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Transcriptional Alteration of Two Metallothionein Isoforms in Mud Loach (*Misgurnus mizolepis*) Fry during Acute Heavy Metal Exposure

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Altered mRNA expression of two metallothionein isoforms (MT-IA and MT-IB) in response to acute heavy metal exposure was examined in mud loach, *Misgurnus mizolepis*, fry using a real-time RT-PCR assay. Sublethal exposure (1 or 5 μM) to Cd, Cr, Fe, Mn, Ni, and Zn resulted in highly variable transcriptional responses of the two MT isoforms to the heavy metal ions, including upregulation, a steady state, and downregulation. Overall, the most potent inducer of both MT isoforms was Cd (up to 6-fold). Another exposure experiment using a series of doses of Cu revealed that the stimulation patterns of the two MT isoforms differed: MT-IA transcription was soon saturated at higher concentrations (about 2-fold at 1-4 μM of Cu), whereas the activation of MT-IB was more dependent on the treatment dose (increased up to 5-fold at 3 μM). The isoform-specific allotment of constitutive and inducible functions was not as clear in fry as in adult tissues. Coordinated interaction between the MT-IA and MT-IB isoforms was hypothesized based on the finding that MT-IA represented a primary action under 'less stressful' or 'sublethal' conditions, whereas the activation of MT-IB became important under 'more stressful' or 'lethal' circumstances in this species.

Key words: Metallothionein, *Misgurnus mizolepis*, Fry, Heavy metal, Gene expression

Introduction

Metallothionein (MT), a low-molecular-weight (6-7 kDa), cysteine-rich protein, plays crucial roles in not only the homeostatic regulation of essential metal ions, but also the detoxification of overloaded or excess heavy metals (Andrews, 2000). Genetic determinants for metallothionein have been exploited in various fish species in a wide array of taxonomic positions, and fish MTs are readily inducible at both the RNA and protein levels during heavy metal exposure (Chen et al., 2004; Cho et al., 2005). Due to their high inducibility upon heavy metal exposure, fish MTs are considered a versatile biomarker for addressing or warning of the ecological risks associated with metal pollution in aquatic environments.

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Despite the proposed use of MT as a biomarker, it has also been claimed that the modulation of *MT* genes in fish tissues is affected by a number of biotic and abiotic factors (Haq et al., 2003; Bourdineaud et al., 2006). As shown in the study of a cypriniform species, MT transcription was induced more sensitively in fry than in adults during metal exposure (Bang et al., 2007; Cho et al., 2008), suggesting that the age or developmental stage of a given fish species should be considered for the finer use of the MT biomarker. Collectively, it is widely agreed that a series of evaluations of the effects of various parameters on MT expression is a prerequisite for the mechanistic application of this protein to biomarker assays. Furthermore, many teleosts, especially cypriniform species, possess multiple isoforms of *MT* genes in their genomes through gene or genome duplication (Knapen et al., 2005). Two noteworthy studies have also reported the differential or inter-

active responses of MT isoforms to metal exposure (Hermesz et al., 2001; Vergani et al., 2007).

Recently, we isolated two tandemly organized *MT* genes from the mud loach (*Misgurnus mizolepis*; Cypriniformes), a potential sentinel organism for the Korean peninsula (Cho et al., 2009). Previously, we found that the basal expression of the two mud loach MT isoforms (MT-IA and MT-IB) differed greatly, and, more importantly, that the stimulation patterns of these two MT isoforms in adult tissues differed significantly; MT-IB was the preferentially induced isoform, whereas MT-IA was a more constitutively expressed type (Cho et al., 2009).

As part of our long-term goal to profile the expression of the MT isoforms in this species from an ecotoxicological perspective, this study examined the expression characteristics of the MT isoforms at the mRNA level in mud loach fry during acute heavy metal exposure. For this, we tested the transcriptional alteration of the two MT isoforms during exposure to the heavy metals cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), and zinc (Zn), and examined whether the isoform-specific responses seen in adult tissues also occurred in the whole body of fry of this species.

Materials and Methods

Fish specimens

Mud loach (*M. mizolepis*) fry used in this study were laboratory stock maintained in the Institute of Marine Living Modified Organisms (IMLMO), Pukyong National University (PKNU). They were full siblings produced by artificial propagation of selected broodfish. The fry were fed a commercial diet (Woosung Feed, Korea) and reared in laboratory tanks at 22-25°C. Details of conditions used for fish rearing and management are in Kim et al. (1994).

In vivo heavy metal exposure

Four-week-old fry (average body weight 84.4± 17.3 mg; total length 15.2±2.1 mm) were exposed to various metals. First, sublethal treatments were performed by exposing the mud loach fry to one of two doses (1 or 5 μM) of Cd, Cr, Fe, Mn, Ni, or Zn. Fry (n=16 each) were allocated into one of two 5-L replicate tanks each containing 3 L of 10-μm-filtered tap water containing each heavy metal ion at the desired dose. A non-exposed control group was prepared similarly with two replicate tanks. Second, based on our preliminary examination of the adverse effect of Cu on the viability of mud loach fry, we exposed fry (n=20 per replicate) to a series of Cu doses (0, 0.5, 1, 2, 3, and 4 μM), with other treatment conditions the same as described above. For both experimental treatments, the exposure duration was 24 h, and no food was supplied during the exposure. The temperature was 25±1°C, and the dissolved oxygen was 4.0±0.5 ppm. After the exposure, the in-tank concentration of each heavy metal was checked as described by Cho et al. (2008) to confirm that there was no significant deviation (less than 10%) from the nominal value. When the exposure treatment was finished, six fry were chosen randomly from each replicate tank, anesthetized with 300 ppm of lidocaine-HCl (Sigma-Aldrich, St. Louis, MO, USA), frozen immediately on dry ice, and stored until RNA preparation.

Nucleic acid preparation and gene expression assay

Whole-body total RNA was extracted with Tripure Regent (Roche Applied Science, Mannheim, Germany). Total RNA was purified again using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) including the DNase treatment. The RNA integrity of each sample was checked based on the 28S:18S rRNA ratio in an ethidium–bromide (Et-Br)-stained MOPS agarose gel. All the procedures for RNA preparation followed the manufacturers' instructions. A fraction of the total RNA (2 μg) was reverse transcribed to complementary DNA (cDNA) using the OmniScript Reverse Transcription kit (QIAGEN) according to the manufacturer's recommendation. To prepare the normalization control, a mud loach 18S rRNA reverse primer (5'-TCTAGCGGCGCAATA CGAAT-3'; Cho et al., 2009) was included in the reverse transcription (RT) reaction at a final concentration of 0.1 μM. The RT product was diluted 1/4 (for MT genes) or 1/8 (for 18S rRNA external control), and two μl of diluted cDNA template was subjected to each PCR amplification. The primer pair used to amplify the MT-IA isoform was qMLMT-A 1F (5'-TGCAAGTGCACTAACTGCA-3') and qMLMT-A 1R (5'-TGAAGACACGAAGTTCGGA-3'), whereas qMLMT-B 1F (5'-GCGTTTGTAAGGG AATA-3′) and qMLMT-B 1R (5'-ACAGTTGCACGA CTGATC-3′) were used for the MT-IB isoform. The expected sizes of the PCR products for MT-IA and MT-IB were 188 and 258 bp, respectively. The normalization control (18S rRNA fragment; 241 bp) was amplified using primer pair qML18S 1F (5'-GCGGTAATTCCAGCTCCAAT-3') and qML18S 1R (5'-CCTAGCTGAGATATTCAGGC-3′) (Cho et al., 2009). As a preliminary assay, semi-quantitative "end-point" PCR bands were visualized with

ethidium bromide (Et-Br) staining and analyzed with the Quantity-One™ software implemented in Versa Doc 4000 (Bio-Rad, Hercules, CA, USA) to verify whether specific amplification occurs for every gene (data not shown). After the "end-point" RT-PCR assay, further quantitative analysis of MT transcripts was performed using real-time PCR. The template cDNA and primer pairs were the same as described above, and the PCR efficiencies of both the target and control genes were determined with standard curves. For real-time monitoring of the amplification, a reaction in a volume of 25 μL containing 2× iQ SYBR Green SuperMix (Bio-Rad) was run for 45 cycles with the iCycler iQ Real-Time Detection System (Bio-Rad). The thermal cycling conditions were 20 s at 94°C, 20 s at 58°C, and 20 s at 72°C with an initial denaturation step at 94°C for 2 min for all three genes. Fluorescence readings were taken after each elongation step, and the threshold line was generated automatically using the default setting of the instrument. The expression levels of the metal-exposed groups relative to those of the non-exposed control based on normalization against control gene transcripts were determined using the comparative C_T method (Schmittgen and Livak, 2008; Cho et al., 2009). Triplicate assays were performed in an independent fashion.

Statistics

Differences in the relative expression levels among groups were assessed using analysis of variance (ANOVA) followed by Duncan's multiple range test or Student's *t*-test using SPSS software. Differences were considered to be significant when $P<0.05$.

Results

Altered MT expression in fry on Cd, Cr, Fe, Mn, Ni, and Zn exposure

Based on the normalization against 18S rRNA, both MT isoforms (MT-IA and MT-IB) were significantly modulated, and the change in the expression levels varied greatly among the heavy metal inducers. When exposed to low metal concentrations (1 μM), MT-IA was significantly induced by Cd (6.6-fold), Fe (2.3-fold), Ni (2.5-fold), and Zn (2.8-fold) ($P<0.05$). The expression of MT-IA on exposure to a dose of 5 μM was significantly upregulated by Cd (6.1-fold), Cr (1.9-fold), Mn (2.9-fold), and Zn (3.4-fold) ($P<0.05$), whereas Fe and Ni exposures at 5 μM caused a decrease in MT-IA from the levels observed on treatment with 1 μM. Conversely, Cd exposure resulted in a similar level of MT-IA between 1 and 5 μM (*i.e.*, highly inducible, but not dose-dependent), and the Cr- or Mn-exposed groups showed notable induction only at 5 μM. By contrast, the Fe- or Ni-exposed groups showed an inverse relationship of the MT-IA levels with the metal doses. Finally, the induction of MT-IA in the Zn-exposed groups was dose-dependent, although the elevation with increased dose was not great (Fig. 1a).

Conversely, MT-IB showed a different pattern of modulation under the same exposure conditions (Fig. 1b). In the treatments with 1 μM, MT-IB was sig-

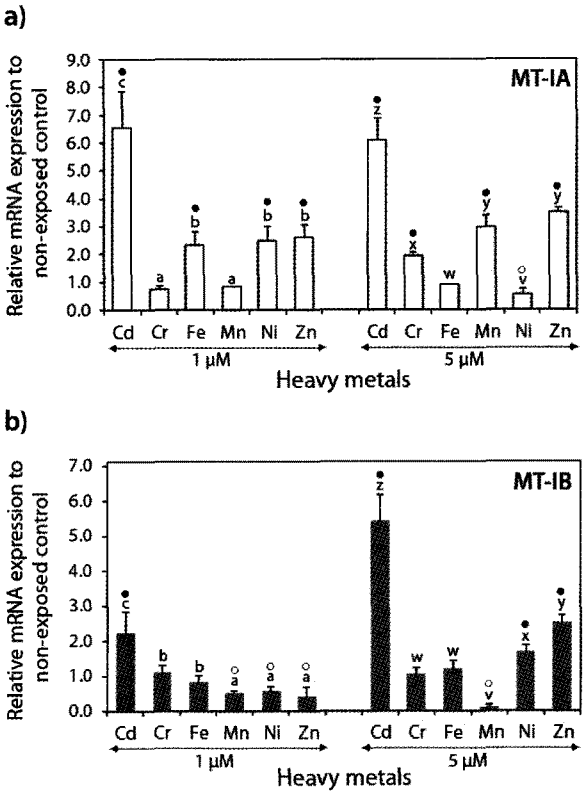

Fig. 1. Altered mRNA expression of mud loach MT-IA (a) and MT-IB (b) isoforms in whole body fry during acute sublethal exposures (1 or 5 μM for 24 h) to different heavy metals. Expression level of MT isoforms in each exposed group relative to that of non-exposed control (*i.e.*, control level=1.0) was determined using real-time RT-PCR assay based on the normalization against 18S rRNA level. Mean± SDs were represented by histograms with T-bars based on two replicate exposures per dose and triplicate expression assay per sample. Means with different letters within dose (a-c or v-z) were significantly different based on ANOVA ($P<0.05$). Significant difference from the value observed in non-exposed control group was indicated either by closed circle (for up regulation) or open circle (for down regulation) based on Student's *t*-test ($P<0.05$).

nificantly activated only by Cd (2.2-fold) ($P<0.05$), whereas the other heavy metals resulted in no apparent change (for Cr and Fe; $P>0.05$) or down-regulation (Mn, Ni, and Zn; $P<0.05$) of the MT-IB transcripts. When the dose was increased to 5 μM, the transcription of MT-IB was stimulated markedly by Cd (5.4-fold) and Zn (2.5-fold) ($P<0.05$). Fry exposed to Ni also exhibited a moderate, but significant, increase (1.6-fold) in MT-IB transcripts. Again, the groups treated with Fe or Cr showed a level similar to that observed in the non-exposed control. Interestingly, the exposure to 5 μM Mn resulted in pronounced repression (only 10% of the control value) of MT-IB transcripts ($P<0.05$).

Viability and transcriptional alteration of MT during exposure to Cu

The viability of mud loach fry was significantly affected by Cu exposure, and the adverse effects were dose-dependent. During the exposure period (24 h), only the groups exposed to low concentrations of Cu (0.5 and 1 μM) showed no mortality, like the non-exposed control, whereas fry belonging to the remaining three groups (2, 3, and 4-μM treatments) suffered from mortality as high as 65% ($P<0.05$) (Fig. 2a). The real-time RT-PCR assay showed that both MT-IA and MT-IB were induced by Cu exposure, although their stimulation patterns differed. The MT-IA transcripts were induced 1.4-fold in the group treated with the lowest dose (0.5 μM) ($P<0.05$), whereas all the groups exposed to higher concentrations (1 to 4 μM) showed similar levels of MT-IA transcripts (2.2- to 2.3-fold relative to the control value) ($P>0.05$). Conversely, the transcription of MT-IB was already stimulated up to 2.3-fold at 0.5 μM, and this level was elevated to more than 5-fold at the 3-μM dose ($P<0.05$). However, the MT-IB level in the fry belonging to the group treated with the highest dose (4 μM) was only increased 2.6-fold (Fig. 2b).

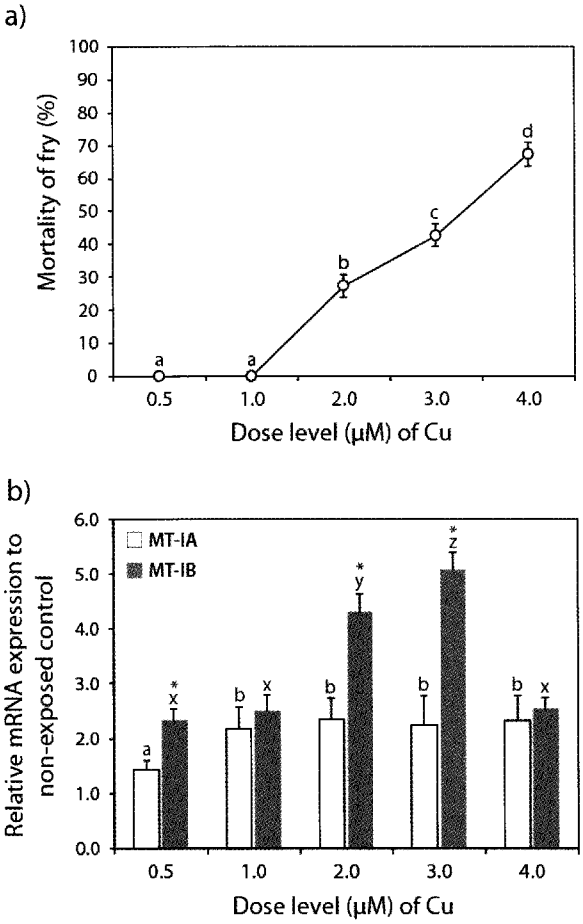

Fig. 2. Mortality (a) and transcriptional alteration (b) of two MT isoforms in mud loach whole body fry during exposure to a series of Cu concentrations from 0 to 4 μM. Cu doses (0.5 and 1.0 μM) showed no mortality (0%) as like the non-exposed control group (control group not shown). From real-time PCR assays, the Cu exposures resulted in the significant stimulation of both MT isoforms ($P<0.05$ based on ANOVA) in all of exposed groups (control level = 1.0). Mean ± SDs were represented by histograms (open histograms for MT-IA and closed for MT-IB) with T-bars based on two replicate exposures per dose and triplicate expression assay per sample. Means with different letters within isoform (a, b or x-z) were significantly different based on ANOVA ($P<0.05$). Significant difference in fold induction between MT-IA and MT-IB at a given dose level was indicated by asterisk on MT-IB based on Student's *t*-test ($P<0.05$).

Discussion

Metallothionein is a multifunctional protein involved in many cellular pathways associated with host defense mechanisms, and this protein is modulated by a number of stimulatory treatments (Coyle et al., 2002). Based on the real-time RT-PCR analysis, the patterns of MT-IA and MT-IB induction differed between the two isoforms and were highly variable depending on the inducer (*i.e.*, the heavy metal). In the sublethal treatments with Cd, Cr, Fe, Mn, Ni, and Zn, the most potent inducer of both MT isoforms was Cd, whereas the MT isoforms were not clearly responsive to Cr in general, except for a 1.9-fold induction of MT-IA at 5 μM. The expression patterns in the groups exposed to Fe, Mn, or Ni were more complex, as the two MT isoforms were regulated in opposite directions. For example, MT-IA

expression was inversely regulated with the higher dose in the fry exposed to Fe and Ni, whereas MT-IB transcription in the same fry was positively stimulated with the increased dose. In addition, the expression of MT isoforms in response to Mn exposure was clearly opposite to the regulatory pattern observed in the exposure to Fe and Ni. Metal-specific stimulation or repression of MT expression has been widely reported in other fish species (Langston et al., 2002; Lin et al., 2004; Cho et al., 2005) as well as in the adults of *M. mizolepis* (Cho et al., 2009). These findings are generally explained by the metal-specific influx/efflux rates and differential availability of the metals (Kock et al., 1995; Olsson et al., 1998; Van Campenhout et al., 2004). Furthermore, several metals inhibit *MT* gene transcription, as shown with the exposure to Fe and Ni in this study, which does not clearly fit the classical dogma of MT induction (Bi et al., 2006).

Although a direct comparison between tissue-specific expression in adults and whole-body expression in fry is impractical, our data indicate that the isoform-specific action in adult tissues (*i.e.*, the preferential induction of MT-IB over MT-IA; Cho et al., 2009) was not as clear in the fry stage of this species. Unlike adult tissues, the preferential activetion of MT-IB was not clear in fry, and, unexpectedly, the induction of MT-IA was higher than that of MT-IB in many treatments, especially those involving exposure to low metal concentrations (1 μM). By contrast, MT-IB transcription was activated only in the groups treated with the higher metal doses. This suggests that a coordinated or compensatory interacttion exists between the two MT isoforms. The predominant isoform, MT-IA, is more responsible for treating low amounts of excess metals, and the activation of MT-IB transcription may be unnecessary at this time. However, when the metal-mediated toxicity or tissue burden was pronounced, the role of MT-IB seemed to become important, and its transcription was stimulated to relieve the stress (Vergani et al., 2007).

In the exposure experiment with varying dose levels of Cu, the two MT isoforms showed different patterns of induction. MT-IA expression was rapidly induced by the lowest dose (0.5 μM in this study), but was soon saturated at higher concentrations (*e.g.*, a maximum 2-fold induction irrespective of the dose of Cu). Conversely, MT-IB transcripts increased gradually up to 5-fold at 3 μM, before declining to 2.6-fold at 4 μM. Because mud loach fry remained alive only in 0.5- and 1-μM Cu, the activation of MT-IB was very significant under lethal conditions. This observation also concurs, at least in part, with our hypothesis on the coordinated orchestration of the two MT isoforms. The drop in MT-IB transcription at the highest dose of Cu could result from the delivery of too much stress or toxicity to fry. Namely, the cellular stress would exceed the regulatory capability of the fry, and the fry would become exhausted trying to maintain homeostatic control of the Cu overload. The depression of metal-coordinating enzymes in fish exposed to high concentrations of heavy metals has also been reported (Yan and Chan, 2004; Cheung et al., 2005).

In summary, the transcriptional responses of the two MT isoforms in mud loach fry to acute heavy metal exposure varied among the metal ions tested; significant induction, a steady state, or downregulation occurred depending on the metal. Based on the hypothesis of the coordinated interaction of MT-IA and MT-IB, MT-IA may play a primary role in sequestrating the excess metals under 'less stressful' or 'sublethal' conditions, whereas the MT-IB is put into action when fry are exposed to 'more stressful' or 'lethal' conditions.

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