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Response of antioxidant enzymes to Cd and Pb exposure in water flea *Daphnia magna*: Differential metal and age — Specific patterns

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ABSTRACT

To investigate oxidative stress responses to cadmium and lead, the freshwater water flea *Daphnia magna* was exposed to Cd and Pb for 48 h. Following treatment with sub-lethal concentrations, intracellular reactive oxygen species (ROS) levels, as well as modulation of multiple biomarker, such as superoxide dismutase (SOD) activity, glutathione (GSH) contents, glutathione *S*-transferase (GST) activity, antioxidant enzyme - coding genes (three GST isoforms, glutaredoxin [*GRx*], glutathione peroxidase [*GPx*], and thioredoxin [*TRx*]), and stress-response proteins (heat shock protein 70 [Hsp70] and Hsp90) were examined. The results showed that intracellular ROS level was not changed at 24 h, but reduced at 48 h. Levels of total GSH content were reduced by Cd, but highly induced by Pb. SOD and GST activities were stimulated 48 h after exposure to Cd and Pb. A significant modulation of oxidative stress marker genes was observed after exposure to each element with different expression patterns depending on the metal and developmental stages. In particular, the expression levels of *GST-sigma*, *HSP70,* and *HSP90* genes were enhanced in Cd - and Pb - exposed neonates. These findings imply that oxidative stress markers appear to be actively involved in cellular protection against metal-induced oxidative stress in *D. magna*. This study would facilitate the understanding of the molecular response to Cd and Pb exposure in water fleas.

1. Introduction

Cadmium and lead are nonessential metals that are widely distributed as natural trace components in the aquatic environment (Tchounwou et al., 2012). However, anthropogenic sources, such as steel industries, agriculture, sewage, and mining activities have increased their background levels in the environment (Fairbrother et al., 2007; Naja and Volesky, 2009). In particular, metals are of great concern in aquatic ecosystems because of their persistence and accumulation properties in the tissues of aquatic organisms (Seebaugh et al., 2005; Naja and Volesky, 2009; Singh et al., 2011). Several studies suggest that metals have adverse effects on the growth, reproduction, physiology and biochemistry of aquatic invertebrates (Sarma et al., 2000; Sharma and Agrawal, 2005; Juárez-Franco et al., 2007; Das and Khangarot, 2011).

Most elements can be regulated by specific mechanisms in organisms (Rutherford and Bird, 2004; Singh et al., 2011). Thus, the toxic effects of metals are dependent on excretory, metabolic storage, and detoxification mechanisms. However, this capacity also varies between different species and metals (Ercal et al., 2001). One of the well-known mechanisms of metal-induced responses is the generation of reactive oxygen species (ROS) in oxygen-consuming organisms. Cd and Pb, are redox-inactive metals that induce oxidative stress indirectly by depleting major cellular sulfhydryl reserves (Stohs and Bagchi, 1993). Particularly, Cd and Pb have electron-sharing affinities, resulting in the formation of covalent attachments between metals and sulfhydryl groups of proteins, such as glutathione (GSH) (Bondy, 1996). In animals, Cd binds to the thiol group of GSH and form a bis(glutathionato)-cadmium complex (Cd-GS2) (Pastore et al., 2003). Continuous depletion of GSH by interaction with organismal metals following chronic exposure is a key part of the toxic response of numerous metals (Hultberg et al., 2001). GSH is recovered by glutathione reductase (GR), which catalyzes the reduction of the oxidized form (GSSG) to the reduced form (GSH), thereby maintaining the balance of GSH:GSSG ratio. GSH is also a substrate of glutathione peroxidase (GPx) and glutathione S-transferase (GST). Other thiol-containing proteins, thioredoxin (TRx)

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and glutaredoxin (GRx), also play a key role in maintaining the cellular redox state (Holmgren et al., 2005). In particular, GSH- and NADPH-dependent GR comprise the GRx system.

Several antioxidant enzymes including superoxide dismutase (SOD) are involved in cellular protection; however, toxic metals can directly attack enzymes with sulfhydryl groups in their active sites. Consequently, increase in intracellular ROS levels causes oxidation of cellular macromolecules, such as lipids (lipid peroxidation and membrane damage), proteins (protein oxidation and dysfunction), and DNA (oxidation, impairment of DNA repair, and mutagenesis/carcinogenesis), leading to cell death (reviewed by Ercal et al., 2001).

The freshwater water flea *Daphnia magna* has a key part as an energy transmitter in the food webs of freshwater ecosystems. Particularly, their feeding strategies, such as non-selective filter feeding, might increase their exposure to xenobiotics in aquatic environments (Geller and Müller, 1981; DeMott, 1982). In ecotoxicological studies, *D. magna* has several advantages, such as easy maintenance in the laboratory, short life cycle, transparent nature, and sensitivity to various chemicals. Therefore, this species has been used as a representative model species in ecotoxicology, risk assessment, and toxicogenomics (Wollenberger et al., 2000; Santore et al., 2001; Jemec et al., 2016). For example, the intensive studies have been published showing the alterations of growth, reproduction, and metabolisms in *D. magna* exposed to various metals in the 1970s (Biesinger and Christensen, 1972) and a variety of researches have shown the possibility of being a model species that can show various trials for environmental monitoring, particularly on metals (Pollard et al., 2003; Schamphelaere and Janssen, 2004). In addition, investigation of the growth and reproduction of *D. magna* exposed to several antibiotics following the standard procedures of the International Organization for Standardization (ISO) and Organization for Economic Co-operation and Development (OECD) also showed it has potential as a model species in aquatic environmental studies for other toxic compounds (Wollenberger et al., 2000). Furthermore, *Daphnia* species have received considerable attention and used as a target species as much as fish in toxicokinetic studies (Santore et al., 2001). However, although genome sequences of *Daphnia pulex* and, more recently, *Daphnia magna* have been publicly released, molecular studies on chemical-induced toxic effects are still lacking for Daphnids. A few recent studies identified and characterized *D. magna* SODs (Lyu et al., 2013, 2014), GST (Lyu et al., 2016), and catalase (CAT) (Kim et al., 2010a). More recently, ecotoxicogenomic studies using a custom *D. magna* cDNA microarray showed upregulation of *GST* in the Cd-exposed group, indicating possible Cd-induced oxidative stress (Poynton et al., 2007). However, studies on the multiple expression and activation of other antioxidant enzymes and stress proteins following exposure to metals are limited in this species.

In this study, we examined intracellular ROS levels of the freshwater water flea *D. magna* after exposure to Cd and Pb for 24 h and 48 h. The non-enzymatic antioxidant GSH and antioxidant GST and SOD enzyme activities were also measured. In addition, transcriptional profiles of eight genes, consisting of antioxidant - related (*GPx, GRx, TRx, GST-mu, GST-sigma*, and *GST-zeta*) and stress response (*Hsp70* and *Hsp90*) genes were investigated. This study will provide insight into the dose response relationships for oxidative stress biomarkers to metal exposure in this species.

2. Materials and methods

2.1. Culture and maintenance

The freshwater water flea *D. magna* was supplied by the National Institute of Environmental Research (NIER) in South Korea and transported to the Laboratory of Molecular Toxicology in Sangmyung University, Seoul, South Korea. They were reared and maintained in ISO medium, according to the ISO 6341(2012) guideline, when the media had pH 6–9, DO 4–5 mg/L, and hardness 160–165 mg/L (as $CaCO_3$). The culture conditions were a temperature of 22 ± 1 °C and a light/dark cycle of 12:12 h. The media was changed every 3 days. The green algae, *Chlorella vulgaris* ($3.0–3.5 \times 10^{6–8}$ cells/mL, 1 mL/day) was supplied daily as a food source.

2.2. Chemical exposure

All the chemicals and reagents used in this study were of molecular biology grade and were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise specified. For the metal exposure test, stock solutions (1 g/L) of Cd (as cadmium chloride [$CdCl_2$]) and Pb (as lead nitrate [$Pb(NO_3)_2$]) were prepared by dissolving in distilled water. For ROS level and antioxidant enzyme activity determination, juvenile (< 5-day-old, 30 individuals) *D. magna* were exposed to Cd (0.4 and 10 μg/L) and Pb (16 and 400 μg/L) for 24 h and 48 h. For gene expression analysis, neonates (< 24-h-old, 300 individuals) and juvenile (< 5-day-old, 30 individuals) were treated with Cd (0.4, 2 and 10 μg/L) and Pb (16, 80 and 400 μg/L), for 24 h and 48 h. Sublethal concentrations of Cd and Pb were calculated based on the acute test by Kim et al. (2017). All experiments were performed in triplicates.

2.3. Total RNA extraction and cDNA synthesis

After exposure to Cd and Pb, the *D. magna* were harvested, homogenized in 5 volumes of TRIZOL® reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) in a grinder, and stored at −80 °C until further use. Total RNA was isolated from the homogenized sample according to the manufacturer's instructions. The total RNA quality and quantity were confirmed by 1% agarose gel electrophoresis and ultraviolet (UV) transilluminator (DU730, Beckman Coulter Inc., Brea, CA, USA) and a Nanodrop (MaestroNano Pro, NaestroGen Inc., Taiwan). The cDNA was synthesized from 2 μg total RNA using oligo$(dT)_{18}$ primer and ReverTra Ace® quantitative polymerase chain reaction (qPCR) reverse transcription (RT) Master Mix (Toyobo Corp.).

2.4. PCR amplification

Partial sequences of *D. magna* genes were obtained from GenBank to identify the full-length sequences of seven genes (*GPx, GRx, GST-mu, GST-sigma, GST-zeta, TRx*, and *HSP90*). The primer sets used in this study are listed in Suppl. Table 1. Full-length cDNA sequences of *D. magna HSP70* were retrieved from GenBank and confirmed using PCR. A PCR analysis was performed to confirm each gene sequence in MyCyclerTM (Bio-Rad Inc., Hercules, CA, USA). All PCR reactions consisted of 1 μL cDNA and a 0.2-μM primer set (see Suppl. Table 1). The PCR reaction conditions were as follows: 95 °C/5 min; 35 cycles of 95 °C/1 min, 57 °C/1 min, 72 °C/1 min; and 72 °C/10 min. The PCR product was visualized on a 1% agarose gel and purified using QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany) for sequencing.

2.5. Rapid amplification of cDNA ends (RACE)

To obtain the full-length cDNA, a SMARTer RACE 5′/3′ kit (Takara Bio USA Inc. (formerly Clontech Laboratories Inc. CA, USA) was used. Each 5′ and 3′ RACE cDNA was synthesized according to the manufacturer's instructions. Gene-specific primers (GSPs) were designed based on each predicted gene sequence (Suppl. Table 1). The PCR products were purified using QIAquick gel extraction kit for sequencing.

2.6. Sequence analysis

Deduced amino acid sequences were produced using the Genetyx Version 7.0.3 (Genetyx Co., Tokyo, Japan). Conserved domains were found using National Center for Biotechnology Information (NCBI)