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Alterations of protein profile in zebrafish liver cells exposed to methyl parathion: A membrane proteomics approach

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ABSTRACT

Methyl parathion (MP) is an extensively used organophosphorus pesticide, which has been associated with a wide spectrum of toxic effects on environmental organisms. The aim of this study is to investigate the alterations of membrane protein profiles in zebrafish liver (ZFL) cell line exposed to MP for 24 h using proteomic approaches. Two-dimensional gel electrophoresis revealed a total of 13 protein spots, whose expression levels were significantly altered by MP. These differential proteins were subjected to matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry analysis, and nine proteins were identified to be membrane proteins, among which seven were up-regulated, while two were down-regulated. In addition, the mRNA levels corresponding to these differential membrane proteins were further analyzed by quantitative real-time PCR. And the differential expression of arginase-2 was specially validated via Western blotting. Regarding the physiological functions, these proteins are involved in molecular chaperon, cytoskeleton system, cell metabolism, signal transduction, transport and hormone receptor respectively, suggesting the complexity of MP-mediated toxicity to ZFL cell. These data could provide useful insights for better understanding the hepatotoxic mechanisms of MP and develop novel protein biomarkers for effectively monitoring MP contamination level in aquatic environment.

1. Introduction

Organophosphorus pesticides (OPs) are widely used for agriculture and domestic purposes when controlling insect pests (Videira et al., 2001). Unfortunately, it is claimed that only 1–3% of an agrochemical reaches the target site of action (Plimmer, 1990), resulting in the presence of its residues in the various environmental matrices. There is evidence that OPs are sufficiently persistent in reaching the aquatic environment at concentrations high enough to pose a potential threat to non-target animals (Hai et al., 1997). Methyl parathion (*O*,*O*-dimethyl *O*-4-nitro-phenylphosphorothioate, MP) is an extensively applied OP in agriculture, food storage shelters and pest control programs due to its low cost and high efficacy (Ma et al., 2003). However, during the last few decades, the excessive use of MP has resulted in high concentration of residues in water, which is likely harmful to fish survival (Doong and Chang, 1998).

The primary effect of MP on organisms carries out the irreversible inhibition of acetylcholinesterase (AChE) activity, which leads to accumulation of acetylcholine in the synaptic cleft, resulting in a variety of neurotoxic effects (Rubin et al., 2002). It has been reported that MP could induce oxidative stress by depletion of antioxidants or excessive generation of reactive oxygen species (ROS), or both, leading to lipid peroxidation, DNA damage and protein oxidation (Celik and Suzek, 2008). In addition, MP possesses mutagenic properties such as gene mutations, chromosomal alterations and DNA damage, and induces reproductive toxicity (Dearfield et al., 1999; Salazar-Arredondo et al., 2008).

At present, many types of fish cell lines including EPC cell line from carp (Ruiz-Leal and George, 2004), FG-9307 cell from flounder gill (Li and Zhang, 2001), hepatoma cell PLHC-1 (Knauer et al., 2007), gonad fibroblast cell RTG-2 and hepatic cell RTL-W1 from rainbow trout (Babin and Tarazona, 2005), have been established and applied to test the toxicity of pesticides in an *in vitro* system. In addition, the liver has been identified as a major target organ for most toxins. Therefore, zebrafish liver (ZFL) cell line was selected for the present study.

Proteins are the primary effector molecules of all living systems, and any adaptive responses to environmental, physiological or pathological conditions will be reflected by alterations in protein activity or content (Bradley et al., 2002; Huang and Huang, 2011). Global techniques such as proteomics, therefore, provide effective strategies for toxicological studies and are regarded as a

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powerful tool to investigate the cellular responses to environmental pollutants (Dowling and Sheehan, 2006), such as pesticides. Chen and Huang (2011) performed proteomic analysis of *Sparus latus* liver treated with MP, and identified 16 differentially expressed proteins that are involved in cell redox homeostasis, metabolic processes and cytoskeleton system. Sanchez et al. (2009) analyzed the liver proteome response of *Micropterus salmoides* exposed to atrazine, and changes are noted in the expression of proteins associated with energy production including glycolysis and ATP synthesis. However, proteomic studies of proteins in response to pesticide toxicity in cultured fish cell lines have rarely been carried out.

Since membrane proteins carry out the essential functions of biomembranes, and are considered as the targets of most drugs and toxicants (Hopkins and Groom, 2002), they may be the major structural and functional components of the signalling pathways involved in MP toxicity. Therefore, we utilized a subcellular fractionation method to extract membrane proteins in ZFL cells, and used two-dimensional gel electrophoresis (2-DE) to analyze the differential proteomic profiles following MP exposure. Furthermore, the membrane proteins altered by MP were identified by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF MS) analysis. We hope these results will provide new clues to the toxic mechanisms of MP involved, and identify novel protein biomarkers for monitoring the contamination level of MP in water.

2. Materials and methods

2.1. Cell culture

ZFL is an adherent tissue hepatocyte cell line with epithelial-like morphology isolated from zebrafish (*Danio rerio*). It was purchased from the American Type Culture Collection (ATCC, CRL-2643) and maintained in a standard culture medium comprising of 50% L-15 medium, 35% DMEM and 15% Ham's F12 and supplemented with 1.5 g L^{-1} sodium bicarbonate, 15 mM HEPES, 0.01 mg mL^{-1} insulin, 50 ng mL^{-1} epidermal growth factor (EGF), 5% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 28 °C, according to the supplier's protocol.

2.2. MTT assay

ZFL cells were seeded in 96-well plates and incubated overnight. The medium was then removed, and the cells were exposed to medium with different concentrations of MP for 24 h. After exposure, the medium was removed, and 5 mg mL^{-1} MTT was added to the wells and plates were incubated for 4 h. Supernatant was removed and 150 μL of DMSO was added to each well incubated for 10 min. Absorbance of samples was measured using a microplate reader at 570 nm.

2.3. Cell cycle analysis and Annexin V-FITC/PI assay

ZFL cells were cultured in medium containing 0 (control), 200 and 300 μM MP for 24 h. To analyze the distribution of ZFL cells in different cell cycle phases under different treatments, the cells were trypsinized, washed with PBS and fixed in 70% ethanol at 4 °C for at least 18 h. They were then incubated in a DNA-staining buffer that contained 50 μg mL^{-1} ribonuclease A (Sigma–Aldrich) at 37 °C for 30 min and incubated in 50 μg mL^{-1} propidium iodide (PI, Sigma–Aldrich) at 4 °C for another 30 min. The samples were then analyzed using an EPICS XL Flow Cytometer (Beckman Coulter, USA). For each sample, 10000 PI stained cells were captured, and those in different phases of the cell cycle were expressed as a percentage of the total number of cells counted.

The apoptotic and necrotic rates were determined with an Annexin V-FITC staining kit (BioSource International Inc., USA). The cells were incubated in Annexin V-FITC and a PI labeling solution for 10 min at room temperature in the dark, and were then analyzed using an EPICS XL Flow Cytometer (Beckman Coulter, USA).

2.4. Membrane protein enrichment

ZFL cells were cultured in medium containing 0 and 200 μM (IC_{50}) MP for 24 h. After that, cells were washed three times with cold PBS, scraped, and centrifuged at 1500*g* for 3 min. The resulting pellets were homogenized in lysis buffer I (5 mM phosphate buffer, pH 8.0, 1 mM EDTA, and 1 mM PMSF) and incubated for 1 h at 4 °C. The lysate was then centrifuged at 600*g* for 10 min to remove the nuclei and unlysed debris. After the centrifugation of supernatants at 17000*g* for 30 min, the supernatants were collected as cytosolic fractions (CF), and the pellets as membrane fractions (MF). The membrane pellets were washed with buffer I and centrifuged at 17000*g* for 30 min, then repeated. After the washing process, the pellets were resuspended in lysis buffer II (8 M urea, 2 M thiourea, 4% CHAPS, 1% NP-40, 65 mM DTT, 40 mM Tris, 0.5% ampholyte 3–10, and 1 mM PMSF). After a 4 h of incubation at 4 °C, centrifugation was performed at 12000*g* for 30 min. The supernatants were collected for 2-DE or Western blotting, and the protein content was measured using the Bradford assay.

2.5. 2-DE and image analysis

A 100 μg of membrane proteins was mixed with a rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS (w/v), 1% DTT (w/v) and 0.5% IPG-buffer, then loaded onto 18 cm, pH 5–8 IPG strips. Isoelectric focusing (IEF) was conducted using an IPGPhor II system (GE Healthcare), and the focusing protocol was as follows: active rehydration for 13 h, 1 h at 100 V, 1 h at 500 V, 1 h at 1000 V, 1 h at 4000 V, 2.5 h at 4000–8000 V, and 5 h at 8000 V. Focused strips were equilibrated with an equilibration buffer [6 M urea, 50 mM Tris, 2% (w/v) SDS, 30% (v/v) glycerol, and a trace of bromophenol blue, pH 8.8] containing 1% (w/v) DTT for 15 min, followed by an incubation in the same buffer containing 2.5% (w/v) iodoacetamide for another 15 min. The equilibrated strips were applied directly to 10% SDS–polyacrylamide gels and separated at 25 mA gel^{-1} until the bromophenol blue reached the bottom of the gel. For each protein sample (control or MP-treatment), three experimental replicates were subjected to 2-DE.

Proteins on the 2DE gels were visualized using silver-staining. The stained gels were scanned with an Image Scanner II apparatus (GE Healthcare). Digitized images of the gels were analyzed using an ImageMaster 2D Platinum software (Version 5.0, GE Healthcare). Protein spots were detected and matched between different samples, and volume values of corresponding spots were obtained according to the program instructions. To eliminate gel-to-gel variation, the individual spot volume of each gel was normalized relative to the total valid spot volume, and the proteins differentially expressed with statistical differences ($P < 0.05$) were selected for identification.

2.6. In-gel digestion and protein identification

Protein spots of 2-DE were manually excised from the gels and washed with water. After being destained, the proteins were *in-gel* reduced, alkylated and digested with trypsin as reported (Huang et al., 2011). The peptide extracts were redissolved in 5 μL of 0.5% trifluoroacetic acid (TFA) and 1 μL of the peptide mixtures