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Differential protein expression of kidney tissue in the scallop *Patinopecten yessoensis* under acute cadmium stress

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ABSTRACT

Morphological and proteomic changes in the kidney of scallops exposed to acute cadmium chloride ($CdCl_2$) were observed, analyzed and compared with those in the non-exposed control group. Under microscopy the paraffin-embedded sections of the kidney revealed that the microstructure of the tissue had been severely deformed after Cd exposure. Two dimensional electrophoresis, MALDI-TOF mass spectrometry and database searches showed 13 differentially expressed protein spots, of which 11 were up-regulated, while two were down-regulated. Among these proteins, guanylate kinase (GK) and C_2H_2-type zinc finger protein are considered to be tightly connected with Cd toxicity. Further studies using quantitative PCR method validated that the GK mRNA was induced under Cd stress. Other proteins identified which had some relevance to Cd toxicity are also discussed. We suggested that differential proteins such as GK could play a potential role as novel biomarkers for monitoring the level of Cd contamination in seawater.

1. Introduction

Cadmium is a pollutant associated with several modern industrial processes, and is known to have numerous undesirable effects on experimental animals and humans, targeting various organs including the kidney (Järup and Akesson, 2009; Kamunde, 2009; Satarug et al., 2010). Opinions regarding the toxic mechanism of cadmium vary among researchers worldwide, reflecting the high complexity of this mechanism. Cd has a high affinity for the protein-thiol group, and thus can displace Zn^{2+} and Ca^{2+} from metal-binding proteins, thus inhibiting their activities (Baudouin-Cornu and Labarre, 2006; Gagnon et al., 2007). It can also induce increased levels of reactive oxygen species, and enhance lipid peroxidation (Brennan and Schiestl, 1996). Moreover, it has been proved that Cd disrupts physiological signaling processes, resulting in cell apoptosis or carcinogenesis (Waisberg et al., 2003). Various studies have showed that metallothioneins (MTs), in marine invertebrates, are employed as a detoxification strategy (Bebianno and Langston, 1991; Roesijadi, 1992; Viarengo and Nott, 1993). MTs are low molecular weight proteins that can be induced by free cytosolic metal ions, especially Cd, Cu, Zn and Hg, and are involved in defending against metal toxicity (Kägi and Schäffer, 1988). This implies that an increased body burden of metals will not necessarily result in increment of toxic effects.

The search for novel biomarkers for environmental risk assessment and pollution monitoring has become a hot topic during the last few decades (Lagadic et al., 2000). Molecular biomarker development has focused on several toxic chemical targets, such as MT, for heavy metals monitoring (Xu et al., 2005). However, many of the classic biomarkers used in ecotoxicology are non-specific, for they are commonly employed in monitoring general pollutants. In other cases, specific biomarkers such as MT also have some drawbacks. For example, they are influenced by multiple compounding factors in the environment, and the resulting variability often hampers their application when they are transferred from the laboratory to the field (Bourdineaud et al., 2006; Monserrat et al., 2007). Therefore, proteomics has been applied in ecotoxicology to provide a deeper, more detailed understanding of the complex mechanisms underlying the toxic effects of chemical pollutants and to identify new candidate biomarkers (Monsinjon and Knigge, 2007). Compared with the classic singular biomarker measurement, the multi-marker method based on proteomics can indicate toxic effects over a comparatively long period, as well as in a more systematic pattern (Baker, 2005).

The widespread dispersion of Cd at trace level is found in natural waters, especially in bottom sediments (Nordberg, 1974). Cd in natural waters can reach sufficiently high concentration through bioaccumulation in the tissue of aquatic animals and cause toxicity to these animals as well as humans who use them for food. Algae can cause Cd accumulation up to approximately

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11–20 fold (Toumi et al., 2007), while fish up to 10^3–10^5 fold (Uysal et al., 2009), and bivalves even to 10^5–10^6 fold (Serafim and Bebianno, 2007). Because bivalves are mostly benthic and in close contact with the sediment, where most pollutants are usually trapped and concentrated, bivalves such as scallops are chosen as ideal materials for environmental pollution monitoring. *Patinopecten* (*P.*) *yessoensis* is a cold water shellfish and is a commercially valuable scallop species in China and Japan. For environmental toxicological research, *P. yessoensis* has several advantages including matured cultivation technique, high abundance and high stress tolerance. The rapid development of the EST database of *P. yessoensis* during recent years has also supported us in its genetic studies (Wang et al., 2008).

However, there are only a few studies which focus on the regulation pattern in the proteomic level of *P. yessoensis* or similar species so far. Fang et al. (2010) reported that ABC (ATP-binding cassette) transporter was up-regulated, and transcriptional regulator of Crp/Fnr family was down-regulated under acute exposure of Cd^{2+} in the gill of *P. yessoensis*; while Huang et al. (2011) showed that the expression level of NOX (NADH oxidase), GDPH (glycerol-3-phosphate dehydrogenase) and ATPase were significantly changed in the heart tissue of the same species in response to Cd^{2+} stress. Since the kidney is one of the major targets of Cd in aquatic animals (Bebianno and Langston, 1995; Campbell et al., 2005; Robinson and Ryan, 1986), it is desirable to focus on the proteomic alterations in the kidney under Cd^{2+} stress. In our present study, analysis of the kidney proteome was performed in the scallop *P. yessoensis* with or without acute $CdCl_2$ treatment for the identification of differentially expressed proteins, using two dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Our aim was to reveal novel biomarkers for continuously monitoring Cd pollution in seawater as well as exploring possible toxic mechanisms of Cd.

2. Materials and methods

2.1. Biological materials and treatment

P. yessoensis were trapped in inshore areas in Xiamen City, China. Five groups of scallops, 20 in each group, of approximately the same size, were kept in 60 L polyethylene aquaria filled with continuously aerated seawater (pH 8.2 ± 0.1, salinity 30.2 ± 0.2) and with a 12-h photoperiod. Seawater was renewed every 24 h, and scallops were fed with adequate Chlorella as food after seawater renewal to avoid starvation. After being acclimated for 1 week, four groups were exposed to 2.5, 5, 10, 20 mg/L $CdCl_2$ (Sigma, USA) for 24 h, respectively, and the remaining group was cultivated in seawater, as control. Food was avoided during the exposure. The death rates of scallops in each group were calculated and the exposure concentration of 10 mg/L $CdCl_2$ was set in the proteomic research. After that, 6 scallops from the 10 mg/L $CdCl_2$ group and 6 from the control group were collected and killed by stripping their shells. The kidneys of scallops in each group were isolated, and immediately fixed in 4% neutral formaldehyde or placed at -80 °C for further use.

2.2. Tissue sectioning and H.E. staining

The kidneys of scallops in the control and the exposed group were fixed in 4% neutral formaldehyde for 24 h, and embedded in paraffin. Serial sections (5 μm thickness) were deparaffinized routinely and processed for H.E. staining (rehydration, hematoxylin staining, differentiation with 1% HCl in 70% alcohol, eosin staining, dehydration and mounting on slides with neutral resin).

2.3. Protein preparation for 2-DE

Protein extraction from scallop kidneys was carried out using the TCA/acetone method as previously described (Huang et al., 2011). In brief, frozen kidney tissue samples (0.1 g) were homogenized in 5 mL 10% TCA (Sigma, USA) in acetone and stored at -20 °C for 4 h. The homogenates were then centrifuged at 12,000g for 10 min to precipitate proteins. The pellets were washed with acetone and resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 60 mM DTT, 10 mM Tris, 1 mM EDTA, 0.5% CA, and 1% protease-inhibitor cocktail), and placed on ice for 2 h. After sonication, the homogenates were ultra-centrifuged to remove any insoluble debris. The supernatants were collected for 2-DE, and protein concentration was determined using the Bradford assay. Protein samples were kept at -80 °C until use.

2.4. 2-DE

2-DE was performed essentially as reported (Feng et al., 2008). Briefly, 150 μg protein samples were loaded onto 14 cm gel strips, and IEF was performed using carrier ampholyte (pH 5-8, Amersham Biosciences, Sweden) for 10,000 voltage · hour. Before SDS-PAGE, the gel strips were equilibrated for 15 min in an equilibration buffer (6 M urea, 30% glycerol, 50 mM Tris–HCl, 2% SDS, and 1% DTT [pH8.8]). The strips were then placed on top of a 20×20 cm^2 SDS-polyacrylamide gel ($T=12\%$).

2.5. Silver staining and image analysis

Proteins on the gels were visualized using silver staining as described previously (Feng et al., 2008; Huang et al., 2007). The silver-stained gels were scanned in an Image Scanner II apparatus (GE healthcare, USA). Digitized images of the gels were analyzed using ImageMaster 2D Elite software (Version 5.0, GE healthcare, USA). Protein spots were detected and matched between different samples, and individual spot intensity volume percentages (%Volume) were obtained according to the program's instructions. To eliminate gel-to-gel variation, each spot volume was processed by background subtraction and total spot volume normalization. The differentially expressed spots between the control and $CdCl_2$-treatment groups were selected with a criterion of $p < 0.05$ obtained by Student's *t*-test.

2.6. In-gel digestion and MALDI-TOF MS/MS analysis

Protein spots were manually excised from 2D gels and washed with water. In a manner similar to the method described by Huang et al. (2009), protein spots were enzymatically digested in gel using sequencing-grade modified trypsin. Tryptic peptides were recovered by combining the aqueous phase from repeated extraction of gel pieces with 50% ACN in 25 mM NH_4HCO_3. The peptide extracts were redissolved in 5 μL 0.5% TFA, and 1 μL of the peptide mixture was mixed with an equal volume of matrix CHCA saturated with 50% acetonitrile and 0.1% TFA and spotted onto a MALDI target plate. The samples were then analyzed with a Reflex™ III MALDI-TOF mass spectrometer (Bruker, Germany). Peptide mass fingerprints (PMFs) were obtained using reflective and positive ion modes. Laser shots, 200/spectrum, were used to acquire spectra with a mass range of 800–3000 Da. PMF data were used to search for candidate proteins using MASCOT (http://www.matrixscience.com) software. The search parameters were set up as follows: the databases were SWISS-PROT and NCBInr; the minimum number of matched peptides was four; there was a fixed modification of carbamidomethylation and a variable modification of methionineoxidation; monoisotope masses were used; the number of missed cleavage sites allowed was up to 1; and the maximal mass tolerance was 100 ppm (Zhuo et al., 2007).

2.7. RT-PCR and quantitative real-time PCR analysis

The total RNA of fresh scallop kidney tissues were isolated according to the RNAiso™ plus kit (TaKaRa, Japan) instructions. Isolated RNA was reverse transcribed into cDNA using a PrimeScript™ RT-PCR kit (TaKaRa, Japan). A regular PCR was performed before the real-time PCR on an ABI 2720 thermal cycler following the program: held at 95 °C for 5 min, 95 °C for 30 s, 56 °C for 40 s, 72 °C for 25 cycles of 30 s, and then held at 72 °C for 7 min. Electrophoresis of the PCR product was run on a 1.5% agarose gel, and real-time PCR was performed using a RotorGene™ 6000 amplifier (Corbett, United States). Each 25 μL of reaction mixture was prepared according to the SYBR® *Premix Ex Taq*™ kit (TaKaRa, Japan) instructions. The reactions were run in triplicate using the following program: 1 cycle at 95 °C for 30 s, and then 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 40 cycles of 30 s. The endogenous control used to normalize the amount of sample RNA was the *P. yessoensis β-actin* gene. The primers were as follows: *Actin* gene upstream primers: 5′-AGGTCATCACCATCGGAAACG-3′, downstream primers: 5′-CGGTCAGCAATACCAGGGAAC-3′; *GK* gene upstream primers: 5′-TTGTATACTA GACGTGGAAATC-3′, downstream primers:5′-GTAGACTCTCGTCAGATTCTGT-3′. Statistical analysis was performed using an independent Student's *t*-test, and *p*-values of < 0.05 were considered statistically significant.

3. Results

3.1. Morphological changes in the kidney tissues under acute $CdCl_2$ treatment

An animal model of Cd toxicity based on *P. yessoensis* should be established before the proteomic studies. To observe the histological changes in the kidneys of scallops under $CdCl_2$