Short communication

Multiple biomarkers of biological effects induced by cadmium in clam *Ruditapes philippinarum*

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ABSTRACT

Cadmium (Cd) is a known heavy metal pollutant in the Bohai Sea. Manila clam *Ruditapes philippinarum* is an important fishery species along the Bohai coast. In this study, the biological effects induced by two concentrations (20 and 200 μg/L) of Cd were characterized using multiple biochemical indices in the digestive glands of clam *R. philippinarum*. The total hemocyte counts, reactive oxygen species productions and antioxidant enzyme activities exhibited that Cd induced dose-dependent immune and oxidative stresses in clam digestive glands. Metabolic responses indicated that both Cd exposures caused immune stress marked by the elevated branched chain amino acids (valine, leucine and isoleucine), together with the disturbance in energy metabolism. The differential metabolic biomarkers related to osmotic stress, including homarine, betaine, tyrosine and phenylalanine, suggested the differential responsive mechanisms in clam digestive glands induced by Cd exposures. In addition, both Cd treatments enhanced the anaerobiosis metabolism in clam digestive glands via differential metabolic pathways.

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1. Introduction

Heavy metal pollution has become a serious environmental stressor to marine animals due to the adverse biological effects [1]. Among heavy metals, cadmium (Cd) is a non-essential metal element for organisms and naturally found as an impurity with some minerals such as Zn. In the Bohai marine environment, as reported, Cd has become one of the most serious metal pollutants because of the industrial discharge from numerous metal smelteries [2]. It is known that chronic accumulation of Cd may induce the itai–itai disease and carcinogenesis [3]. In addition, Cd can also induce oxidative stress by producing excessive reactive oxygen species in organisms [4].

Since Manila clam *R. philippinarum* is widely distributed along the Bohai coast and has a high tolerance to environmental changes (e.g., temperature, salinity), it has become one of the most important economic species in marine aquaculture in China. Furthermore, *R. philippinarum* is a preferred bioindicator in the "Mussel Watch Programs" for pollution biomonitoring. Therefore, this species is also often used as an experimental animal model on pollutant-induced biological effects [5–8].

Researchers often use a bottom-up approach to study responsive effects and mechanisms of pollutants, in which a few biochemical indices, such as the anti-oxidative enzyme activities, are tested for oxidative stress [9]. With the development of system biology, the omic techniques, including genomics, transcriptomics, proteomics and metabolomics, provide top-down approaches to explore the global profiles related to the biological perturbations induced by environmental stressors in organisms [10–13]. Researchers then can comprehensively profile one type of molecules such as genes, proteins and metabolites and their alterations to characterize the biological responses with high-throughput analyses. Among these omic techniques, metabolomics focuses on the whole set of low molecular weight (<1000 Da) metabolites that are the end products in multiple biological systems including organs, tissues, biofluids, or even whole organisms [14,15]. Since metabolomics can directly characterize the perturbations in metabolic pathways, it may present an insightful view into the pollutant-induced effects in marine animals [16–20].

In this study, the biochemical responses induced by two sublethal concentrations (20 and 200 μg/L) of Cd were studied by using both bottom-up and top-down approaches. In details, these

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biochemical indices, including the total hemocyte count (THC), production of reactive oxygen species, antioxidant enzyme activities and metabolic profile, were characterized to elucidate the biological effects of Cd in clam R. philippinarum after acute exposure for 48 h.

2. Materials and methods

2.1. Experimental animals and conditions

Twenty four adult clams R. philippinarum (shell length: 3.5–3.8 cm, $n = 8$ from White pedigree) were purchased from local culturing farm in Yantai, China. After transported to the culture laboratory, the clams were allowed to acclimate in aerated seawater (25 °C, 33 psu, collected from pristine environment) in the laboratory for 1 week and fed with the Chlorella vulgaris Beij at a ration of 2% tissue dry weight daily. After acclimation, the clams were randomly divided into three tanks (one control and two Cd exposures, respectively) containing eight individual clams in 20 L aerated seawater. Two sublethal concentrations (20 and 200 μg/L) of Cd were selected for the exposures of R. philippinarum. After exposure for 48 h, the hemolymph (approx. 350 μL per sample) was sampled from the adductor muscle by a sterile syringe with the same volume of anticoagulant (50.0 mM Tris, 111.0 mM Glucose, 341.9 mM NaCl, 25.5 mM EDTA, pH 7.4). Samples were immediately filtered through a nylon mesh and kept individually in tubes held on ice to prevent hemocyte clumping until use. Then the digestive gland tissues of all the clams were dissected quickly and divided into three parts for metabolite extraction, assessment of antioxidant enzyme activities and determination of Cd accumulation. The samples of digestive gland tissues were flash-frozen in liquid nitrogen and stored at −80 °C before further procedures.

2.2. Total haemocyte count and reactive oxygen species

The counting of free hemocytes was done by using a Neubauer hemocytometer according to a previous study with some minor modifications [21]. The hemocyte suspension fixed with 4% formalin was vortexed before transferring an aliquot of the suspension to a hemocytometer to quantify the total hemocytes under a light microscope (Olympus BX61, Tokyo, Japan). Three separate aliquots of the hemocyte suspension were counted and averaged for each clam. The data were presented as number of hemocytes/mL hemolymph.

The production of reactive oxygen species (ROS) in hemolymph was assayed using 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma). Inside hemocytes, the -DA radicals is firstly hydrolyzed by esterase enzymes, and converted to 2′,7′-diclorofluorescein (DCFH), which emits fluorescence when reacting with ROS produced by the cell. Hemocyte suspensions diluted with PBS (341.9 mM NaCl, 2.7 mM KCl, 10.1 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4) were allowed to adhere for 20 min before adding DCFH-DA with the final concentration of 10 μM. After 60 min incubation at 18 °C, DCF fluorescence intensity was measured using a flow cytometer (Becton–Dickinson, San Diego, CA, USA). The mean of fluorescence intensity of hemocyte population was expressed as arbitrary unit (AU).

2.3. Metabolite extraction

Polar metabolites in clam digestive gland tissues ($n = 8$ for each treatment) were extracted by the modified extraction protocol as described previously [19]. Briefly, the digestive gland tissue (ca. 100 mg wet weight) was homogenized and extracted in 4 mL g^{-1} of methanol, 5.25 mL g^{-1} of water and 2 mL g^{-1} of chloroform. The methanol/water layer with polar metabolites was transferred to a glass vial and dried in a centrifugal concentrator. The extracts were then re-suspended in 600 μL phosphate buffer (100 mM Na$_2$HPO$_4$ and NaH$_2$PO$_4$, including 0.5 mM TSP, pH 7.0) in D$_2$O. The mixture was vortexed and then centrifuged at 3000 g for 5 min at 4 °C. The supernatant substance (550 μL) was then pipetted into a 5 mm NMR tube prior to NMR analysis.

2.4. ^{1}H NMR spectroscopy

Metabolite extracts of digestive gland tissues from clams were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 25 °C) as described previously [19]. All ^{1}H NMR spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin (version 2.1, Bruker).

2.5. Spectral pre-processing and multivariate analysis

All one dimensional ^{1}H NMR spectra were converted to a data matrix using the custom-written ProMetab software in Matlab version 7.0 (The MathsWorks, Natick, MA) [19]. Each spectrum was segmented into bins with a width of 0.005 ppm between 0.2 and 10.0 ppm. The bins of residual water peak between 4.70 and 5.20 ppm were excluded from all the NMR spectra. The total spectral area of the remaining bins was normalized to unity to facilitate the comparison between the spectra. All the NMR spectra were generalized log transformed (glog) with a transformation parameter $\lambda = 1.0 \times 10^{-8}$ to stabilize the variance across the spectral bins and to increase the weightings of the less intense peaks [19]. As described in details in our previous work, data were mean-centered before principal components analysis (PCA) using PLS Toolbox (version 4.0, Eigenvector Research, Manson, WA) [19]. Furthermore, the supervised multivariate data analysis methods, partial least squares discriminant analysis (PLS-DA) and orthogonal projection to latent structure with discriminant analysis (O-PLS-DA), were sequentially carried out to uncover and extract the statistically significant metabolite variations related to Cd exposures, as described previously [22]. Metabolites were assigned following the tabulated chemical shifts and by using the software, Chenomx (Evaluation Version, Chenomx Inc., Edmonton, Alberta, Canada) [23].

2.6. Antioxidant enzyme activities

The antioxidant enzyme activities in the digestive gland tissues ($n = 8$) of R. philippinarum were assayed using a multimode microplate reader (Infinite M200, TECAN, Switzerland) according to the manufacturer's protocols for enzyme kits (Jiancheng, Nanjing, China). In this work, the antioxidant enzymes for the activity measurement included superoxide dismutase (SOD, EC 1.15.1.1), glutathione S-transferases (GST, EC 2.5.1.18) and glutathione peroxidase (GPx, EC 1.11.1.9). Protein concentration was determined by the Coomassie brilliant blue G-250 dye-binding method with bovine serum albumin as standard [24]. The unit of each enzyme was defined as the activity of an enzyme per milligram of total protein (U/mg protein).

2.7. Determination of Cd in clam digestive gland tissues

The digestive gland tissue samples ($n = 5$) of R. philippinarum were dried at 80 °C to constant weights. The dried tissues were digested in concentrated nitric acid (70%, Fisher Scientific) using a microwave digestion system (CEM, MAR5). The samples were heated in the microwave oven (heating to 200 °C and holding at 200 °C for 15 min). All completely digested samples were diluted