Expression of Cu/Zn Superoxide Dismutase (Cu/Zn-SOD) mRNA in Shark, *Schyliorhinus torazame,* Liver during Acute Cadmium Exposure

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Superoxide dismutase (SOD), an antioxidant enzyme catalyzing the first step for scavenging the reactive oxygen species is important as an early warning indicator to address various biological stresses. For this reason, the monitoring the expressed pattern of SOD gene in fish organs is one of important biomarkers to assess the aquatic pollution caused by many toxic chemicals. Based on the Northern blot hybridization, semi-quantitative and/or real-time RT-PCRs, the alteration of SOD gene transcripts in shark liver was examined during the experimental acute exposures to cadmium. The expression of SOD at mRNA level was up-regulated both by injection (0, 0.5, 1 or 2 mg CdCl2/kg body weight for 48 hours) and by immersion (0 or 5 μM Cd for 0, 1, 4 and 7 days) treatments of cadmium. The transcriptional stimulation of shark SOD gene by cadmium exposure was dependent upon doses and durations: there was a trend toward more increase in higher dose and longer durations of exposure. The hepatic SOD mRNA levels showed also a general agreement with the tissue cadmium concentrations accumulated in immersion exposure. This result may provide useful strategy to develop a fine molecular biomarker at mRNA level for detecting aquatic pollution caused by toxic metals.

Keywords: *Scyliorhinus torazame,* SOD, Cadmium exposure, RNA expression

Introduction

Generation of reactive oxygen species (ROS) is an unavoidable consequence in most aerobic organisms, and organisms express antioxidant enzyme (AOE) system to scavenge the excess of ROS. Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide ion (O^{2-}) to hydrogen peroxide and molecular oxygen, which is the first step in antioxidant defense against the reactive oxygen species (ROS) (den Hartog et al., 2003). Monitoring the activity of this enzyme in fish organs or tissues has been extensively used as an early warning indicator for addressing the ecotoxicological risk of aquatic and/or marine environment, due to its induced expression by various pollutants such as heavy metals and other xenobiotics (Lopes et al., 2001; Basha and Rani, 2003). However, most previous studies have based their results on the SOD activity at protein levels only, and little has been reported on the altered expression at mRNA level. Molecular function of fish SOD has still remained to be studied in detail, with particular emphasis on the transcriptional regulation under the stressful conditions in which ROS may play a role.

Pollution of the coastal areas in Korean peninsula has been gradually increased by a variety of anthropogenic and/or industrial activities, and has given rise to public concern about the safety of foods produced from these areas both by capture and aquaculture. To assess or monitor the quality of aquatic and/or marine environment, fine biomarker system is prerequisite for many purposes (Whitfield and Elliott, 2002). The objective of this study is to examine the transcriptional response of SOD gene in liver of *Scyliorhinus torazame* (Scyliorhinidae), a small cartilaginous shark species that resides in Korean waters, during acute experimental exposure to cadmium, one of the most ubiquitous toxic heavy metals.

Materials and Methods

Animals and experimental *in vivo* exposures to cadmium

The complementary DNA encoding shark SOD was

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isolated from the liver cDNA library, and the full-length SOD cDNA sequence was characterized previously (Ha et al., 2005). For *in vivo* exposure, live sharks were purchased from a local fisheries market, transferred to laboratory and maintained in 200 L tank. To examine the effect of cadmium injection on the transcriptional levels of SOD in liver of shark, the fish (n=12 per dose; 235±34 g of body weight) were injected intraperitoneally with 0.1 ml of $CdCl_2$ in saline at the dose level of 0, 0.5, 1 or 2 mg $CdCl_2$/kg body weight. The injected fish were transferred to one of three replicate tanks (100 L each; 4 individuals per replicate tank). The water temperature throughout the experiment was adjusted at 12±1.0°C, and water change rate was 200% per day. Salinity of the seawater during experiment was 31.8± 1.0 ‰. Liver was surgically obtained from fish at 48 hours post injection and stored at -85°C until the RNA analysis.

In the second experiment, immersion treatment was performed. Sharks (n=14) were immersed in a seawater (200 L) containing 0 or 5 μM of cadmium for 0, 1, 4 and 7 days. Two replicate tanks were prepared both for non-exposed and exposed groups. Three individuals were randomly selected from each replicate tank to obtain liver samples, and hepatic levels of SOD mRNA at each detection point were examined.

RNA isolation

Total RNA was extracted from pooled tissues using TriPure Reagent (Roche Applied Science, Germany) according to the instruction manual from the manufacturer. The integrity of extracted total RNA was verified by rRNA ratio (28S *vs* 18S) in ethidium bromide stained gels. RNA was treated with DNase I (Roche; 10 U/μg RNA) for 30 min at 37°C to remove the any contaminating DNA. DNase was inactivated by heating the reaction at 75°C for 25 min.

RNA blot hybridization

Total RNA was extracted from pooled livers (from 6-12 individuals; approximately 20 mg per fish) using TriPure reagent (Roche). The RNA was treated with DNase I as described above. One μg of total RNA in a volume of 1 μl was spotted on a positively charged

nylon membrane (Roche), and hybridized with digoxygenin-11-dUTP labeled cDNA fragment generated by PCR using shSOD 1F (5'-CAATTTGACCAAGCTG-GAG G-3') and shSOD 1R (5'-ACGACTTCCAG-CATTTCCTG-3'). All the procedures including sample preparation, prehybridization, hybridization, washing and signal detection were carried out according to manufacturer's instructions of DIG DNA Labeling and Detection Kit (Roche, Germany). The hybridized signals were analyzed by the image analysis software (Quantity One; BioRad, USA) using arbitrary values for signal density (INT/mm^2). The membranes were stripped and reprobed with DIG-labeled shark beta-actin cDNA fragment (unpublished data) in order to normalize the SOD mRNA levels.

Semi-quantitative RT-PCR

First-strand cDNA using Superscript II Reverse Transcriptase (Invitrogen, USA) was generated from 2.5 μg of total RNA (DNase-treated) with oligo $(dT)_{18}$ primers. Two μL RT-reaction (cDNA) were subjected to a PCR amplification using ExTaq (Takara) according to the manufacturer's recommendations (see also Cho et al., 2005). The primer pair for shark SOD cDNA was shSOD 1F and shSOD1R as described above. As an internal control, shark beta-actin transcript was amplified by two specific primers, shb-act 1F (5'-CTGTGC-CCATCTACGAAGGT-3') and shb-act 1R (5'-AGAG CGGTGATCTCCTTCTG-3'). PCR was performed using the iCycler (BioRad, USA) under the following conditions: 94°C for 2 min (initial denaturation), 94°C for 45 sec, 58°C for 30 sec and 72°C for 1 min. Numbers of cycles for SOD and actin genes were 28 and 22, respectively, based on the pilot optimization study. PCR reactions were repeated three times for each cDNA sample. Expected sizes of PCR products of SOD and actin are 372 and 475 bp, respectively. PCR products were separated on a 2.0% agarose gels and the ethidium bromide stained bands were analyzed by Quantity-One Image Analysis Software (BioRad) to determine the relative mRNA levels.

Real-time RT-PCR amplification

Real-time monitoring the threshold cycle was carried

out using selected RNA samples. Shark SOD RNA (open reading frame) and partial beta-actin RNA (475 bp) were synthesized *in vitro* from the cDNA-containing linear plasmid, using the Riboprobe *In vitro* Transcription Kit (Promega) according to the manufacturer's recommendations including DNase I treatment. Concentration of the purified transcripts was estimated by light absorbance at 260 nm, and a series of standard concentrations were prepared using yeast RNA carrier solution. Molecular weights of the resultant SOD and beta-actin transcripts were used for copy number calculation. Log copies of transcripts in serial dilutions were adjusted 3 to 8 to draw the standard curves. The standard and unknown RNA samples were reverse transcribed with specific primers: shSOD 1R (for SOD) and/or shb-act 1R (for beta-actin) using Superscript II Reverse Transcriptase (Invitrogen) as described above. Amplification was performed using two µL of RT-product (cDNA) and iQ SYBR Green Supermix (BioRad) according to the manufacturer's recommendation. Oligonucleotide primers for PCR amplification of SOD and beta-actin transcripts were same with those for semi-quantitative RT-PCR as described above. Reaction volume was 25 µL. Amplification condition was 94 for 3 min (initial denaturation) followed by 45 cycles of 94°C 30 sec, 58 for 30 sec and 72°C for 45 sec. Relative fluorescence unit, calculated threshold cycle (C_T), and dissociation curve were monitored by the analysis software of the system (iCycler Real-time Detection System; BioRad). Triplicate assays per cDNA sample were carried out to determine the average C_T values.

Cadmium tissue concentration

Approximately 50 mg of liver tissues was dried at 80 °C for 19-24 hours. Dried samples were weighed and 10 ml of digestion solution (5 : 2 : 3 = HNO_3 : H_2O_2 : H_2O in volumes) was added. All the reagents including water were HPLC-grade. Digestion was performed with a microwave reactor (microwave digestion system ETHOS 1600; Milestone, Italy). The total content of cadmium in the samples was measured by inductively coupled plasma mass spectrophotometer (ICPMS; Elan 6100; Perkin Elmer, USA). When necessary, digested samples were diluted with 1% nitric acid (Blust et al., 1988).

Statistics

Significant differences in the levels of SOD gene transcripts were assessed by Student's t-test and/or ANOVA (followed by Duncan's multiple range tests). Statistical significance was considered at the level of $P < 0.05$.

Results

Mortality and cadmium tissue burden

During the immersion exposure, mortality was negligible in most experimental groups. Injection experiment, no adverse effect on viability of shark was found in both control and injected groups: cadmium exposures were sub-lethal in all the dose levels. Immersion exposure also showed no significant difference in viability among groups including non-exposed control: only one individual was dead at exposed group.

Accumulated amount of cadmium in liver during immersion exposure was increased as a function of duration. The notable elevation of tissue burden was detected from the day 4 and further increased at day 7 (Fig. 1).

Up-regulation of hepatic SOD mRNA by cadmium injection

Transcriptional level of shark SOD in liver was
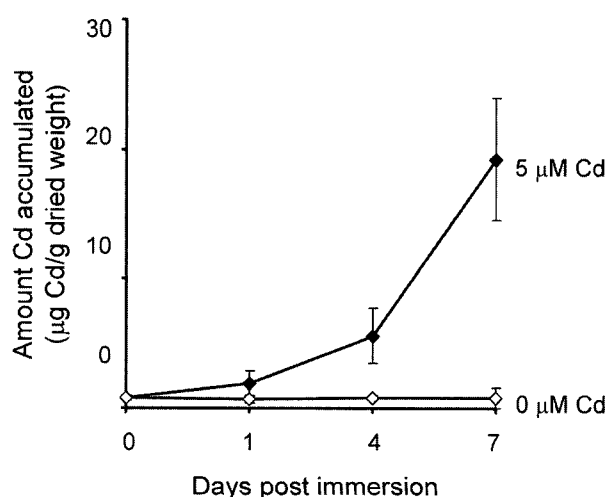
Fig. 1. Cadmium concentrations in liver of fish exposed to either 0 (◇) or 5 µM (◆) Cd up to 7 days. The cadmium concentration was assessed by ICPMS, and expressed as mg cadmium per gram dried weight of tissue.

readily affected by exposures to cadmium (Fig. 2). In RNA dot blot hybridization, the hybridized signal was quite different depending upon the amount of cadmium delivered. Generally, the signal was toward more intense in higher dose of cadmium, although there was variation among individuals. Statistically all the doses except the lowest one (0.5 mg/kg body weight, BW) represented the significantly elevated hybridization signal when compared to the control group (Fig. 2a). Semi-quantitative RT-PCR also showed the dose-dependent amplification of SOD transcripts. Internal control (beta-actin transcript) showed consistent mRNA levels regardless the doses of cadmium injected. How-

ever, hepatic levels of SOD mRNA were significantly stimulated by cadmium exposure, and again, the increase was positively related with the doses of cadmium injected. Relative mRNA levels of SOD (based on the normalization against beta-actin transcripts) observed in non-exposed control group was less than 0.3 (actin ratio), however, Cd-exposed groups except the group treated with the lowest dose (0.5 mg/kg) showed notably increased levels of SOD mRNA ranging 0.3 to 0.9. Consequently, the maximum mRNA level of SOD detected at the group injected with the highest dose (2 mg/kg BW) was more than 3-fold of that observed in non-exposed group (Fig. 2b). In addi-
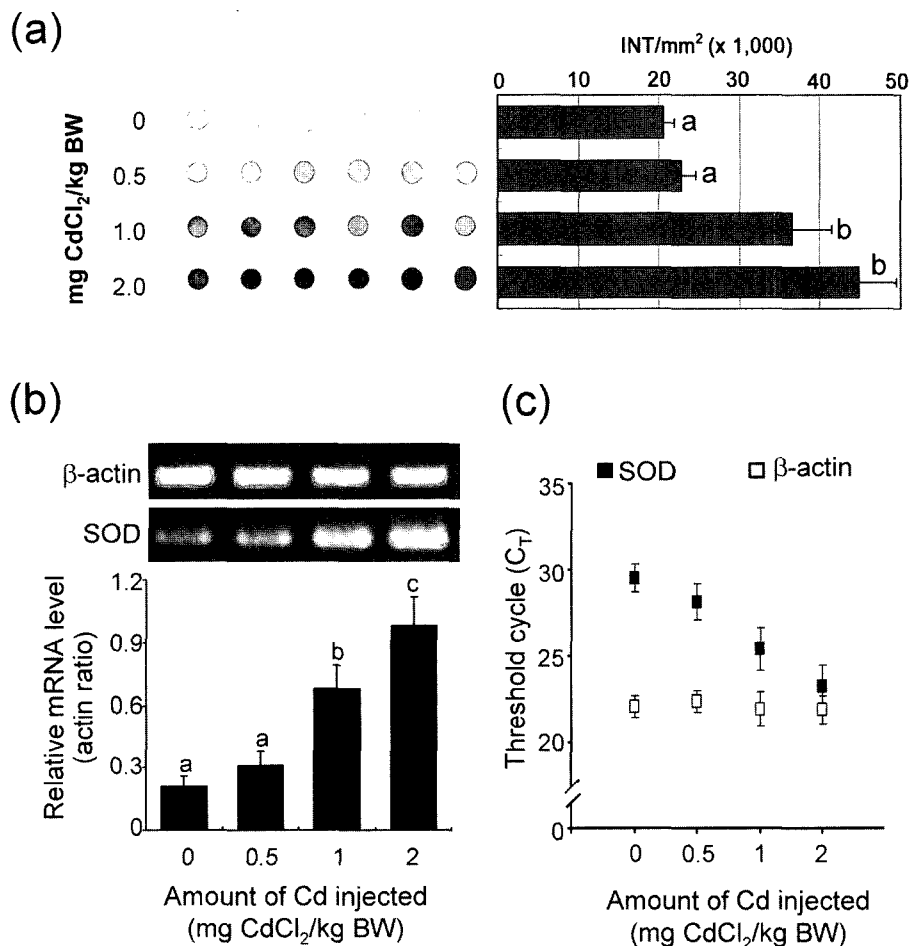

Fig. 2. Stimulated expression of hepatic SOD mRNA during acute exposures to cadmium by injection at different dose levels ranging 0 to 2 mg CdCl₂ per kg body weight. (a) Representative blot (left) and scanning densitometry analysis (right) from RNA dot blot hybridization showing the increased SOD transcripts by cadmium injection. Signals hybridized dots were assayed by the image analysis software (Quantity One; BioRad). Standard deviations are indicated by T bars. Means with same letters did not differ based on the ANOVA test at P=0.05. (b) Semi-quantitative RT-PCR assay of SOD transcripts with beta-actin gene as an internal control. Representative bands amplified by RT-PCR were shown along with the histograms to estimate the relative levels of SOD mRNA against actin transcripts. Statistically different means are noted by different letters (a-c) based on the ANOVA test (P<0.05). (c) Average threshold cycles for SOD and beta-actin genes in experimental groups. Real-time PCR analysis was performed with iQ Real-time PCR Detection System (BioRad).

tion to the end-point RT-PCR, real-time RT-PCR displayed the similar pattern for SOD and actin expression. The linearity R^2 coefficient of threshold cycle (C_T) versus log quantity from standard curve was 0.996 for SOD and 0.995 for beta-actin, respectively (standard curves not shown). Threshold cycle of beta-actin gene was relatively constant in all of the experimental RNAs. However, the C_T values were significantly decreased depending upon the doses of cadmium injected: the lower C_T scores the higher amounts of cadmium treated (Fig. 2c). The threshold cycles (C_T) of beta-actin gene was steady-state ranging from 21.9 to 22.4 in average. On the other hand, the C_T of SOD was markedly reduced with the increase of cadmium doses (from 29.5 in control to 23.2 in 2 mg/kg-injected group) (Fig. 2c).

Time course expression of shark SOD mRNA in liver

Exposure to cadmium by immersion treatment also increased the hepatic SOD transcripts: the increase of the mRNA was toward longer durations in a given concentration of 5 µM (Fig. 3). Based on the semi-quantitative RT-PCR, the internal control gene, actin represented the steady-state mRNA levels throughout the experiments in both non-exposed and exposed groups. However, the significant increase of SOD was detectable at day 4 in the exposed group (5 µM), and the difference in SOD mRNA levels between control and exposed fish was more apparent at day 7. The SOD mRNA level in liver of non-exposed fish was also slightly increased at the end of experiment (day 7) compared to the starting level (day 0), although the difference didn't receive often the statistical support (Fig. 3a&b). The significant elevation of SOD transcripts at day 7 was confirmed by real-time monitoring the C_T values. Individual RNA sample from each specimen ($n=6$) belonging to 0 or 5 µM-treated group was subjected to real-time PCR. Amplification of beta-actin transcripts represented no difference in average C_T between non-exposed (22.2±0.7) and exposed groups (22.3±1.1). However, C_T value of SOD was significantly reduced in exposed group (23.4±1.0) when compared to that observed in non-exposed group (28.9±1.2) (Table 1).
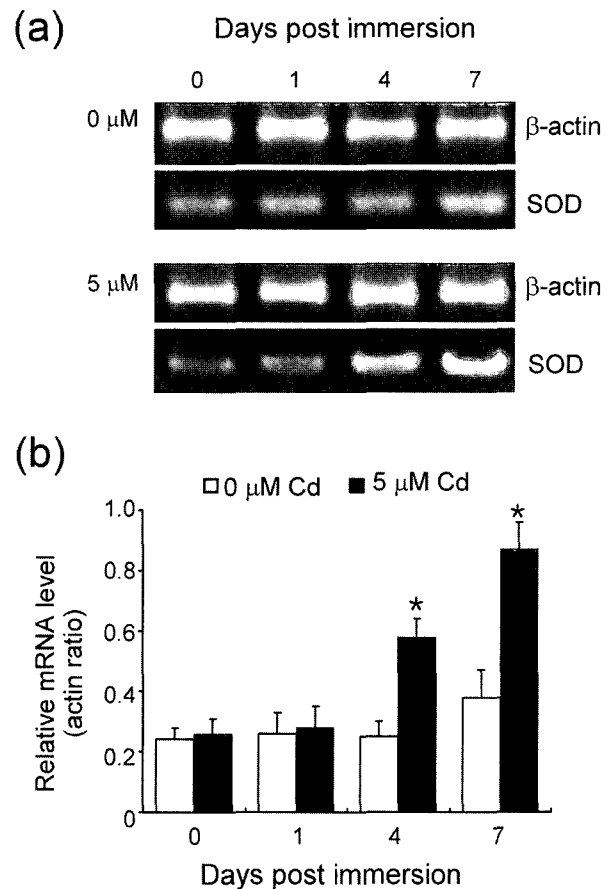
Fig. 3. Time course expression of SOD mRNA during exposure to cadmium at 0 or 5 µM up to 7 days. (a) Representative RT-PCR bands of SOD and beta-actin transcripts amplified from non-exposed and exposed fish. (b) Histograms to show the relative mRNA levels of SOD (actin ratios) in non-exposed and exposed groups, as determined by semi-quantitative RT-PCR. Triplicate assays per cDNA sample were prepared and statistical deviation from the control level was noted by asterisks at $P<0.05$ based on Student's t-test.

Discussion

Altered expression of superoxide dismutase in fish tissues is one of the versatile bioindicators for the monitoring of risks in aquatic environment with especially respect to the pollution caused by toxic chemicals including heavy metals and other inorganic xenobiotics (Basha and Rani, 2003; Pandey et al., 2003). However, despite its importance, its transcriptional regulation in fish has not been completely studied. Expression of SOD mRNA in shark liver was apparently affected by the exposure to cadmium based on the RNA dot blot, semi-quantitative RT-PCR and real-time RT-PCR assays. Induced expression of hepatic SOD mRNA was

Table 1. Average threshold cycle of beta-actin and SOD transcripts in the liver of fish exposed to either 0 or 5 μM of cadmium by immersion for 7 days

Fish	Beta-actin	SOD
Non-exposed control (0 μM Cd)		
#1	22.1	28.7
#2	22.4	26.9
#3	20.9	29.8
#4	21.9	30.1
#5	23.0	29.4
#6	22.6	28.5
Mean	22.2[a]	28.9[a]
SD	0.7	1.2
Exposed group (5 μM Cd)		
#1	21.3	24.5
#2	23.0	23.4
#3	21.4	23.6
#4	21.5	21.8
#5	23.1	22.8
#6	23.7	24.0
Mean	22.3[a]	23.4[b]
SD	1.1	1.0

Means with the same superscript within a column were not significantly different at $P<0.05$ based on the Student's t-test.

in general dependent upon not only the doses of cadmium administered but also the durations of exposure. The cadmium tissue concentrations were also increased with durations in immersion experiment, and showed the positive relationship with the elevated SOD mRNA levels. The increased SOD in high tissue burdens has also been reported in other fish species using the enzymatic analysis (Kock et al., 1995; Almeida et al., 2002). The increase of SOD transcripts without reaching plateau in *S. torazame* liver may suggest that this species would have relatively large capacity to detoxify the heavy metal. However, further study should be needed to compare the mRNA levels with actual enzyme activity of SOD.

Interestingly, non-exposed control fish showed a slight elevation of SOD mRNA levels at the end of immersion exposure, although the amount of increase was not as high as in the Cd-exposed fish. One possible explanation for this phenomenon may be the general stress response of wild stock to laboratory containment, since the period for adaptation to laboratory condition was quite short. Another explanation is that the ROS might be increased by starvation because

we supplied no food to shark throughout the experiment. Starvation has been known as one of factors to increase ROS and consequently the food depletion might induce the antioxidant enzymes in shark of this study. The induced expression of antioxidant enzymes including SOD during starvation has already been reported in fish and mammals (Gomi and Matsuo, 1998; Pascual et al., 2003).

The present study suggests that monitoring of antioxidant enzyme (AOE) transcripts at mRNA levels could also be used as a biomarker system for environmental pollution by heavy metals. Increased sensitivity and robustness of the present gene sequence-based assay may also be useful for examining a specific response of each AOE isotype to pollutants in a fine manner (see Gallagher et al., 1999; Williams et al., 2003), which may be often difficult in many post-mortem studies with AOE at protein levels, since the measuring of an AOE activity in a tissue homogenate have usually detected the pooled activity from numerous isoenzymes (Klumpp et al., 2002; Pandey et al., 2003).

Comparative examination in SOD analysis between mRNA level and protein level would provide valuable information on whether the changes in mRNA could be reflected in enzyme activities, because the transcriptome may not always be presented at proteome levels. Further studies should also be needed using more environmentally realistic conditions such as lower cadmium concentration and longer durations, although the development of optimum condition for the laboratory maintenance of wild shark specimen should be prerequisite. When considering that SOD is biomarker for various biological stresses, the future study to examine the transcriptional response of SOD gene to different pollutants would also be valuable.

Acknowledgement

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