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Hepatic Expression of Cu/Zn-Superoxide Dismutase Transcripts in Response to Acute Metal Exposure and Heat Stress in *Hemibarbus mylodon* (Teleostei: Cypriniformes)

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Hemibarbus mylodon (Cypriniformes) is an endemic freshwater fish species in the Korean peninsula, for which urgent conservation efforts are needed. To understand their stress responses in relation to metal toxicity and thermal elevation, we performed a real-time RT-PCR-based expression assay of hepatic copper/zinc-superoxide dismutase (Cu/Zn-SOD), a key antioxidant enzyme, in response to experimental heavy metal exposure or heat treatment. The transcription of hepatic Cu/Zn-SOD was differentially modulated by acute exposure to Cu, cadmium (Cd), or Zn. Exposure to each metal at 5 μM for 24 h revealed that Cu stimulated the mRNA expression of Cu/Zn-SOD to a greater extent than the other two heavy metals. The elevation in Cu/Zn-SOD transcripts in response to Cu exposure was dose-dependent (0.5 to 5 μM). Time course analysis of Cu/Zn-SOD expression in response to Cd exposure (5 μM) revealed a transient pattern up to day 7. Exposure to thermal stress (an increase from 22 to 30°C at a rate of 1°C/h followed by 30°C for 18 h) did not significantly alter SOD transcription, although heat shock protein 90 kDa (HSP90) transcription was positively correlated with an increase in temperature.

Key words: Cu/Zn-SOD, *Hemibarbus mylodon*, Heavy metals, Heat stress, Gene expression

Introduction

Over the last decade, the natural habitat of *Hemibarbus mylodon* (Teleostei: Cypriniformes), an endemic species in the Korean peninsula, has been significantly disturbed by various anthropogenic activities; as a result, this species is considered an endangered organism with a high risk of extinction (Cho et al., 2008; Kim et al., 2008). Furthermore, the results of two recent molecular analyses indicated that the genetic diversity of currently existing *H. mylodon* populations is extremely low (Kim et al., 2007a; Lee et al., 2008). Thus, conservation and restoration efforts are urgently needed for this species. For the *in situ* and *ex situ* restoration of threatened fish species, a biomarker-assisted understanding of stress physiology at the cellular level is invaluable in the designation of conservation plans.

Copper/zinc-superoxide dismutase (Cu/Zn-SOD; EC 1.15.1.1) is a key antioxidant enzyme with roles in the first line of defense against oxidative stress caused by reactive oxygen species (ROS) (Zelko et al., 2002). Due to its essential function, Cu/Zn-SOD is known as a housekeeper in most aerobic cell types. However, despite its essential function, the expression of Cu/Zn-SOD at both the mRNA and protein levels is known to be modulated by several biotic (*e.g.*, disease or physiological states) and abiotic factors (*e.g.*, environmental pollutants), since those stimulatory factors may enhance the formation of ROS in animal tissues (Cho et al., 2006; Hansen et al., 2006; Kim et al., 2007b). Consequently, studies have proposed that Cu/Zn-SOD be used as a nonspecific biomarker to warn of and/or detect physiological alterations in animals and environmental problems associated with pollution (Almeida et al., 2002; Kunikowska and Jenner, 2003; Pandey et al., 2003; Nam et al., 2005).

The objective of this study was to examine the transcriptional response of hepatic Cu/Zn-SOD to acute metal exposure or heat stress in *H. mylodon* to evaluate the potential usefulness of Cu/Zn-SOD

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mRNA as a candidate biomarker of oxidative stress in this species.

Materials and Methods

Fish specimen and Cu/Zn-SOD cDNA

The fish specimen used in this study was obtained from a laboratory stock maintained at Soonchunhayng University (Asan, Korea). The general conditions of fish management were as described in Bang et al. (2007a). The Cu/Zn-SOD clone was isolated as part of a previous expressed sequence tag (EST) analysis (Bang et al., 2007b); its full-length cDNA sequence is available from the NCBI GenBank database under accession number FJ975147. The *H. mylodon* heat shock protein 90 kDa (HSP90) clone, which was employed as a quality control to validate the effectiveness of the thermal regime used in this study, was a partial cDNA clone identified from the same EST database (Nam YK, unpublished data).

Acute heavy metal exposure

The transcriptional modulation of Cu/Zn-SOD was examined using a series of acute metal exposure experiments. First, to examine the potency of different metals for the stimulation of Cu/Zn-SOD transcripttion, juvenile fish (average body weight, 10.3±1.2 g) were exposed to a given concentration (5 μM) of waterborne heavy metals. The fish (n=18) were allocated to one of two replicate tanks containing 50 L of well aerated (dissolved oxygen=5±1 ppm) tap water. After this period, the experimental groups were exposed to a nominal concentration (5 μM) of cadmium (Cd), Cu, or Zn using 0.5 M stock solutions of $CdCl_2$, $CuCl_2$, or $ZnCl_2$, respectively. The metal reagents were of analytical grade (Sigma, St. Louis, MO, USA). Control groups were prepared, which were identical except for the addition of the heavy metals. The water was kept at 22±1°C throughout the experiment. After 24 h, six individuals were randomly selected from each tank, and the livers were surgically obtained for RT-PCR analysis. Second, fish were exposed to different levels of Cu or Cd to examine the interrelationship between dose strength and the expression of SOD. Twelve individuals of the same size class as above were immersed in Cu (0, 0.5, 1, 2, or 5 μM) or Cd (0, 1, 2, 5, or 10 μM) for 24 h, and the hepatic SOD mRNA levels were examined in five individuals randomly chosen from each tank. All other conditions were as described above. Third, *H. mylodon* individuals were exposed to Cd for different durations (1, 2, 4, and 7 days). Fifteen individuals were allocated to each of 50-L tanks containing 5 μM Cd. Non-exposed controls were also prepared. The water exchange rate was 50% per day, and the metal was renewed at the time of each water change. Three individuals were randomly obtained from each tank at each detection point. The liver was removed from each individual, pooled within the group, and subjected to RNA extraction for RT-PCR analysis.

Experimental heat stress

Fish were exposed to a thermal regime to examine whether the mRNA expression of SOD is affected by heat stress. Using fish (4.5±1.2 g body weight) that had been maintained in a tank (200 L) at 22±1°C, 30 individuals were transferred to one of two 50-L tanks containing well aerated water (dissolved oxygen= 5.0±1.5 ppm) at 22°C. The fish were allowed to acclimate for 24 h. Afterward, the water temperature of one tank was raised to 30°C at a rate of 1°C/h using an electrically controlled heating device, whereas the other tank was kept at 22°C (control group). The fish (n=4) were sampled at the starting point (22°C) and at 4 (26°C), 8 (30°C), 14 (6 h after reaching 30°C), 20 (12 h after reaching 30°C), and 26 h (18 h after reaching 30°C) post-temperature elevation. At the same time, an equal number of fish was sampled from the control tank (22°C). Livers were surgically obtained, pooled within the group prior to RNA extraction, and used for RT-PCR analysis.

Real-time RT-PCR assay

Total RNA was purified from each liver using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations plus DNase treatment. For real-time RT-PCR, 4 μg of purified total RNA was reverse transcribed at 37°C for 60 min in a 40-μL reaction volume using Omniscript RTase (Qiagen) and oligo $d(T)_{20}$ primer (1 μM final concentration). To prepare the normalization control, Fi18S rRNA 1R primer (5'-CAAGAATTTCA CCTCTAGCGGC-3'), a conserved reverse primer for fish 18S rRNA, was included in the RT reaction at 0.1 μM for coapplication reverse transcription (Co-RT) (Zhu and Altmann, 2005). When the reaction was finished, the product was diluted twofold with sterile water and 1 μL of the diluted cDNA was subjected to real-time PCR. Standard curves of the target (SOD) and control (18S rRNA) were prepared as threshold cycles versus the log number of positive cDNA templates (Schmittgen and Livak, 2008). The primers used were the following: HM Cu/Zn-SOD 1F (5′-CGCACTTCAACCCTCACAAT-3') and HMCu/Zn-SOD 1R (5′-CTCTACCCAGTGATGCCAAT-3′) for Cu/Zn-SOD, and HM18S 1F (5′-AGAAACGGCTAC

CACATCCA-3′) and HM18S rRNA 1R (5′-GGAC ACCCAGTTAAGGGCAT-3′) for the 18S rRNA control (Nam YK, unpublished data). The expected sizes of the products were 280 and 338 bp for SOD and 18S rRNA, respectively. PCR primers, qHMhsp90 1F (5′-ATGAAGACGTGCCTGTGGAA-3′) and qHMhsp90 1R (5′-AGACAATACTGCAGC AACCC-3′) were used to amplify 330 bp from the *H. mylodon* HSP90 cDNA fragments as a control. Reaction volume was 25 μL. The reaction included iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and was run with the iCycler iQ Real-Time Detection System (Bio-Rad). An initial incubation at 95°C for 5 min was followed by 45 cycles of 20 s at 94°C, 20 s at 58°C, and 25 s at 72°C for all three transcripts. Fluorescence readings were taken at 72°C after each cycle to calculate the average threshold cycles (C_t). The level of SOD mRNA in each sample was normalized against its own level of 18S rRNA, and the fold-changes in SOD transcription in the treated group relative to the untreated controls were estimated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

Statistics

Triplicate real-time RT-PCR was performed per cDNA sample in an independent manner. The relative mRNA levels of SOD were assessed by ANOVA followed by Duncan's multiple range tests. If needed, Student's *t*-test was used to examine the statistical difference between the stimulated and control groups. Differences were considered to be significant at $P<0.05$.

Results and Discussion

The viability of the experimental groups did not differ under the present exposure conditions ($P>0.05$, data not shown); however, the hepatic mRNA expression of Cu/Zn-SOD was readily influenced by exogenously administered heavy metal ions. The standard curves prepared in our real-time PCR analysis were fairly close to linear, with linearity coefficients ≥0.991 for both the target (SOD) and control (18S rRNA) genes. The expression of the 18S rRNA control was without notable fluctuations, regardless of the experimental group (C_t values ranging from 13.3 to 13.8). At 5 μM, Cu was more potent than the other metals in terms of the increase in hepatic Cu/Zn-SOD transcription. Cu/Zn-SOD expression in the Cu-exposed group was 3.8 times the level in the unexposed (control) group. Cd exposure also moderately induced Cu/Zn-SOD transcription up to 1.7-fold, whereas Zn did not alter the mRNA level of Cu/Zn-SOD (Fig. 1A). Our finding of Cu/Zn-SOD stimulation in response to metal exposure is congruent with previous reports in other fish species (Pandey et al., 2003; Cho et al., 2006; Nam et al., 2006), suggesting that heavy metals are a strong prooxidative factor. The differential effects of the metals on the expression of metal-binding proteins could be explained by the metal-specific accumulation rate, which is associated with the amount of binding ligands, the influx/efflux rate, and the tissue-specific availability of each metal ion (Kock et al., 1995; Maes et al., 2005). One possible, but undefined, explanation for the lack of stimulated expression in the Zn-exposed fish is that other Zn-binding proteins, particularly metallothioneins (major reservoirs of intracellular Zn), might bind the Zn competitively, thereby diminishing the Zn-mediated tissue burden and forestalling the need for additional Cu/Zn-SOD transcription in the liver. Furthermore, our relatively short exposure period (24 h) despite the high treatment concentration may also explain this finding (see Haq et al., 2003). However, a comparative expression analysis of Cu/Zn-SOD along with other zinc-binding proteins (*e.g.,* MTs) for an extended period should be carried out in the future.

In the second exposure experiment utilizing varying concentrations of Cu (0.5 to 5 μM) or Cd (1 to 10 μM), the expression pattern of Cu/Zn-SOD was different between the two heavy metal-treated groups. With Cu, a rise in hepatic Cu/Zn-SOD transcription was observed at all four doses, and the increase was fairly dose-dependent ($P<0.05$). On the other hand, Cd upregulated the mRNA expression of Cu/Zn-SOD at 5 and 10 μM but not at lower doses (1 and 2 μM; Fig. 1B). Furthermore, no dose-dependency was observed between the 5 and 10 μM-treated groups. This result (*i.e.,* the preferential induction of SOD by Cu) suggests that the accumulation rate of Cd in the liver might be slower than that of Cu in *H. mylodon* livers; a longer duration should be tested to validate this preliminary finding. A study previously reported that SOD activity could be "saturated" without further elevation by increasing the dose of Cd (Almeida et al., 2002).

From the Cd-exposure experiment used to examine the effects of treatment duration (5 μM up to 7 days) on SOD expression, the control group showed no significant fluctuation in the hepatic level of Cu/Zn-SOD transcription during the experimental period. On the other hand, the expression of Cu/Zn-SOD in fish exposed to Cd was upregulated as early as day 1 (1.9-fold; $P<0.05$), further stimulated at day 4 (4.2-fold;

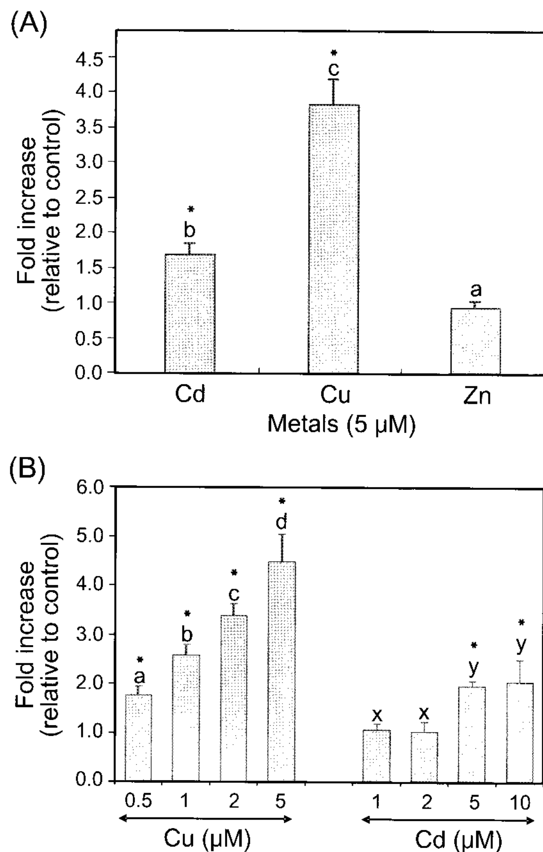

Fig. 1. Effects of heavy metal exposure on the hepatic expression of *Hemibarbus mylodon* Cu/Zn-SOD transcripts. A: Expression of Cu/Zn-SOD in fish exposed to three different heavy metals (Cd, Cu, or Zn) at 5 μM for 24 h. B: Transcriptional response of Cu/Zn-SOD to various doses of Cu (0.5 to 5 μM) or Cd (1 to 10 μM) for 24 h. By real-time RT-PCR analysis, the relative mRNA level expression of SOD was estimated by normalization against the 18S rRNA control; it is expressed as the fold-increase relative to that in the control groups. The means ± SD based on triplicate examinations are shown as histograms with T-bars. Means with different letters (a–c in A and a–d or x–y in B) were significantly different when assessed by ANOVA ($P<0.05$). The means indicated by asterisks are significantly different from the basal level in the control group (Student's *t*-test, $P<0.05$).

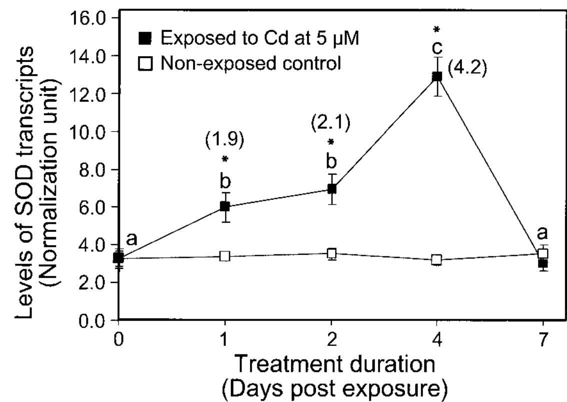

Fig. 2. Time course of Cu/Zn-SOD mRNA expression in the liver of *Hemibarbus mylodon* exposed to 5 μM Cd for 1, 2, 4, and 7 days. The controls showed no notable fluctuation in SOD expression regardless of the time point (ANOVA, $P>0.05$). Significantly different means (ANOVA, $P<0.05$) are indicated by different letters (a-c). Means showing a significant difference from the controls are noted by asterisks (Student's *t*-test, $P<0.05$); the fold-increases (relative to the control values) at days 1, 2, and 4 are noted in parentheses.

$P<0.05$), and then returned to the control level at day 7 ($P>0.05$; Fig. 3). The diminished expression or even inverse regulation of antioxidant enzymes has been reported following acute exposure to relatively high concentrations of heavy metals (Geret et al., 2002; Matz et al., 2006; Nam et al., 2006). This phenomenon may be associated with the acclimation process following an initial shock phase, in which the physiology establishes a new steady state (McDonald and Wood, 1993; Hansen et al., 2006). Alternatively, such a decrease could result from detrimental effects related to increased stress, in which the intensity of stress is beyond the regulatory capacity such that the fish reach a stage of exhaustion (Guecheva et al., 2003).

During heat treatment, the quality control, HSP90, was significantly upregulated during Phases I (from 22 to 30°C at 1°C/h) and II (6 to 18 h after reaching 30°C) of the thermal regime. The maximum fold-increase in HSP90 expression was 7.6 relative to the control group (maintained continuously at 22°C) as assessed by real-time PCR analysis. However, in contrast, the level of Cu/Zn-SOD transcription was not altered during the elevation or the static incubation phase ($P>0.05$; Fig. 3). This was unexpected since the temperature applied in the present study (30°C) was higher than the generally agreed optimum temperature for *H. mylodon* culture (about 24-25°C). Water temperature is the primary abiotic factor modulating various physiological pathways in poikilothermal animals, and thermal stress is believed to be one cause of oxidative stress in those organisms (Buckley et al., 2006; Kim et al., 2007b). The lack of a transcriptional response by SODs to heat stress in the present study may have been due to the relatively short period of exposure, which might be insufficient for generating excessive ROS to be scavenged by SODs. Many previous studies have reported that the

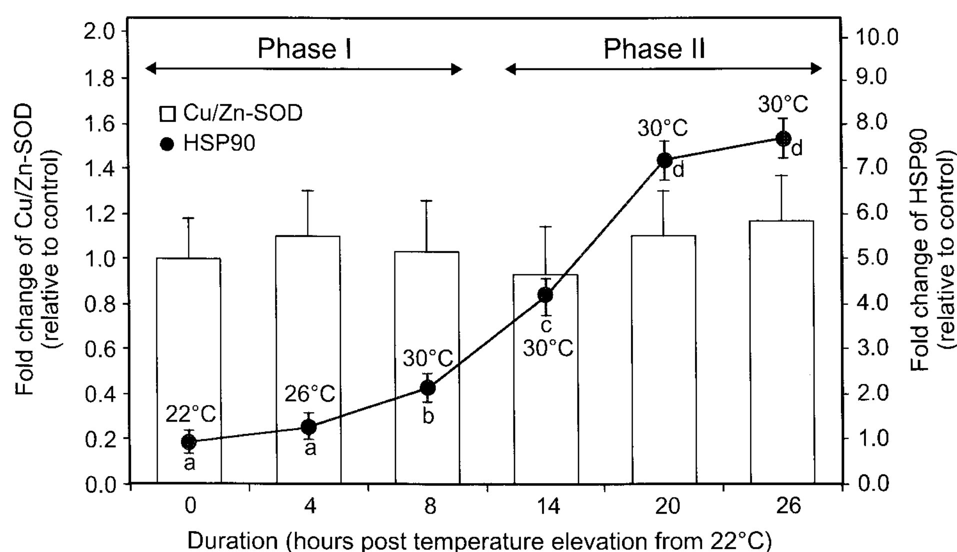

Fig. 3. Expression of Cu/Zn-SOD and HSP90 transcripts during thermal treatment. In Phase I, the water temperature was elevated from 22 to 30°C at a rate of 1°C/h; fish were sampled immediately when the temperature reached the desired point (22, 26, and 30°C). In Phase II, the water temperature was maintained at 30°C for 18 h. During Phase II, fish were sampled at 6-h intervals. No significant alteration was observed in Cu/Zn-SOD whereas a notable elevation was detected in HSP90. Means (HSP90) with different letters are significantly different based on ANOVA/Duncan's test (P=0.05).

most responsive proteins to the acute phase of heat stress in teleosts are HSPs rather than SOD (Murtha and Keller, 2003; Deane and Woo, 2005; Buckley et al., 2006). Our examination of the profound upregulation of *H. mylodon* HSP90 mRNA under the same experimental conditions (particularly in Phase II; Fig. 3) is also, at least in part, supportive of this explanation. Hence, further study to examine the expression of SODs along with other heat stress-responsive proteins following extended thermal treatment are needed to elucidate the cross talk between SODs and other heat stress-related proteins.

In summary, the transcriptional response of Cu/Zn-SOD to stimulation with heavy metals and heat was examined in the liver of *H. mylodon*, a threatened fish species. The results of this study can be used to evaluate Cu/Zn-SOD as a candidate biomarker for the altered physiology of *H. mylodon* in response to toxic pollutants. However, additional studies should be initiated to test the effects of other biotic and abiotic factors on the expression of SOD under more environmentally robust conditions.

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