative stress-inducing effect, Cd^{2+} itself is unable to generate free radicals directly (Nemmiche et al., 2007), but an indirect generation of superoxide radicals and hydroxyl radicals has been reported (Galan et al., 2001). Cd^{2+} is also known to be a promoter of oxidative stress by inducing the formation of reactive oxygen species (ROS), including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO^-) (Tamás et al., 2009). Oxidative stress induced by Cd^{2+} can negatively affect DNA, RNA, and ribosome synthesis, and consequentially inactivate enzyme systems (Stohs et al., 2000). Most of the ROS are neutralized by the cellular self-defense system, antioxidant enzymes, and free antioxidants such as glutathione (GSH). To date, several antioxidant defense enzymes such as glutathione *S*-transferase (*GST*), superoxide dismutase (*SOD*), catalase (*CAT*), glutathione reductase (*GR*), glutathione peroxidase (*GPx*) have been found in most organisms including teleost (Basha and Rani, 2003; Abele and Puntarulo, 2004). In the antioxidant defense system, the superoxide anion, arising either through metabolic processes or oxygen activation by physical irradiation, is dismutated into H_2O_2 by superoxide dismutase. Subsequently, catalase initiates the decomposition of H_2O_2 into H_2O and O_2. The SODs are classified

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doi:10.1016/j.cbpc.2010.08.002

according to the metal content such as manganese SOD (*Mn-SOD*) and copper/zinc SOD (*Cu/Zn-SOD*). SOD functions in conjunction with H_2O_2-reducing enzymes such as CAT and GPx (Schreibelt et al., 2007). GSH is also an effective antioxidant, which can bind metals that otherwise induce oxidative stress. GSH is instantly oxidized by ROS, and is converted to glutathione disulfide (GSSG) (Vandeputte et al., 1994). The reduction of GSSG to yield GSH is catalyzed by *GR* (Stohs and Bagchi, 1995).

The genus *Takifugu* has attracted the attention of scientists for their peculiar biology, physiology, and genomics (Van de Peer, 2004; Kai et al., 2005; Yamanoue et al., 2009). *Takifugu* spp. offers a suitable test system for the study of stress responses, as these species are ecophysiologically adapted to a wide range of habitat changes that correspond with pollution (Kato et al., 2005). During their migration for spawning from the ocean to the river, these fish are exposed to a number of diverse pollutants in their respective environments. They might, therefore, be an interesting model in order to understand and characterize adaptive mechanisms under stress conditions. In our previous studies, Kim et al. (2008a, 2010) has cloned the cDNA coding for metallothionein (*MT*) and seven *GSTs* from *Takifugu obscurus*, and determined of the modulatory pattern of mRNA expression after exposure to Cd chloride. They reported that Cd induced those mRNA expressions in a time- and dose-dependent manner. In addition, they verified the usefulness of the *T. obscurus MT* and *GST* genes as a biomarker for evaluating Cd pollution.

Along with previous findings, in this paper, we discussed the role of other antioxidant genes that are involved in the antioxidant defense mechanism against Cd-induced oxidative stress. The aims of this study were to characterize the mode of expression of several antioxidant genes in different tissues from *T. obscurus,* and to examine its transcriptional responses upon Cd exposure. In this paper, we suggest that the river pufferfish, *T. obscurus* antioxidant defense enzymes would be a good biomarker for use in marine environment toxicity assessment.

2. Materials and methods

2.1. Fish

The juveniles *T. obscurus* (body length, 12.6 ± 2.3 cm; body mass 35.5 ± 3.8 g) were obtained from the Yangchon fish hatchery (Gimpo, Gyounggi-do, South Korea). The fish were reared and maintained in aquarium (Department of Chemistry, Hanyang University, Seoul, South Korea) at 23 ± 1 °C with 12 h/12 h light/darkness cycles, pH 7.88, dissolved oxygen, 6.0 mg/L and 15 ± 0.5‰ salinity for 2 weeks in 100 liter aquaria. The commercial fish diet S7 (Higashimaru Foods, Inc., Kagoshima, Japan) was supplied to satiation twice a day. Tank water was replaced every day to minimize the contamination from metabolic wastes, and the fish were used according to animal care committee agreements.

2.2. Total RNA extraction and cDNA synthesis

Fish tissue was homogenized in TRIZOL® reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Total RNAs were extracted according to the manufacturers' protocol. RNA qualities were confirmed spectrophotometrically. Single-stranded cDNA was synthesized from 2 μg total RNA using an oligo $(dT)_{20}$ primer and the SuperScript™ III RT kit (Invitrogen™, Carlsbad, CA, USA) by reverse transcription according to the manufacturers' protocol.

2.3. Cloning and sequencing of cDNAs for T. obscurus antioxidant genes

Several degenerative primers were designed using the conserved domains after multiple alignments of full-length or partial cDNA sequence of each antioxidant gene reported from other species. For amplifying the partial sequences in *T. obscurus* antioxidant genes, the RT-PCR was carried out using 2 μM of each primer and hepatic cDNA as a template using *Taq* polymerase (TaKaRa Bio Inc., Japan) under the following conditions: 5 min at 95 °C, 40 cycles of 98 °C for 25 s, 55 °C for 30 s, and 72 °C for 60 s, and 10 min at 72 °C. Information of primers used in this study is shown in Table 1. The RT-PCR products were purified from 1% agarose gel using the Gel Extraction kit (Qiagen, Hilden, Germany), ligated into pCR2.1TA plasmid vector, and transformed into the competent *Escherichia coli* (Invitrogen™, Carlsbad, CA, USA) cells. The recombinant plasmid DNA was isolated from bacterial cultures using the Plasmid Purification Kit (Promega, Madison, WI, USA), and further subjected to DNA sequencing analysis (Bionix, Seoul, Korea).

Table 1
PCR primers used in this study.

Gene	Oligo name	Sequences (5′→3′)	Remarks
TO-Mn-SOD	F	GACCTACGACTACGGAGCCTTGG	cDNA amplification
	R	ATGCCAAGGAGCGGGATGAGACC	
	3GSP1	CTGCGCTGAAGTTTAACGGAGG	3CCGACAG
	3GSP2	CCAGAAGATGAAGGAGAAGATG	
	5GSP1	GATAGTAGGCGTGCTC	5ATAGTA
	5GSP2	CGCCGTTTGGAGAGAGGTTC	
	5GSP3	GCGCAGGCTGGAGAGCAACC	
	Mn S-F	AGATGTCCGCCGCTACAGTTGC	Real-time PCR
	Mn S-R	GCCAAGGAGCGGGATGAGACC	
TO-Cu/Zn-SOD	F	GTGCGTGTTAAAAGGAGCCG	cDNA amplification
	R	GATGCCGATGACTCCACAGG	
	3GSP1	GCCGACAGGCATGTTGGAGACC	3GTAGTT
	3GSP2	ATCCACGAGAAGGCCGACGAC	
	5GSP1	TCTACGGGCTGATTAC	5CTACGG
	5GSP2	AATAGGGGCCAGTGAGGGTCAAC	
	5GSP3	AATCTCCCCGGTCAGCTTCACAG	
	Cu/Zn S-F	GCCGACAGGCATGTTGGAGACC	Real-time PCR
	Cu/Zn S-R	CAGGTCGTCGGCCTTCTCGTG	
TO-GR	F	AGGTGATGTGGAATGCTGC	cDNA amplification
	R	TGAAGCATCTCATCACAGCCCAGG	
	3GSP1	CTCACACCTGTTGCCATTGCTGC	3TCACAC
	3GSP2	CAGTGGGACTAACAGAAGAGGAGG	
	5GSP1	CCTGGATAGTTCTGAG	5CTCTTC
	5GSP2	CCGCACCAACCACAACGCTG	
	5GSP3	CCAGTGGCGATGAGGATGTGAGG	
	GR-F	CTCACACCTGTTGCCATTGCTGC	Real-time PCR
	GR-R	GCCTCCTCTTCTGTTAGTCCCAC	
TO-CAT	F	CCATCCCATTGGGGACAAACTG	cDNA amplification
	R	GCGAACCTGAGTGACATTATCC	
	3GSP1	TGAGCCAAGCCCTGACAAGATGC	3GAGCCA
	3GSP2	CAGCGCGATGGTCCGATGTGC	
	5GSP1	CTCCAATGGCATAGTC	5TCCAAT
	5GSP2	GGATGAAGGACGGGAACAACAGG	
	5GSP3	TGACTGCAAAGCCTCGTGGATCTC	
	CAT-F	TGAGCCAAGCCCTGACAAGATGC	Real-time PCR
	CAT-R	GGTAGTTGGCCACACGGGTTCTG	
TO-GPx1a	F	GGGTGTCCCCTGCAATCAGTTTGG	cDNA amplification
	R	GGCTCGCCGTCGGGACCAATG	
	3GSP1	GTCCGTCCTGGAGGTGGTTTCG	3TCCGTC
	3GSP2	CCCTCTGATAATTCCATGGCTCTC	
	5GSP1	CATCGTTCCTGTTGAC	5ATCGTT
	5GSP2	GAGAGCCATGGAATTATCAGAGG	
	5GSP3	GGTGGGCATTCTTTCCATTTACG	
	GPx a-F	TGTCCGTCCTGGAGGTGGTTTCG	Real-time PCR
	GPx a-R	ACTTTGGGTCAGCCATGAGAGC	
TO-GPx1b	F	GGGTGTCCCCTGCAATCAGTTTGG	cDNA amplification
	R	GGCTCGCCGTCGGGACCAATG	
	3GSP1	CCTGCAATCAGTTTGGCCATCAG	3CTGCAA
	3GSP2	GGACGTGAACGTGAAGGATGC	
	5GSP1	AGTGAGCACAGGTTTG	5GTGAGC
	5GSP2	CCTGGCTGAGGAGCTTCTTTATG	
	5GSP3	CCAGGGCGGACATATTTCAGAG	
	GPx b-F	CCTGCAACCAGTTCGGCCATCAG	Real-time PCR
	GPx b-F	GTGGGCATCCTTCACGTTCACG	
TO-β-actin	RT-F	CCTCTTCCAGCCATCCTTCC	
	RT-R	GTTGGCATACAGGTCCTTACG	

TO = Takifugu obscurus.