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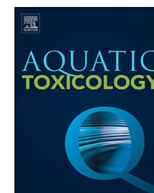

Identification and molecular characterization of two Cu/Zn-SODs and Mn-SOD in the marine ciliate *Euplotes crassus*: Modulation of enzyme activity and transcripts in response to copper and cadmium

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ABSTRACT

The superoxide dismutase (SOD) family is a first line antioxidant enzyme group involved in transformation of the superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) and O_2. SOD gene expression patterns and enzyme activities therefore have a role as molecular biomarkers in evaluating the oxidative stress status of aquatic organisms. However, antioxidant enzyme systems are yet to be fully explored in the marine ciliates. In this study, we identified and characterized two types of Cu/Zn SODs (Ec-Cu/ZnSOD1 and Ec-Cu/ZnSOD2) and Ec-Mn SOD in the marine ciliate *Euplotes crassus*. Subsequently, SOD activity and transcriptional modulation of the relevant genes were investigated after the exposure to Cd and Cu for 8 h. All Ec-SODs showed conserved domains and metal binding sites on their active sites. Total SOD activity was induced at 1 h after exposure to Cd (125 and 1000 μg/L), and showed a marginal increase at 1-h exposure to Cu (10 and 100 μg/L). However, SOD activity was maintained at a steady level under Cd and decreased under Cu exposure conditions at 3 h and 8 h. mRNA expression of both the *Ec-Cu/Zn-SODs* and *Mn-SOD* were remarkably elevated after the exposure to Cd (250–1000 μg/L, maximum 4-fold, $p < 0.05$) and, in particular, Cu (25–100 μg/L, maximum > 20-fold, $p < 0.05$), in a concentration – dependent manner. These findings suggest that Ec-SODs may be actively involved in cellular protection against metal – mediated oxidative stress. This study is therefore helpful in understanding the molecular responses for metal toxicity in the ciliates.

1. Introduction

The continuous input of metals derived from mining, refinery industry and oil spills into aquatic ecosystems is of a great concern, because of their persistency and accumulation in the tissue of aquatic organisms through water, food, and sediment (Phillips, 1977; Hsu et al., 2017; Sánchez-Quiles et al., 2017). The most important issues regarding metal pollution in the environment is that metals may catalyze reactions in organisms that generate reactive oxygen species (ROS) which may lead to environmental oxidative stress. In the cell, in particular, imbalances between the amount of ROS and the antioxidant system cause oxidative damage, such as lipid peroxidation, protein oxidation, and DNA damage, which in turn lead to aging, disease, and cell death (Ercal et al., 2001; Bhattacharya, 2014; Pisoschi and Pop, 2015; Sara et al., 2016). Metals are divided into two groups according to the mechanisms that generate ROS: redox active metals (e.g., copper, zinc, iron), and redox inactive metals (e.g., cadmium, lead, mercury). The two groups have different mechanisms of metal-induced oxidative stress (Hobman et al., 2007). Redox-active metals produce reactive oxygen species (ROS) by involvement in cellular redox cycles, whereas several metals induce oxidative stress through indirectly attacking the thiol-group of glutathione (GSH) (reviewed by Ercal et al., 2001). GSH plays an important role in maintaining homeostasis and preventing oxidative stress in cells by scavenging hydroxyl radicals, singlet oxygen, and numerous electrophiles (Lushchak, 2011; Hellou et al., 2012)

Copper is a redox-active metal that produces hydroxyl radicals from hydrogen peroxide via the Haber-Weiss and Fenton reactions; these radicals are highly reactive and break down lipid peroxides into peroxyl and alkoxyl radicals, leading to the propagation of lipid oxidation (Ziouzenkova et al., 1998). Cadmium is a redox-inactive metal that

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binds to the thiol group of GSH, a substrate of glutathione peroxidase (GPx) and glutathione reductase (GR), which eliminates peroxides. In particular, GR catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH)-driven reduction of glutathione disulfide (GSSG) to GSH (Franco and Cidlowski, 2012). Glutathione mediates the ROS process by donating reduced equivalents to GPx (Espinosa-Diez et al., 2015). When superoxide dismutase (SOD) shows superoxide reductase activity, GSH acts to remove cellular radicals in concert with SOD (Winterbourn et al., 2002).

SODs are widely found in prokaryotes and eukaryotes. They are classified into three types according to the kinds of metals located on the active sites: copper/zinc-SOD (Cu/Zn-SOD), manganese SOD (Mn-SOD), and iron-SOD (Fe-SOD). Cu/Zn-SODs are located in the cytoplasm of eukaryotes, including plants, and prokaryotes, and bind Cu and Zn for the catalytic reaction. Two types of Cu/Zn-SOD, intracellular Cu/Zn-SOD and extracellular Cu/Zn-SOD, are found in eukaryotes (Fattman et al., 2003; Plantivaux et al., 2004; Miller, 2012; Ferro et al., 2015). Mn-SODs are found in prokaryotes and in the mitochondria of eukaryotes. In contrast, Fe-SODs are present only in prokaryotes, plants, and protozoa. Mn- and Fe-SODs show a high degree of similarity in sequence and structure, suggesting that they have a common ancestry (Wintjens et al., 2004).

SODs play an essential role in cellular defense systems against oxidative stress by catalyzing the dismutation of superoxide anions to hydrogen peroxide and molecular oxygen. They form a first line antioxidant enzymatic defense, acting as scavengers of endogenous and exogenous ROS. Thus, SOD activities and gene expression are considered as molecular biomarkers for evaluating the oxidative stress status of aquatic organisms exposed to various contaminants (reviewed by Valavanidis et al., 2006). Cu/Zn- and Mn-SODs have been identified and characterized in several aquatic invertebrates, such as water fleas (Lyu et al., 2014), copepods (Kim et al., 2011a), shrimps (Gómez-Anduro et al., 2006), prawns (Cheng et al., 2006), and rotifers (Yang et al., 2013). However, although their presence in a few frewshwater species of single-celled eukaryotes has recently been reported, including *Tetrahymena thermophila* (Ferro et al., 2015) and *Paramecium caudatum* (Sara et al., 2016), more research is required into the identification and characteristics of SODs in ciliates.

Eukaryotic single-celled ciliate protozoa are widely distributed in terrestrial, freshwater, and marine environments. They are important components of planktonic and benthic microecosystems and act as energy transmitters in aquatic environments (Fenchel, 1987). Their eukaryotic characteristics and sensitive responses of single cells to various stressors have facilitated getting results indicating exposure effects in ecotoxicological studies (Gutierrez et al., 2003; Dayeh et al., 2005). Furthermore, they are considered suitable for predicting the responses of higher organisms as protozoa are the eukaryotic ancestors of all metazoans (Trielli et al., 2007). Additional advantages, such as small size, short generation time (−10 h) (Roth et al., 1985), production of genetically homogeneous progeny by asexual reproduction, and ease of maintenance under laboratory conditions, make them a potential eukaryotic model species for evaluating the effects of contaminants on aquatic communities (Gomiero et al., 2012, 2013). Our previous studies suggest that Cd and Cu act to generate ROS and induce oxidative stress by increasing the antioxidant enzyme activities (i.e., glutathione S-transferase (GST), GPx, and GR) and mRNA expression of genes encoding them along with changes in GSH content in *Euplotes crassus* (Kim et al., 2011b, 2014). More recently, we analyzed the transcriptome of *E. crassus* after exposure to Cd and Cu, and obtained differentially expressed genes (DEGs), including *SODs* and *heat shock proteins* (Kim et al., 2018).

In the present study: 1) the full-length cDNAs of intracellular Cu/Zn-SOD, extracellular Cu/Zn-SOD, and Mn-SOD were first cloned and sequenced from the marine ciliate *E. crassus*; 2) transcriptional modulation of these genes in response to Cd and Cu was investigated; and 3) enzyme activity of SOD was further assayed both with and without Cd and Cu. The aims of this study were to: identify SODs and characterize their molecular response when exposed to Cd and Cu; investigate the effects of metals on mRNA and enzymatic activity of SODs; highlight the usefulness of SODs as potential molecular biomarkers for risk assessment; and, finally, contribute additional ecotoxicological data in ciliate field.

2. Materials and methods

2.1. Cell culture and maintenance

Euplotes crassus was cultured in 0.2 μm-filtered artificial seawater (Reef Crystals, Aquarium Systems, OH, USA), salinity 30 psu, at 22.5 °C under a 12 h: 12 h light/dark photoperiod, according to the method of Kim et al. (2011b). A bacto-yeast exract (0.2 g per day) (Laboratorios Conda S.A., Madrid, Spain) was used as the food source for *E. crassus* cells growth. Identification of the species was performed using morphological characteristics and a molecular marker (18S rRNA) (Kim et al., 2011c).

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 indicated. For stock solution Cd (as 1.0 g/L with $CdCl_2$) and Cu (as 0.1 g/L with $CuCl_2$) were dissolved in sterile distilled water. For gene expression, after a 24-h acclimation period exponential phase *E. crassus* cells (2.5×10^4 cells/10 mL) in 15 mL conical tubes were exposed to Cd (0, 125, 250, 500 and 1000 μg/L) and Cu (0, 10, 25, 50 and 100 μg/L) for 8 h. For measurements of total SOD activity, after a 24-h acclimation period exponential phase *E. crassus* cells (4×10^4 cells/10 mL) in 15 mL conical tubes were exposed to Cd (0, 125 and 1000 μg/L) and Cu (0, 10 and 100 μg/L) for 1, 3 and 8 h.

2.3. Total RNA extraction and cDNA synthesis

After exposure to Cd and Cu, cells (2.5×10^4 cells) were harvested in 5 vols of TRIZOL reagent (Thermo Fisher Scientific Inc., Ambion.) and stored −80 °C until use. The total RNA was extracted from the cells according to manufacturer's instructions. Extracted RNA quality and quantity were checked using a 1% agarose gel electrophoresis and UV transilluminator (DU730, Beckman Coulter, USA), and a spectrophotometer (DU®730, Beckman Coulter Inc., Brea, USA). Samples with the ratio of A260/A280 of 1.8 ~ 2.0 were used. The cDNA was synthesized from 2 μg of the total RNA by using ReverTra Ace® qPCR RT Master Mix (Toyobo Corp.).

2.4. Polymerase chain reaction (PCR) amplification

In order to identify the ORF sequence of the *SOD* genes, partial sequences were obtained from the local database of *E. crassus* transcriptome (Genetic laboratory, Sangmyung University). A polymerase chain reaction (PCR) analysis was performed to confirm each gene sequence. All PCR reactions included 1 μL of cDNA and a 0.2-μM primer set (Table S1). The PCR reaction conditions were as follows: 95 °C/5 min; 35 cycles of 95 °C/30 s, 55 °C/30 s, 72 °C/1 min; and 72 °C/10 min. The PCR proceeded in MyCycler™ (Bio-Rad Inc., CA, USA), and 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 ORF from partial sequences, 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

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