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Metabolic responses in gills of Manila clam *Ruditapes philippinarum* exposed to copper using NMR-based metabolomics

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ABSTRACT

Copper is an important heavy metal contaminant with high ecological risk in the Bohai Sea. In this study, the metabolic responses in the bioindicator, Manila clam (*Ruditapes philippinarum*), to the environmentally relevant copper exposures were characterized using NMR-based metabolomics. The significant metabolic changes corresponding to copper exposures were related to osmolytes, intermediates of the Krebs cycle and amino acids, such as the increase in homarine, branched chain amino acids and decrease in succinate, alanine and dimethylamine in the copper-exposed clam gills during 96 h exposure period. Overall, Cu may lead to the disturbances in osmotic regulation and energy metabolism in clams during 96 h experimental period. These results demonstrate that NMR-based metabolomics is applicable for the discovery of metabolic biomarkers which could be used to elucidate the toxicological mechanisms of marine heavy metal contaminants.

1. Introduction

Copper (Cu) is an essential element for organisms, since it is a cofactor of the prion protein and many enzymes. It acts as a catalyst for many enzyme systems, and is important as an electron carrier in intracellular structures (Hay, 1984). However, excessive copper is highly toxic due to its high affinity for thiol groups. Cu can participate in redox reactions that generate highly reactive hydroxyl radical enhancing the production of reactive oxygen species (ROS) through Fenton reactions associated with free Cu to catalyze the reaction between superoxide anion and H_2O_2 and direct binding to free thiols of cysteines, which can cause catastrophic damage to lipids, proteins and DNA (Halliwell, 1999; Cecconi et al., 2002). Copper pollution along the Bohai Sea mainly arises from mining and electroplating, which has posed severe ecological risk on the marine ecosystem of the Bohai Sea (Ma et al., 1995). Ma et al. reported that excessive copper had become one of most severe pollutants in the Jinzhou Bay of Bohai Sea due to the high levels of copper accumulated in the invertebrates listed in the "Mussel Watch Program" (Ma et al., 1995). It is therefore necessary to assess the toxicological effects and subsequent ecological risk of copper in the marine and coastal environments.

Marine bivalves can accumulate heavy metals in direct proportion to their environmental levels (Roesijadi, 1980). Therefore, marine mussels and oysters have often been used as sentinel organisms in many countries for heavy metal pollution monitoring since "Mussel Watch Program" was proposed in the late 1970s (Goldberg, 1975). Manila clam, *Ruditapes philippinarum*, is consumed as economic seafood and distributed in the natural environment along the coasts of the Bohai Sea. It has been considered a good sentinel organism for the heavy metal pollution monitoring of marine and coast ecosystems and assigned as a bioindicator in the marine ecotoxicology due to its wide distribution, long life cycle, high tolerance to salinity and temperature, ease of collection and high bioaccumulation of heavy metals (Park et al., 2006; Park and Tsutsumi, 2008; Ji et al., 2006).

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Traditional toxicological studies on heavy metals have usually focused on the measurement of specific responses such as the activity of acetylcholinesterase to test for neuro-toxicity or anti-oxidant enzyme levels to test for oxidative stress induced by heavy metals (Matozzo et al., 2005; Elbaz et al., 2010; Geret et al., 2002). Therefore, some of these enzymatic parameters such as activity of acetylcholinesterase have been used as biomarkers for the heavy metal monitoring (Matozzo et al., 2005). With the development of system biology, metabolomics, one of the techniques of system biology, has been widely applied in drug toxicity studies, disease diagnosis, functional genomics and environmental sciences (Wu et al., 2005; Waters et al., 2001; Marchesi et al., 2007; Gavaghan et al., 2002; Griffin et al., 2004; Brindle et al., 2002; Bundy et al., 2004; Viant et al., 2006a,b; Katsiadaki et al., 2009). The application of proton nuclear magnetic resonance (NMR) spectroscopy combined with pattern recognition methods to detect the responses of low molecular weight metabolites (<1000 Da) to toxicants or environmental contaminants has been demonstrated in both terrestrial vertebrate and invertebrate systems relevant to environmental toxicology (Bundy et al., 2002; Griffin et al., 2000; Fiehn, 2002). ^{1}H NMR spectroscopy is suitable for the detection of a large range of endogenous low molecular weight metabolites in an organ or cells, since practically all metabolite molecules contain protons. Additionally, NMR is a rapid, non-destructive analytical approach that delivers rich structural and quantitative information and can allow the metabolites to be analyzed simultaneously (Jones et al., 2008; Tuffnail et al., 2009; Viant et al., 2003).

Gill tissue is a main target tissue for heavy metal accumulation in marine bivalve invertebrates (Panfoli et al., 2000; Viarengo et al., 1994). Hence, it is potentially sensitive and suitable for the detection of metabolic biomarkers induced by accumulated heavy metal. In this study, therefore, ^{1}H NMR-based metabolomics was applied to the gill tissue extracts from Manila clam *R. philippinarum*, one of the bioindicators for marine heavy metal monitoring, to detect the metabolic responses in gill tissues as to copper exposure. Previously, Jones et al., (2008) reported the toxicological effects induced by nickel at the concentration of EC_{50} in blue mussel (*Mytilus galloprovincialis*). However, the concentration (770 μg L^{-1}) of heavy metal was not environmentally relevant. In this work, the aim was to detect metabolic biomarkers characterizing the toxicological effects of copper with environmentally relevant concentrations in adult Manila clams with various exposure times.

2. Materials and methods

2.1. Sample collection

All the adult Manila clams *R. philippinarum* (shell length: 3.0–4.0 cm, Zebra pedigrees) were purchased from local culturing farm. Animals were allowed to acclimate in aerated seawater (25 °C, 32 psu, collected from clean environment) in the laboratory for 10 days and fed with the *Chlorella vulgaris Beij* daily. After acclimatization, a total 60 clams were randomly divided into 3 flat-bottomed rectangular tanks with 20 individual animals respectively.

For the challenge experiment, clams were exposed with copper at following concentrations, 10 and 40 μg L^{-1} (as $CuCl_2$, 10 and 40 μg L^{-1}). The concentrations of Cu can be found in some heavily polluted sites of Bohai Sea (Ma et al., 1995). Clams cultured in the normal fresh seawater (FSW) were used as control samples. The gill tissues of five clams from each tank were randomly sampled for metabolomics analysis after exposure for 24, 48 and 96 h respectively. After collection, the samples were flash-frozen in liquid nitrogen immediately and stored at −80 °C prior to metabolite extraction.

2.2. Metabolite extraction

Polar metabolites were extracted from the gill tissues using methanol/chloroform solvent system (Bligh and Dyer, 1959; Lin et al., 2007; Wu et al., 2008). Briefly, the gill tissue (~100 mg) was homogenized in 4 mL g^{-1} (solvent volume/tissue mass) of methanol and 0.85 mL g^{-1} of water using a high throughput homogenizer, Precellys 24 (Bertin, France). The homogenate was then transferred to a glass vial. A total of 2 mL g^{-1} of chloroform and 2 mL g^{-1} of water was added to the homogenate, and the mixture was vortexed and centrifuged (10 min, 2000 g, 4 °C). The methanol/water layer with the polar metabolites from clam gill tissue was removed and dried in a centrifugal concentrator and then stored at −80 °C. The metabolite extracts were subsequently resolvated in 600 μL of 150 mM phosphate buffer (Na_2HPO_4 and NaH_2PO_4, pH 7.0) with 0.5 mM sodium 3-trimethylsilyl-2,2,3,3-*d*4-propionate (TSP) as chemical shift standard in D_2O. The mixture was vortexed and then centrifuged at 2500 g for 5 min at 4 °C. The supernatant (550 μL) was pipetted into a 5 mm NMR tube prior to NMR measurement.

2.3. NMR spectroscopy

The gill tissue extracts were analyzed using a Bruker AV 500 NMR spectrometer operated at 500.18 MHz at 298 K. Basic one-dimensional (1-D) ^{1}H NMR spectra were obtained using a 11.9 μs pulse, 6009.6 Hz spectral width, mixing time 0.1 s, and 3.0 s relaxation delay with standard 1D NOESY pulse sequence, with 128 transients collected into 16, 384 data points. Data sets were zero-filled to 32, 768 points, and exponential line-broadenings of 0.3 Hz were applied before Fourier transformation. All ^{1}H NMR spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin (version 2.1, Bruker). NMR spectral peaks were assigned following tabulated chemical shifts (Fan, 1996; Viant et al., 2003) and using the software, Chenomx (Evaluation Version, Chenomx Inc., Canada). Some of the metabolites were confirmed by the 2D NMR method, ^{1}H–^{1}H homonuclear correlation spectroscopy (COSY).

2.4. Spectral pre-processing and multivariate data analysis

All the NMR spectra were converted to a format for pattern recognition (PR) analysis using custom-written ProMetab software based on the Matlab software package (version 7.0; The MathWorks, Natick, MA) (Purohit et al., 2004). Each ^{1}H NMR spectrum was segmented into 0.01 ppm bins between 0.2 and 10.0 ppm with bins from 4.60 to 5.20 ppm (the residual water peak) excluded. The area of each segment was calculated and normalized using the total integrated spectral area of the spectrum. All the NMR spectra were log transformed (with transformation parameter, $\lambda = 1 \times 10^{-8}$) to stabilize the variance across the spectral bins and to enhance the weightings of the less intense peaks (Purohit et al., 2004; Parsons et al., 2007). The data sets were preprocessed using mean-centering before either principal components analysis (PCA) or partial least-squares discriminant analysis (PLS-DA) was applied using PLS Toolbox software (version 4.0, Eigenvector Research, Manson, WA).

Two well-developed pattern recognition methods, PCA and PLS-DA, were used in this work to separate the sample groups. PCA is an exploratory unsupervised pattern recognition technique which is blind to the status of each sample, and serves to reduce the dimensionality of the data and summarize the similarities and differences between multiple NMR spectral sets. The algorithm of this pattern recognition method calculates the highest amount of correlated variation along PC1, with subsequent PCs containing