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Tissue-specific molecular and cellular toxicity of Pb in the oyster (*Crassostrea gigas*): mRNA expression and physiological studies

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ABSTRACT

Lead (Pb) is one of the ubiquitous and toxic elements in aquatic environment. In oysters, gills and digestive glands are the main target organs for Pb-induced toxicity, but there is limited information on the molecular mechanisms underlying its toxicity. The present study investigated the Pb-induced toxicity mechanisms in the Pacific oyster (*Crassostrea gigas*) based on transcriptome, phenotypic anchoring, and validation of targeted gene expression. Gene ontology and pathway enrichment analyses revealed the differential Pb toxicity mechanisms in the tissues. In the gills, Pb disturbed the protein metabolism, with the most significant enrichment of the "protein processing in endoplasmic reticulum" pathway. The main mechanism comprised of a Pb-stimulated calcium (Ca^{2+}) increase by the up-regulation of transporter-Ca-ATPase expression. The disturbed Ca^{2+} homeostasis then further induced high expressions of endoplasmic reticulum (ER) chaperones, leading to ER stress in the oysters. Unfolded proteins induced ER associated degradation (ERAD), thereby preventing the accumulation of folding-incompetent glycoproteins. However, Pb mainly induced oxidative reduction reactions in the digestive gland with high accumulation of lipid peroxidation products and high expression of antioxidant enzymes. Further, Pb induced fatty acid β-oxidation and CYP450 catalyzed ω-oxidation due to increased metabolic expenditure for detoxification. The increased content of arachidonic acid indicated that Pb exposure might alter unsaturated fatty acid composition and disturb cellular membrane functions. Taken together, our results provided a new insight into the molecular mechanisms underlying Pb toxicity in oysters.

1. Introduction

Metal pollution in many parts of the world has increased over the last decades due to their increasing usages in agricultural, chemical, and industrial processes. Lead (Pb) is one of most ubiquitous and toxic elements in the aquatic environment (Cheng and Hu, 2010; Flora et al., 2012), and ranks second among the 275 substances comprised by the Agency for Toxic Substances and Disease Registry (ATSDR)/Environmental Protection Agency (EPA) Priority List of Hazardous Substances published in 2001 (Stevens et al., 2002). Bivalves (Mollusca: Bivalvia), and in particular oysters, are known to bioaccumulate and tolerate high concentrations of Pb and other metals, and are thus used as biomonitors for metal pollution and in ecotoxicological studies. Because Pb is not biodegradable, it persists in the aquatic environment, becoming a source of pollution and presenting risks to human beings and other living organisms (Hariharan et al., 2016). Therefore, it is essential to study the detrimental effects of Pb and formulate strategies for safeguarding aquatic organisms.

Lead accumulates in various living organisms and causes various toxicity symptoms, both directly and indirectly. Several studies have been conducted on vertebrates and invertebrates to investigate the Pb toxicity mechanisms, including neurotoxic effects (Mason et al., 2014; Sanders et al., 2009), oxidative stress (Agrawal et al., 2014; Ercal et al., 2001), antioxidant defenses (Eroglu et al., 2015; Regoli and Giuliani, 2014), lipid oxidation (Shenai-Tirodkar et al., 2017; Vlahogianni and Valavanidis, 2007), endoplasmic reticulum (ER) stress (Qian and Tiffany-Castiglioni, 2003). One of the most important Pb toxicity mechanisms is the induced oxidative stress (Ahamed and Siddiqui, 2007). Exposure to excess Pb induces the production of reactive oxygen species (ROS), which in turn results in cell membrane damage and lipid peroxidation. In addition, Pb is considered to impair nervous system functions and its toxic effects are mainly localized in the ER (Moreno

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and Tiffany-Castiglioni, 2015; Qian and Tiffany-Castiglioni, 2003), which is the major storage site and regulating organelle for intracellular calcium (Ca^{2+}) (Masoliva, 1989). However, there is currently limited information regarding Pb exposure in bivalves (Shenai-Tirodkar et al., 2017). Although a few studies observed the effects of Pb exposure on antioxidant enzyme systems (Dafre et al., 2004), none has mentioned the potential contribution of Pb for ER stress response and effects on lipid metabolism.

The advent of omics technologies, such as genomics and transcriptomics, have enabled the simultaneous assessment of the expression profiles of thousands of genes that respond to a toxic compound within a particular cell type, tissue, or organism (Ung et al., 2010). Transcriptome analysis has been used to detect metal toxicity mechanisms in Mollusca, and it revealed several biomarkers for detecting zinc (Zn) and cadmium (Cd) exposure in oysters (Meng et al., 2017; Zhang et al., 2012). Shi et al. (2015) analyzed the transcriptome of oysters exposed to copper (Cu) and revealed that the gamma-aminobutyric acid (GABA) transporter can control the action of GABA transmitter in the nervous system, thereby affecting Cu accumulation in the gills and mantle. Transcriptome responses to Cu stress have also been examined in the mussel *Mytilus coruscus* (Xu et al., 2016), but no transcriptome analysis has been conducted for Pb toxicity in Mollusca.

In the present study, we investigated the messenger RNA (mRNA) expression profiles in the gills and digestive gland of Pacific oyster *(Crassostrea gigas*) under Pb stress using transcriptome comparisons, quantitative real-time PCR analysis, and physiological methods. Gills and digestive gland were selected in this study because these tissues are the active sites of metal accumulation, oxy-radical generation, and enzyme biotransformation. The key genes in ER stress and fatty acid oxidation were identified, which provided a better understanding of the molecular mechanisms underlying oyster's response to Pb stress. This first illustration of Pb toxicity mechanism in oysters is essential not only for developing approaches to protect Mollusca from toxicities due to metal stress, but also to obtain biomarkers that can be used in metal pollution monitoring programs.

2. Materials and methods

2.1. Oyster collection and experimental treatments

Two-year-old Pacific oysters (shell length, 9–12 cm) were purchased from a farm in Weihai, China, in January 2011, and acclimated for one week. After this period, 180 oysters contained in three replicate tanks (60 per tank) were exposed to dissolved Pb at a concentration of 5 μg/L. At the beginning of experiment (0 h) and after 9 d of P b exposure, total RNA was extracted from the gills and digestive glands of nine oysters (three per tank) and used for transcriptome analysis. At the same time, the gills and digestive glands of five oysters per tank were collected and prepared for quantitative real-time PCR (qRT-PCR) and physiological experiments. Salinity (30 ± 1 ppt), temperature (20 ± 0.5 °C), and pH (8.0 ± 0.3) were maintained throughout the exposure period and oysters were fed 0.5% *Isochrysis galbana* every two days. No mortality of oysters was observed during the period of exposure.

2.2. Measurement of Pb concentration in tissues

Gills and digestive glands of five oysters per replicate tank were separately pooled. Homogenates of similar weight were freeze-dried and thoroughly digested in concentrated HNO_3 (5 mL) and HCl (1 mL). This mixture was boiled, and, after cooling, Pb contents were determined by inductively coupled plasma-mass spectrometry (ICP-MS) using an Agilent 7700x (Agilent Technologies, Santa Clara, CA, USA) as previously described (Liu et al., 2012b).

2.3. RNA extraction

Total RNA was extracted from each individual using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The yield and purity of RNA were determined spectrophotometrically (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 and 280 nm. The RNA integrity was assessed via electrophoresis in a 1.2% agarose gel. The RNA collected from the nine individuals from each tank at each time point was then pooled for RNA sequencing (RNA-seq) analysis in an Illumina HiSeq2000 (Illumina, San Diego, CA, USA) platform. For the qRT-PCR analysis, the RNA extracted from the five individuals within each replicate tank was pooled and then used for determining the gene expression. Three biological replicates per time point were analyzed.

2.4. Transcriptome data analysis

Gene expression profiling was measured by mapping reads to oyster assembled sequences (Zhang et al., 2012) in Tophat (Trapnell et al., 2009), and gene expression level was measured in reads per kilobase per million mapped reads (RPKM; (Mortazavi et al., 2008). The Audic's method was used for pairwise comparisons of cDNA libraries and differential gene expression analysis. Significant *P*-values were calculated as described in Chen et al. (2010). The false discovery rate (FDR) cutoffs method was used to control the *P*-value (Chen et al., 2010). Differentially expressed genes were set at FDR < 0.01. Fold changes were calculated as $(RPKM_{Pb\_9dexposure})/(RPKM_{control})$.

Gene ontology (GO) functional annotation was conducted in the Blast2GO program using the non-redundant protein database from the National Center for Biotechnology Information (nrNCBI) (Gotz et al., 2008). The Kyoto Encyclopaedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) database was used for metabolism pathway analysis (Chen et al., 2015). Heat map of gene expression was drawn using module "HierarchicalClustering" at the GenePattern server (http://genepattern.broadinstitute.org/).

2.5. Quantitative real-time PCR

For qRT-PCR analysis, RNA samples were reverse transcribed using a cDNA synthesis kit (DRR420, TaKaRa Biotechnology, China), and gene expression was detected in the ABI7500 fast Real-Time Detection System (Applied Biosystems, Foster City, CA, USA), using the primers specified in Table S1. The elongation factor (*EF*) gene was chosen as the internal reference. The qRT-PCR amplifications were carried out in triplicate in a total volume of 20 μL containing 10 μL SYBR Green 2 × Supermix (TaKaRa), 1 μL 1:100 diluted cDNA, 0.4 μL each primer, 0.4 μL ROX Dye II (TaKaRa), and 7.8 μL diethylpyrocarbonate-treated (DEPC) H_2O. The qRT-PCR program comprised of 30 s at 95 °C followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. Gene expression was based on the cycles to threshold (Ct) values of PCR products. A melting curve analysis was performed at the end of each PCR amplification to confirm that only one PCR product was amplified and detected. Differences between the Ct values of amplified genes and *EF* (ΔCt) were calculated. A blank sample was used as the reference sample (i.e., the calibrator). The ΔCt of each sample was then subtracted from the ΔCt of the calibrator, and this difference (i.e., the ΔΔCt value) was used to calculate the expression level of the target genes as $2^{-\Delta\Delta Ct}$.

2.6. Phylogenetic analysis of genes encoding CYP450s

To examine the divergence of genes encoding CYP450s, we conducted a phylogenetic analysis from oyster and the well-studied human. From NCBI protein database, we obtained CYP450 gene product sequences from oyster and human. Protein sequences were aligned by MUSCLE. Phylogenetic trees were constructed using neighbor-joining (NJ) as implemented in TreeBeST (http://treesoft.sourceforge.net/treebest.shtml). Robustness of the NJ tree was assessed using