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Cadmium affects the expression of metallothionein (MT) and glutathione peroxidase (GPX) mRNA in goldfish, *Carassius auratus*

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Abstract

Cadmium (Cd) is a widespread non-essential heavy metal that enters the aquatic environment as a result of natural and human-caused activities, including industrial effluent, mining, and agricultural runoff. In the present study, we investigated time and dose-related effect of $CdCl_2$ on metallothionein (MT) and glutathione peroxidase (GPX) mRNA levels in a number of goldfish tissues, *in vivo*. Basal MT and GPX mRNA levels remained unchanged in the tissues tested throughout the experiment. Injection with $CdCl_2$ significantly increased MT mRNA levels in the brain, liver, kidney and intestine in a dose-dependant manner at all time tested (6, 12, 24 and 36 h). We isolated the full length GPX cDNA from goldfish kidneys, and found it to contain 785 nucleotides, including an open reading frame, predicted to encode a protein of 142 amino acids. In contrast, injection with $CdCl_2$ significantly decreased GPX mRNA levels in the liver and kidney in a time-, and dose-, dependant, and became undetectable after 12, 24 and 36 h. The findings provide molecular characterization of MT and GPX in goldfish and suggest that exposure to Cd results in significant physiological changes in goldfish.

Keywords: Cadmium; *Carassius auratus*; Gene expression; Glutathione peroxidase; Metallothionein

1. Introduction

Cadmium (Cd) is a non-essential and potentially highly toxic element to humans, animals, and plants even at low doses (Benavides et al., 2005). Cd can be released into and concentrated in aquatic environments from industrial sources such as mining, refining of ores, and plating processes, or from natural sources such as rocks and soils (Vido et al., 2001; Henkel and Krebs, 2004; Huang et al., 2004). The uptake of Cd by living cells can have a considerable effect, inducing oxidative stress, and normally leads to cell death depending on the metal dose and time of exposure (Benavides et al., 2005). There is evidence that exposure to Cd increases the formation of reactive oxygen species (ROS) and promotes oxidative stress in organisms from yeasts (Brennan and Schiestl, 1996) to vertebrates (Stohs et al., 2000) by generating free radicals and active oxygen (Toppi and Gabbrielli, 1999; Hegedus et al., 2001; Geret et al., 2002; Company et al., 2004). Reactive oxygen species are produced naturally during oxygen metabolism and include superoxide (O^-_2), hydrogen peroxide (H_2O_2), hydroxyl radicals (HO^-), and singlet oxygen ($^1O_2$). Oxidative stress by reactive oxygen species can increase lipid peroxidation, oxidation of proteins, and DNA damage. It can also affect cell viability by causing membrane damage and enzyme inactivity (Mount, 1996; Dalton et al., 1999; Singh et al., 2006), and then accelerate cell senescence and apoptosis (Kim and Phyllis, 1998).

To protect themselves against heavy metals and other toxic materials generating oxidative stress, aerobic organisms have evolved complex antioxidant defense systems. Antioxidant systems are beginning to receive attention as a biological means to reduce damage to aquatic organisms (McFarland et al., 1999; Wedderburn et al., 2000; Lionetto et al., 2003; Pandey et al., 2003; George et al., 2004). Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and antioxidant materials with low molecular weights such as glutathione (GSH), ascorbic acid, metallothionein (MT), and α-tocopherol have been found in the livers and kidneys of marine organisms (Basha and Rani, 2003; Hansen et al., 2006).

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MT is the most well known antioxidant that protects against metal toxicity (Chan and Cherian, 1992; Klaassen and Liu, 1998). It was suggested that production of cysteine-rich MT and/or binding of free metal ions to glutathione (GSH) play a cooperative role in protecting against metal toxicity; glutathione seems to be the first defense, appearing before MT (Chan and Cherian, 1992). MTs are small proteins (~7 kDa) that bind and are induced by free cytosolic metal ions, especially Cd, Cu, Zn, and Hg (Kägi and Schäffer, 1988), and are involved in defending against metal toxicity (Park et al., 2001). These properties indicate that MT is an important factor affecting metal toxicity among aquatic invertebrates (Levinton et al., 2003; Xie and Klerks, 2004).

GPX breaks down hydrogen peroxide as an antioxidant enzyme (Di Giulio et al., 1995; Halliwell and Gutteridge, 1999; Matés, 2000; Gomes-Junior et al., 2006) and is closely involved in glutathione enzymatic changes (Świergosz-Kowalewska et al., 2006). GPX activity is believed to play an important role in cellular antioxidant defense by reducing hydrogen peroxide and various hydroperoxides using glutathione as a reducing agent to water (Wendel, 1980).

The aim of this present work was to investigate and evaluate MT and GPX transcript levels as potential sensitive biomarkers of Cd toxicity in goldfish.

2. Materials and methods

2.1. Experimental fish

Goldfish (*Carassius auratus*; length: 10~13 cm) were obtained from Aquatic Imports (Calgary, Alberta, Canada) and maintained in semi-recirculating tanks in a laboratory. During the experimental period, water temperature was maintained at 17–18 °C, with a 16L: 8D photoperiod. All animals were treated in accordance with the principles and guidelines of the Canadian Council of Animal Care.

2.2. $CdCl_2$ treatment and sampling

Goldfish were anesthetized with 3-aminobenzoic acid ethyl ester (Sigma, USA) and injected with $CdCl_2$ (0, 0.01, 0.05 and 0.1 μg/g body mass, BW). The tissues (brain, liver, kidney and intestine) were sampled from five different fish at each of the following time periods: 0, 6, 12, 24 and 36 h. All tissues were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until total RNA was extracted.

2.3. Identification of GPX cDNA

Mixed primers for GPX were designed using highly conserved regions of rainbow trout GPX (GenBank accession no.AY622862) and zebrafish GPX (GenBank accession no.BC083461): gfGPX forward primer (5′-TAC ACC CAG ATG AAC GAG C-3′) and gfGPX reverse primer (5′-AGG AAC TTY TCA AAG TTC CAG GA-3′). Total RNA was extracted from kidney tissue using the Total RNA Isolation System (Promega, USA). Reverse-transcription and PCR amplification were conducted using Accupower® RT/PCR Premix (Bioneer, Korea) according to the manufacturer's instructions. The reverse-transcription was conducted at 42 °C for 60 min. PCR was subsequently carried out for 35 cycles as follows: one cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and an extension at 72 °C for 30 s, followed by one cycle of 5 min at 72 °C for the final extension. Amplified PCR products were processed by electrophoresis in 1% agarose gels containing ethidium bromide (0.5 μg/μL). The PCR product was excised and ligated into the pGEM-T Easy Vector (Promega, USA) according to the manufacture's instructions, and sequenced.

2.4. Rapid amplification of GPX cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the PCR reaction, total RNA was extracted from kidney tissue using the Total RNA Isolation System (Promega, USA). With 3 μg of total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full length cDNA Premix Kit (Seegene, Korea). First-strand cDNA synthesis was conducted using oligo (dT) anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA $T(T)_{18}$-3′) and a CapFishing™ adaptor (Seegene, Korea).

GPX-specific primers were selected from the PCR product obtained by RT-PCR. For 3′ RACE, the 50 μL of PCR reaction mixture contained 5 μL of 3′ RACE cDNA, 1 μL of 10 mmol L^{-1} 3′ target primer (5′-CTG TGA ATG CTG CGA

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gfGPX   1:MNELHSRYADQGLVVLGAPCNQFGHQENTKNDEILLSLKYVRPGNGFEPNFQLLEKLEVN  60
zfGPX   1:MNELHSRYADQGLVVLGAPCNQFGHQENCKNEEILQSLKYVRPGNGFEPKFQILEKLEVN  60
rbGPX   1:MNELHNRYSAKGLVILGVPCNQFGHQENCKNEEILKALKYVRPGNGFEPKFQLLEKVDVN  60
rtGPX   1:MNELHERYADKGLVILGVPCNQFGHQENCKNEEILMSLKYVRPGNGFEPKFQLLEKVDVN  60

gfGPX  61:GVNAHPLFVFLKEKLPQPSDDSVSLMGDPKFIIWSPVNRNDISWNFEKFLIGPDGEPFKR 120
zfGPX  61:GENAHPLFAFLKEKLPQPSDDPVSLMGDPKFIIWSPVCRNDISWNFEKFLIGPDGEPFKR 120
rbGPX  61:GQDAHPLFVFLKEKLPFPCDDAMALMTDPKFIIWSPVSRNDVSWNFEKFLVSPDGEPYKR 120
rtGPX  61:GKDAHPLFVYLKDKLPFPSDEPMALMNDPKCIIWSPVCRTDIAWNFEKFLIGPAGEPFKR 120

gfGPX 121:YSRRFLTIDIEADIKELLKRAK                                       142
zfGPX 121:YSRRFLTIDIDADIKELLKRTK                                       142
rbGPX 121:YSRNFLTTDIEADIKELLKRVK                                       142
rtGPX 121:YGRRFLTSNIEGDIKELLNTAN                                       142
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Fig. 1. Comparison of amino acid sequences of goldfish, *Carassius auratus* GPX (gfGPX), zebrafish, *Danio rerio* GPX (zfGPX), rock bream, *Oplegnathus fasciatus* GPX (rbGPX), and rainbow trout, *Oncorhynchus mykiss* GPX (rtGPX), optimally aligned to match identical residues (indicated by the shaded box). The GPX sequences used for alignment are goldfish GPX (DQ983598), zebrafish GPX (BC083461), rockbream GPX (AY734530) and rainbow trout GPX (AY622862). The sequences were taken from the GenBank/EMBL/DDBJ sequence databases.

CTA CGA T-3′), 1 μL of 10 mmol L^{-1} 3′ RACE gene specific primer (5′-TGT GTT CCT CAA GGA GAA GCT GCC TCA G-3′), and 25 μL of SeeAmp Taq Plus master mix. PCR was carried out for 35 cycles as follows: one cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by one cycle of 5 min at 72 °C for the final extension.

For 5′ RACE, the 50 μL of PCR reaction mixture contained 5 μL of 5′ RACE cDNA, 1 μL of 10 mmol L^{-1} 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL of 10 mmol L^{-1} 5′ RACE gene specific primer (5′-TCC AGG AGA TGT CAT TCC TGT TCA CCG GA-3′), and 25 μL of SeeAmp Taq Plus master mix. PCR was carried out for 35 cycles as follows: one cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by one final extension cycle of 5 min at 72 °C. Amplified PCR products were processed by electrophoresis in 1% agarose gels containing ethidium bromide (0.5 μg/μL). The PCR product was purified and then cloned into pGEM-T Easy Vector (Promega, USA). The colony formed by transformation was cultivated in DH5α (RBC, Korea), and then plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Korea) and EcoR I (Fermentas, USA). Based on the plasmid DNA, GPX cDNA sequence data were analyzed using an ABI DNA Sequencer (Applied Biosystems, USA).

2.5. Expression patterns of MT and GPX mRNA

RT-PCR was conducted to determine the relative expression of MT and GPX mRNA in goldfish. Total RNA was extracted from brain, liver, kidney and intestine. MT-specific primer for RT-PCR was designed from the published sequence as follow: MT forward primer (5′-AAT GCG CCA AGA CTG GAG CT-3′) and MT reverse primer (5′-TGA AGA ACA ACA GGG AGG TCG T-3′). This primer was based on the sequence of the goldfish MT

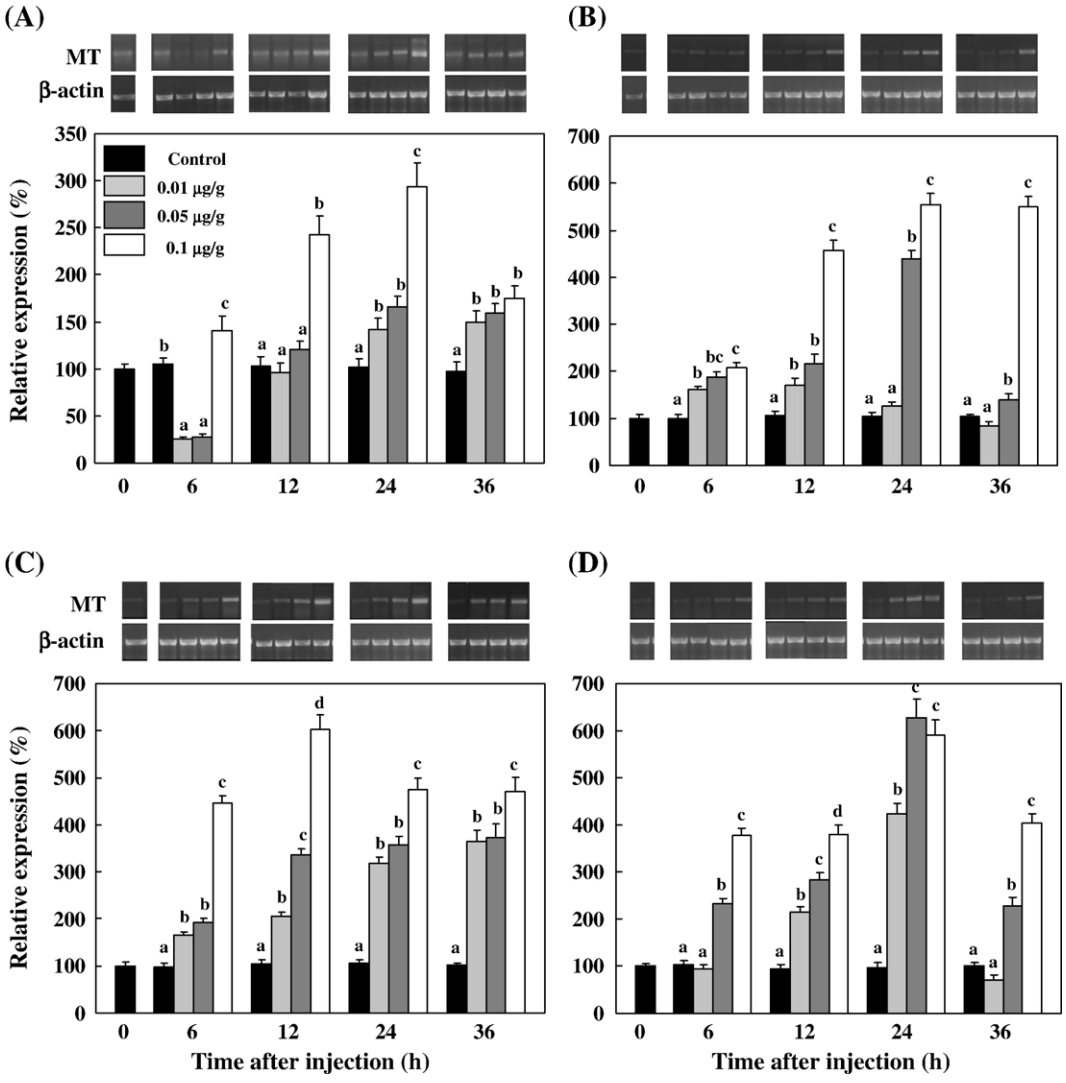

Fig. 2. Expression of MT mRNA in goldfish tissue by $CdCl_2$ treatment. One μg of total RNA prepared from brain (A), kidney (B), liver (C) and intestine (D) was reverse-transcribed and amplified using goldfish MT-specific primer. Tissue distribution of goldfish MT was analyzed using RT-PCR. Values with dissimilar letters indicate a significant difference ($P<0.05$) among $CdCl_2$ concentrations in the same time of sampling. Values are mean±SD ($n=5$).

(Genbank accession no.S75039). GPX-specific primer for RT-PCR was designed from the above results in this study: GPX forward primer (5′-TAC ACC CAG ATG AAC GAG C-3′) and GPX reverse primer (5′-AGG AAC TTC TCA AAG TTC CAG GA-3′). Total RNA was extracted using the Total RNA Isolation System (Promega, USA) from brain, liver, kidney, and intestine. RT-PCR was conducted using SuperScript III One-Step RT-PCR with Platinum Taq (Invitrogen, USA) with 2.5 μg of total RNA as a template. A reverse-transcription was conducted at 50 °C for 60 min, after which PCR was carried out for 35 cycles as follows: one cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and an extension at 68 °C for 30 s, followed by one cycle of 5 min at 68 °C for the final extension. Amplified PCR products were processed by electrophoresis in 1% agarose gels containing ethidium bromide (0.5 μg/μL).

Quantification of PCR amplified fragment was carried out by high resolution scanner and the band densities were estimated by a computer program (KBT Co., Korea). In each case the loading was controlled by amplification of β-actin. The densitometric process from ethidium bromide-stained gel was optimized for linearity as described previously (Kermouni et al., 1998).

2.6. Statistical analysis

The data from each experiment were tested for significant differences using a one-way analysis of variance (ANOVA; least significant difference [LSD] test) with the SPSS statistical package (version 10.0) at a significance level of $P<0.05$.

3. Results

3.1. Identification of GPX cDNA

RT-PCR was used to clone a fragment of a GPX cDNA using total RNA extracted from goldfish kidney tissue. A single PCR product of the expected size (338 base pairs, bp) was obtained. A PCR-based cloning strategy (RT-PCR followed by 3′- and 5′ RACE) was used to clone a full length cDNA encoding a putative GPX from goldfish kidney tissue. GPX cDNA contained 785 nucleotides, including an open reading frame (ORF), predicted to encode a protein of 142 amino acids. Fig. 1 compares the amino acid sequence of goldfish GPX to previously reported GPX amino acid sequences. The deduced amino acid sequence of goldfish GPX had the closest identity to zebrafish GPX (89%; GenBank accession no.BC083461), followed by rock bream (79%; GenBank accession no. AY734530) and rainbow trout (78%; GenBank accession no. AY622862).

3.2. Levels of MT transcripts

The MT transcript level did not change significantly in the control groups at different time intervals (Fig. 2). Treatment with $CdCl_2$ significantly increased MT transcript level in the brain, kidney, liver and intestine in a dose and time related manner. Maximal response was observed at the highest dose of $CdCl_2$ tested (0.1 μg/g), although differences were observed between different tissues. In the brain, lower doses of $CdCl_2$ (0.01 and 0.05 μg/g) resulted in significant reduction in MT transcript level following 6 h of treatment. After 12 h, however, no reduction was observed and $CdCl_2$ at the highest dose (0.1 μg/g) resulted in significant increase in MT transcript level in the brain (Fig. 2). In the kidneys, all doses of $CdCl_2$ increased MT transcript level following 6 and 12 h of treatment, and significant increase was observed only at higher doses after 24 and 36 h of treatment. In the liver, all doses of $CdCl_2$ significantly increased MT transcript level at all time points tested, although the extent of stimulation was somewhat greater at 12~36 h following treatment with $CdCl_2$. In the intestine, treatment with $CdCl_2$ increased MT transcript in a dose and time

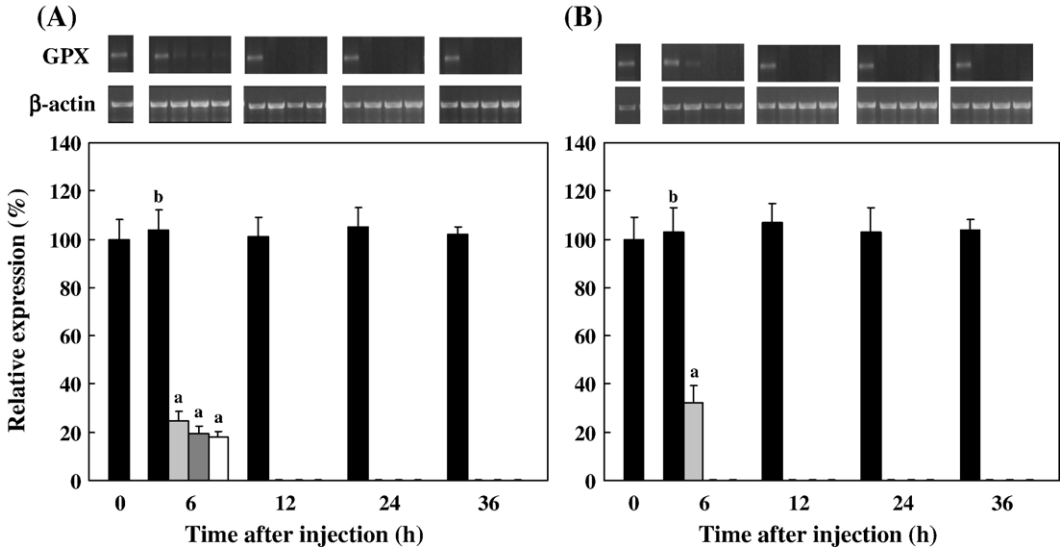

Fig. 3. Expression of GPX mRNA in goldfish tissue by $CdCl_2$ treatment. One μg of total RNA prepared from liver (A) and kidney (B) was reverse-transcribed and amplified using goldfish GPX-mixed primer. Tissue distribution of goldfish GPX was analyzed using RT-PCR. Values with dissimilar letters indicate a significant difference ($P<0.05$) among $CdCl_2$ concentrations in the same time of sampling. Values are means±SD ($n=5$).

related manner with greatest level of expression following 24 h of treatment with $CdCl_2$.

3.3. Levels of GPX transcripts

The expression of GPX was only present in the goldfish liver and kidney, and no GPX mRNA was detected in the brain and intestine (Fig. 3). Treatment with $CdCl_2$ significantly inhibited GPX expression at all doses after 6 h of treatment (Fig. 3). The expression of GPX became totally undetectable following 12~36 h of treatment with $CdCl_2$ in both liver and kidney (Fig. 3). No results are shown for the brain and intestine as the levels were undetectable.

4. Discussion

Heavy metals induce reactive oxygen species in animals by binding with electrons, preventing transport of oxygen during respiration and affect physiology, growth, and protection against pathogens. Because reactive oxygen species have high reaction abilities, abnormally high amounts can result in various kinds of oxidative damage (Kim and Phyllis, 1998). With the exception of MT, very few studies have examined antioxidant gene expression in fish (Hansen et al., 2006). In the present study, we examined changes in MT and GPX mRNA levels following treatment with Cd in goldfish. Expression analysis of the GPX gene is becoming a basic research tool for studying antioxidants as an indication of environmental pollution or degree of stress among fish (Aurélie et al., 1997; Peters et al., 2001; Lionetto et al., 2003; Pandey et al., 2003).

In general, MT binds to heavy metals and reduces their toxicity. Treatment of goldfish with $CdCl_2$ significantly increased MT mRNA levels in brain, liver, kidney and intestine. This is consistent with previous studies demonstrating that black goby (*Gobius niger*) treated with Cd had higher levels of MT mRNA level than control (Migliarini et al., 2005). In rainbow trout, higher MT mRNA was observed in the liver after 14 days of exposure to Cd (Lange et al., 2002). Similarly in shark (*Scyliorhinus torazame*) and specimens of *Seriola dumerilli*, treatment with $CdCl_2$ exposure resulted in increased MT mRNA levels in the liver according to time- and dose-dependence (Cho et al., 2005; Jebali et al., 2006). In general, it is evident that exposure to Cd significantly increases MT expression in various species, with some differences depending on concentration and exposure time. The findings collectively support the hypothesis that Cd increases synthesis of MT, which plays a role in heavy metal detoxication by reducing oxidative stress (Baudrimont et al., 2003).

GPX is closely involved with glutathione, playing a role in detoxication and removing hydrogen peroxide from cells by glutathione oxidation (Świergosz-Kowalewska et al., 2006). In this study, we isolated GPX cDNA from goldfish kidneys. Goldfish GPX cDNA is composed of 785 nucleotides, including an open reading frame (ORF), predicted to encode a protein of 142 amino acids. GPX expression was observed in the goldfish liver and kidney, but the transcript was not detectable in the brain and intestinal tissues. Expression of GPX in the liver and kidney was significantly reduced by treatment with all doses of $CdCl_2$ tested, reaching undetectable levels at post treatment of 12 h and greater. This is consistent with previous studies demonstrating considerably reduced levels of GPX mRNA expression in Asiatic clam (*Corbicula fluminea*) and gastropod (*Achatina fulica*) by increasing the amounts of $CdCl_2$ (Chandran et al., 2005; Legeay et al., 2005). There appear to be an interaction between Cd and Cu as well as Zn which increase GPX mRNA expression levels as well as superoxide dismutase and catalase (Chung et al., 2005; Hansen et al., 2006). Cd treatment was found to decrease superoxide dismutase and GPX activity by inhibiting enzyme-defending mechanisms (Geret et al., 2002; Company et al., 2004). While in a number of species Cd reduce GPX mRNA level, in tilapia exposure to sublethal levels of Cd^{2+} was found to increase GPX in the liver and kidney tissue indicating that the observed response may not be present in all species (Basha and Rani, 2003).

Overall, we observed increased levels of MT mRNA and decreased levels of GPX mRNA following treatment presumably due to $CdCl_2$ induced oxidative stress, suggesting MT plays a role in detoxifying Cd. Measurement of MT provide a suitable biomarker for the presence of Cd and possibly other heavy metals in goldfish.

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