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Proteome profiles in medaka (*Oryzias melastigma*) liver and brain experimentally exposed to acute inorganic mercury

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ABSTRACT

Mercury is a widespread and persistent pollutant occurring in a variety of forms in freshwater and marine ecosystems. Using the proteomic approach, this study examined the protein profiles of the medaka (*Oryzias melastigma*) liver and brain exposed to an acute mercuric chloride ($HgCl_2$) concentration (1000 μg/L) for 8 h. The results showed that acute exposure of medaka to inorganic mercury enhanced metal accumulation in both the liver and brain, and a higher content of mercury was detected in the latter. Comparison of the two-dimensional electrophoresis protein profiles of $HgCl_2$-exposed and non-exposed group revealed that altered protein expression was quantitatively detected in 20 spots in the brain and 27 in the liver. The altered protein spots were subjected to matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry analysis, with the resultant identification of 46 proteins. The proteins identified were involved in oxidative stress, cytoskeletonal assembly, signal transduction, protein modification, metabolism and other related functions (e.g. immune response, ionoregulation and transporting), highlighting the fact that inorganic mercury toxicity in fish seems to be complex and diverse. This study provided basic information to aid our understanding of the possible molecular mechanisms of acute inorganic mercury toxicity in aquatic organisms, as well as potential protein biomarker candidates for aquatic environmental monitoring.

1. Introduction

Mercury is a widespread and persistent pollutant occurring in a variety of forms in freshwater and marine ecosystems (Satoh, 2000). Since mercury is ubiquitous in the environment, it is almost impossible for most humans and animals to avoid exposure to some form of mercury, be it elemental, organic or inorganic (Ung et al., 2010). The principal sources of exposure to inorganic mercury (Hg^{2+}) are water, food and air, while exposure to other forms of mercury is from dental amalgam in tooth fillings (elemental Hg^0) and from the consumption of fish and other seafood (organic mercury Hg^+) (Lorschieder et al., 1995). Mercury pollution, which is mainly attributable to anthropogenic activities including industrialization, has resulted in several catastrophic mercury poisoning events in Japan (Kudo et al., 1998), the Amazon Basin (Pfeiffer and Lacerda, 1988) and Iraq (Bakir et al., 1973). Recently, many high-risk sites with mercury pollution have been reported in Asia, since this area has become the largest contributor of anthropogenic atmospheric mercury, and is responsible for over half of the global emission (Li et al., 2009). For example, during the evaluation of mercury pollution of the water and sediment in Agusan River basin, eastern Mindanao, which has several centers of artisanal gold mining, Appleton et al. (1999) find that drainage downstream of Diwalwal is characterized by extremely high levels of total mercury (T-Hg) in solution (maximum 2906 μg/L) and in the bottom sediment (20 mg/kg).

Mercury compounds are very toxic and all forms of mercury are possible human carcinogens (ATSDR, 1999). Due to its great affinity for the SH groups of biomolecules, such as glutathione (GSH) and sulfhydryl proteins (Hansen et al., 2006), mercury is considered to display multiple toxicity (e.g. hepatotoxicity and neurotoxicity) (Castoldi et al., 2001; Ung et al., 2010; Zalups, 2000). One of the main and most common mechanisms behind mercury toxicity is ascribed to oxidative stress. Mercury induces oxidative stress and the production of reactive oxygen species (ROS) by binding to intracellular thiols (GSH and sulfhydryl proteins) and acting as a catalyst in Fenton-type reactions (Stacchiotti et al., 2009), with concomitantly begetting oxidative damage. However, other mechanisms (e.g. the mimicking of calcium signaling, cytoskeletonal disruption and the degeneration of the DNA repair system) are also related to the initiation of mercury toxicity (Castoldi et al., 2001; Crespo-López et al., 2009). Despite numerous studies, the biochemical mechanisms whereby mercury exerts its negative effects in organisms are not yet fully understood.

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Toxicoproteomics is a relatively new discipline that applies global proteomic technologies to toxicological studies, and is intended to detect critical proteins and pathways disrupted by exposure to harmful chemicals and environmental stressors (Merrick, 2006). Recently, proteomic-based approaches have been applied to investigate the mechanisms of toxicity involved in the effects of methylmercury in fish (Berg et al., 2010; Keyvanshokooh et al., 2009). Mercuric chloride ($HgCl_2$) is one of the most toxic forms of mercury because it easily forms organomercury complexes with proteins (Lorschieder et al., 1995). However, few efforts have been devoted to the study of inorganic mercury in aquatic organisms, although many studies have been conducted on organic mercury (e.g. methylmercury) toxicity as well as its neurotoxic outcomes and mechanisms. So in this study, the medaka (*Oryzias melastigma*), a model marine fish for aquatic toxicological studies, were acutely exposed to a high concentration of $HgCl_2$ (1000 μg/L) for 8 h, and mercury accumulation in the liver and brain was examined after the exposure. The protein expression profiles of the liver and brain of exposed and non-exposed medaka were also analyzed using the proteomic approach, and the altered expression proteins were identified using matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS) analysis. The purpose of this study was to investigate inorganic mercury toxicity to the medaka (e.g. hepatotoxicity and neurotoxicity) at the proteomic level so as to understand the damage and/or detoxification mechanisms involved, and to identify new protein biomarkers.

2. Materials and methods

2.1. Medaka exposure experiment

Medaka (*O. melastigma*) were acclimatized in aerated seawater tanks for 15 days prior to the experiment at a water temperature of 25 °C under a 12 h light/dark cycle, and fed twice a day, 9:00 am and 3:00 pm, with commercial artemia dry bait. Then, fish (weighing 0.5 ± 0.05 g) were randomly assigned to two experimental groups: seawater control and mercury treatment ($HgCl_2$ was added to the seawater to achieve a final Hg^{2+} concentration of 1000 μg/L). Each treatment included two groups with 25 fishes for each group. The experiments were carried out in four glass tanks (40 cm × 20 cm × 20 cm), each with 14-L filtered water and lasted for 8 h under the same conditions as described above during acclimation. No mortality was found in the control group, but 20% was observed to be dead in the treatment group. At the high dose used, the experiment focused on the early response in the medaka to acute contamination by inorganic mercury. At the end of the exposure, the tissues (i.e. liver and brain) of 16 medaka (8 fish per tank) were dissected and pooled. Then the pooled tissues were randomly divided into three parts, and solely followed by the subsequent proteomic analysis, with presenting three independent replicates. For mercury accumulation analysis, 12 fish per tank was collected for mercury concentration analysis, with concomitantly producing two biological replicates for each treatment. It should be noted that the medaka used in this study were mixed-sex adult fish of the same age. All seawater used was filtered through 0.45 μm acetate fiber membranes, with the background concentration of T-Hg being 0.0051 μg/L. The seawater characteristics were described as follows: dissolved oxygen, 6.2–6.7 mg/L; salinity, 29–30 PSU; and pH, 8.0–8.1.

2.2. Mercury concentration analysis

T-Hg concentrations in medaka tissues (i.e. liver and brain) were measured using the EPA 7474 method, with a few modifications. After freeze-drying for 2 days, the tissues were digested in 70% nitric acid in a heating block at 80 °C overnight. In the hydrochloride/bromate/bromide mixture (Sigma–Aldrich), mercury was oxidized by stannous chloride (Wako) and analyzed using cold vapor atomic fluorescence spectrometry (CVAFS, Brooks Rand Model III). Standard reference materials (Mussel Homogenate IAEA 142 and Tuna Fish Flesh homogenate IAEA 436) were concurrently digested and measured for T-Hg, and the recoveries were >90% in the standards. T-Hg content in medaka tissues was measured as ng/g dry weight (DW), and the data were expressed as mean values ± semi-range.

2.3. Proteomic analysis

2.3.1. Protein extraction

Frozen fish livers or brains were homogenized in 1.0 mL of 20% TCA/acetone (w/v) lysis buffer with 20 mM dithiothreitol (DTT) using an ultrasonic disrupter. The supernatant was removed by centrifugation at 18000 g for 30 min at 4 °C, and the pellet was washed twice with 80% acetone (v/v) and twice with ice-cold acetone containing 20 mM DTT. The pellet was recovered by centrifugation at 18,000 × *g* for 30 min at 4 °C each time. Residual acetone was removed in a SpeedVac for about 5 min. The pellet was dissolved in 150 μL rehydration buffer containing 8 M urea, 2% CHAPS, 2.8 mg/mL DTT, 0.5% immobilized pH gradient (IPG) buffer and a trace of bromophenol blue. The solution was centrifuged at 20,000 × g for 30 min at 15 °C and the supernatant was collected for two-dimensional electrophoresis (2-DE) analysis. The protein content was quantified using the 2D Quant kit (GE Healthcare).

2.3.2. 2-DE analysis

400 μg of each protein sample was mixed with a rehydration buffer and then loaded onto IPG strips of linear pH gradient 4–7 (GE Healthcare). Rehydration and subsequent isoelectric focusing were conducted using the Ettan IPGphor III Isoelectric Focusing System (Amersham Biosciences, USA). Rehydration was performed overnight in a strip holder with 340 μL of rehydration buffer. After rehydration, isoelectric focusing was performed in the following manner: 2 h at 100 V, 2 h at 200 V, 1 h at 500 V, 2 h at 1000 V, 2 h at 4000 V and 6 h at 8000 V. After the first dimension was run, each strip was equilibrated with about 10 mL equilibration buffer containing 50 mM Tris (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 1% DTT and a trace amount of bromophenol blue, for 17 min. The strip was then placed in fresh equilibration buffer containing 2.5% iodoacetamide (instead of DTT) for another 17 min. Subsequently an 11.5% SDS–PAGE second dimension was performed. Electrophoresis was carried out at 10 mA/gel for 15 min, followed by a 6 h run at 200 V until the bromophenol blue front reached the edge of the gels. The proteins were visualized by colloidal Coomassie staining and three independent 2-DE gels were performed for each treatment.

2.3.3. Coomassie staining

Colloidal Coomassie staining was carried out following the method of Candiano et al. (2004). Briefly, the gel was initially fixed for 1 h in a fixation solution containing 40% (v/v) methanol and 10% (v/v) acetic acid, followed by four Milli-Q water washes (15 min each time). The gel was then stained overnight in the working colloidal "blue silver" solution with 0.12% Coomassie Brilliant Blue G-250, 10% ammonium sulfate, 10% phosphoric acid, and 20% methanol. Finally, the gels were destained for 2 h via six Milli-Q water washes (20 min each time).

2.3.4. Image capture and analysis

Images were made using a Gel-documentation system on a GS-670 Imaging Densitometer from Bio-Rad (USA) and 2-DE electrophoretogram matching software. Images were saved in TIFF

format before analysis. Computerized 2-DE gel analysis (spot detection, spot editing, pattern matching, database construction) was undertaken using the ImageMaster 2D Elite (GE Life Science, USA) and Melanie IV. After spot detection and matching, spot intensities were normalized with total valid spot volume in order to minimize nonexpression related variations in spot intensity and hence accurately provide semiquantitative information across different gels. Differences of ≥1.75 in expression (ratio %V) between matched spots were considered significant whenever a spot group passed statistical analysis (an independent-samples *t*-test, $P < 0.05$) and a second manual verification of the spots in the gel images.

2.3.5. Mass spectrometric analysis

The altered protein spots were manually excised from 2-DE gels. The gel pieces were washed sequentially with Milli-Q water, and 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate until they were completely destained. After dehydration with 100% ACN, the dry gel pieces were digested by adding 10.0 ng/μL trypsin (Promega, Madison, WI) in 10 mM ammonium bicarbonate overnight at 37 °C. For MALDI-TOF/TOF MS analysis, 1 μL of the digest mixture was mixed on-target with 0.5 μL of 100 mM α-cyano-4-hydroxy-cinnamic acid in 50% ACN and 0.1% trifluoroacetic acid on the target plate before being dried and analyzed with a MALDI-TOF/TOF mass spectrometer (5800 Proteomics Analyzer, Applied Biosystems, Foster city, CA). MALDI-TOF MS and TOF-TOF tandem MS were performed and data were acquired in the positive MS reflector mode with a scan range from 900 to 4000 Da, and five monoisotopic precursors (S/N > 200) were selected for MS/MS analysis. For interpretation of the mass spectra, a combination of peptide mass fingerprints and peptide fragmentation patterns were used for protein identification in an NCBI nonredundant database using the Mascot search engine (http://www.matrixscience.com). All mass values were considered monoisotopic, and the mass tolerance was set at 75 ppm. One missed cleavage site was allowed for trypsin digestion; cysteine carbamidomethylation was assumed as a fixed modification, and methionine was assumed to be partially oxidized. Results with C.I. % (Confidence Interval %) values greater than 95% were considered to be a positive identification. The identified proteins were then matched to specific processes or functions by searching Gene Ontology (http://www.geneontology.org/).

3. Results

3.1. Mercury accumulation in medaka liver and brain

After exposure to $HgCl_2$ for 8 h, the medaka liver and brain were subjected to T-Hg analysis. T-Hg concentrations in liver and brain from unexposed animals were 87.3 ± 11.3 and 182.7 ± 35.4 ng/g DW, respectively. T-Hg concentrations in liver and brain from $HgCl_2$-treated animals were 3812.2 ± 708.1 and 17091.4 ± 2307.3 ng/g DW after 8 h exposure.

3.2. Protein expression profile in medaka liver exposed to $HgCl_2$

The 2-DE gels of mercury-exposed and non-exposed medaka livers are shown in Fig. 1, and quantitative spot comparisons were made with image analysis software. On average, more than 800 protein spots were detected in each gel using the colloidal Coomassie staining and the ImageMaster 2D Elite software. Compared with the 2-DE gels of the non-exposed livers, a total of 27 protein spots from the mercury-exposed livers were found to be significantly altered in abundance (≥1.75-fold, $P < 0.05$). Among these altered proteins, fourteen protein spots were significantly downregulated in the treatment, while two protein spots disappeared. Thirteen protein spots were noticeably upregulated in the treatment, including two newly-induced protein spots. The results from MALDI-TOF/TOF MS analysis showed that 26 protein spots were successfully identified with C.I. % values greater than 95% (Table 1), and all the matched proteins came from the NCBI database for fish species. Of them, eight protein spots (spots 5, 6, 7, 10, 15, 16, 17 and 24) were involved in cell structure. Four protein spots (spots 21, 22, 23 and 27) were concerned with the oxidative stress response. Three protein spots (spots 19, 25 and 26) were related to signal transduction; two (spots 8 and 20) played a role in protein modification (i.e. proteolysis); eight (spots 1, 2, 3, 11, 12, 13, 14 and 18) participated in metabolism, and the remaining protein spot (spot 4) was correlated with immune defense.

3.3. Protein expression profile in medaka brain exposed to $HgCl_2$

The 2-DE images from the brain are shown in Fig. 2, and on average, more than 600 protein spots were detected in each gel. Compared with the control group, a total of 20 protein spots from the mercury-exposed medaka brains were observed to show remarkable changes in expression (≥1.75-fold, $P < 0.05$). Five protein spots (including two newly induced protein spots) were significantly upregulated in the treated group. The other fifteen protein spots were significantly depressed, and seven of them were not detected in the mercury-exposed brain. All the altered protein spots were successfully identified with C.I. % values greater than 95% (Table 2), and all the matched proteins came from the NCBI database for fish species. Among them, four protein spots (spots 30, 33, 39 and 43) were involved in cell structure; two (spots 38 and 42) were related with the oxidative stress response; two (spots 35 and 47) played a role in signal transduction; and two (spots 36 and 41) were concerned with protein modification (e.g. the protein metabolic process). In addition, seven protein spots (spots 29, 31, 32, 40, 44, 45 and 46) participated in metabolism, and the other three (spots 28, 34 and 37) were correlated with other functions, such as acid–base balance and transporting.

4. Discussion

Many studies have been devoted to the toxicity of mercury in various aquatic organisms, however most of them are focused on organic mercury (e.g. methylmercury) toxicity, and little is known about inorganic mercury toxicity. Our study aimed to be the first to investigate the biochemical mechanism involved in the acute toxicity of inorganic mercury of the fish liver and brain at the proteomic level. Our results showed that the presence of inorganic mercury facilitated metal accumulation in both the liver and brain, which is in line with a previous study which shows that mercury evidently accumulates in several tissues (e.g. liver) of the fish *Brycon amazonicus* under mercury chloride treatment (Monteiro et al., 2010). Additionally, higher T-Hg content was found in the brain than in the liver. Gonzalez et al. (2005) report that in the zebrafish (*Danio rerio*), higher mercury contents accumulate in the brain than the liver after 63 days of methylmercury exposure. It should be noted that the T-Hg contents in the mercury-exposed tissues in our study were within the span of mercury concentrations in several fish species in the environment (Cardellichio et al., 2002; Frodello et al., 2000), hence enabling our study to show an environmentally related significance.

Our proteomic analysis demonstrated significant proteomic responses in both the liver and brain of mercury-exposed medaka (Figs. 1 and 2), and this might have been attributable to high mercury accumulation in the tissues under mercury treatment. The altered proteins identified in our study were involved in oxidative stress, cytoskeletonal assembly, signal transduction, protein modification, metabolism and other related functions (e.g. immune

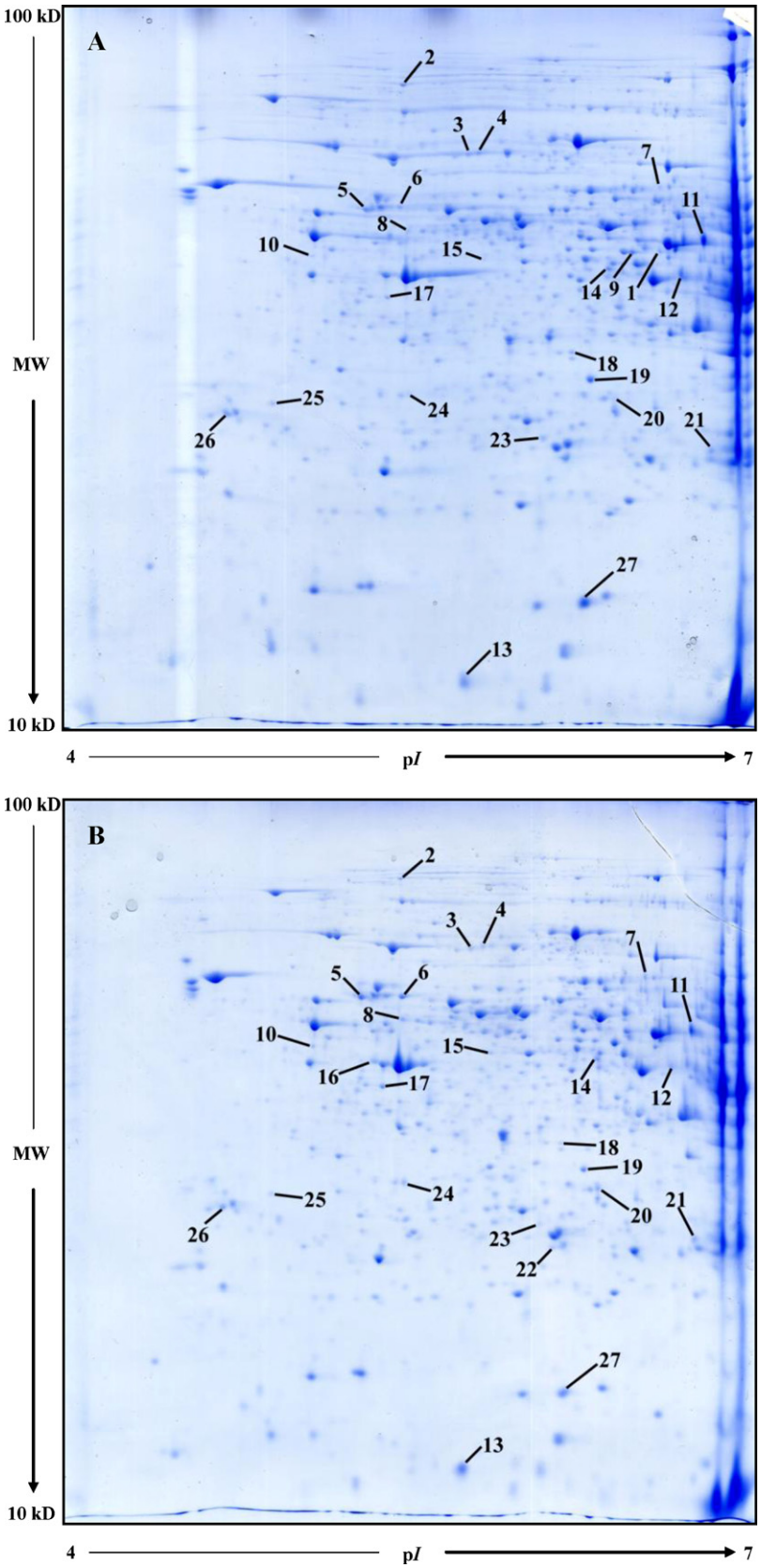

Fig. 1. Representative 2-DE gels of liver proteins in the medaka *Oryzias melastigma* after mercury exposure. (A) Control and (B) 1000 μg/L. The soluble proteins from medaka liver were separated by 2-DE and visualized using the colloidal Coomassie G-250 staining. The protein spots altered by mercury exposure are labeled with numbers. The molecular weights (MWs) and p*I* scales are indicated. Each gel is representative of three independent replicates.

Table 1
A detailed list of protein spots identified using MALDI-TOF/TOF MS from the liver of medaka *Oryzias melastigma* following $HgCl_2$ exposure.

Spot id.	Protein identity	Accession number	Protein score	Protein score C.I. %	Peptide count	MW/p*I*	Organism	Fold change
Cell structure								
5	α-Tubulin 1	gi|220678413	1330	100	20	50.62/4.97	*Danio rerio*	2.03
6	Keratin 8	gi|41056085	427	100	20	57.78/5.15	*Danio rerio*	1.88
10	Keratin 8	gi|41056085	312	100	17	57.78/5.15	*Danio rerio*	1.96
7	α-Actin	gi|8895765	289	100	5	42.33/5.30	*Ictalurus punctatus*	1.94
15	Keratin 18	gi|82191536	168	100	8	49.21/5.60	*Carassius auratus*	2.06
16	β-Actin	gi|157278351	1060	100	19	42.05/5.29	*Oryzias latipes*	I[b]
17	Type I keratin-like protein	gi|224796285	195	100	9	35.52/4.94	*Sparus aurata*	1.82
24	Lamin type B	gi|32452113	66	96.02	16	68.29/5.98	*Acipenser baerii*	8.41
Oxidative stress response								
21	Peroxiredoxin 4	gi|148226847	222	100	8	29.43/6.30	*Danio rerio*	2.50
22	Peroxiredoxin 6	gi|209733404	345	100	7	24.66/5.46	*Salmo salar*	I[b]
23	Glutathione S-transferase	gi|183604400	70	98.22	4	26.03/8.24	*Channa maculata*	−3.41
27	Superoxide dismutase [Cu–Zn]	gi|40218091	354	100	6	16.08/5.94	*Oreochromis mossambicus*	−1.93
Signal transduction								
19	Annexin 4	gi|32401412	231	100	9	35.95/6.07	*Danio rerio*	−2.00
25	14-3-3E1 protein	gi|185134340	101	99.99	6	29.41/4.67	*Oncorhynchus mykiss*	−1.84
26	14-3-3 protein	gi|50844461	543	100	12	27.68/4.67	*Oreochromis mossambicus*	−1.80
Protein modification								
8	Cytosolic nonspecific dipeptidase	gi|76362269	251	100	4	53.23/5.54	*Oreochromis niloticus*	2.37
20	Proteasome alpha 1 subunit	gi|51010945	133	100	5	29.51/6.20	*Danio rerio*	4.87
Metabolism								
1	Homogentisate 1,2-dioxygenase	gi|148298760	75	99.48	5	50.65/6.20	*Danio rerio*	D[a]
2	Alanyl-tRNA synthetase, cytoplasmic	gi|213512300	183	100	14	107.92/5.35	*Salmo salar*	−1.83
3	Dihydrolipoamide S-acetyltransferase	gi|47086703	121	100	8	69.68/8.8	*Danio rerio*	−2.70
11	Adenosylhomocysteinase	gi|213513453	752	100	16	48.51/6.43	*Salmo salar*	−2.05
12	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	gi|223648696	142	100	9	44.59/6.52	*Salmo salar*	−4.06
13	Brain-type fatty acid binding protein	gi|171544945	128	100	5	15.04/5.80	*Oryzias latipes*	1.89
14	Methionine adenosyltransferase-like	gi|94536641	101	99.99	3	43.55/6.38	*Danio rerio*	−2.87
18	S-formylglutathione hydrolase	gi|225706590	113	100	4	31.54/6.06	*Osmerus mordax*	−2.07
Other related functions								
4	Complement component C3-1	gi|157311655	239	100	12	186.08/5.90	*Oryzias latipes*	−2.74
9	Unidentified							D[a]

MW: molecular weight; p*I*: isoelectric point. *Note*: Variations were calculated as treated/control spot volume ratio and if the result was below 1, it is reported as – control/treated ratio.

[a] Indicates a protein which disappeared in the treated group.

[b] Represents a protein newly induced in the treatment.

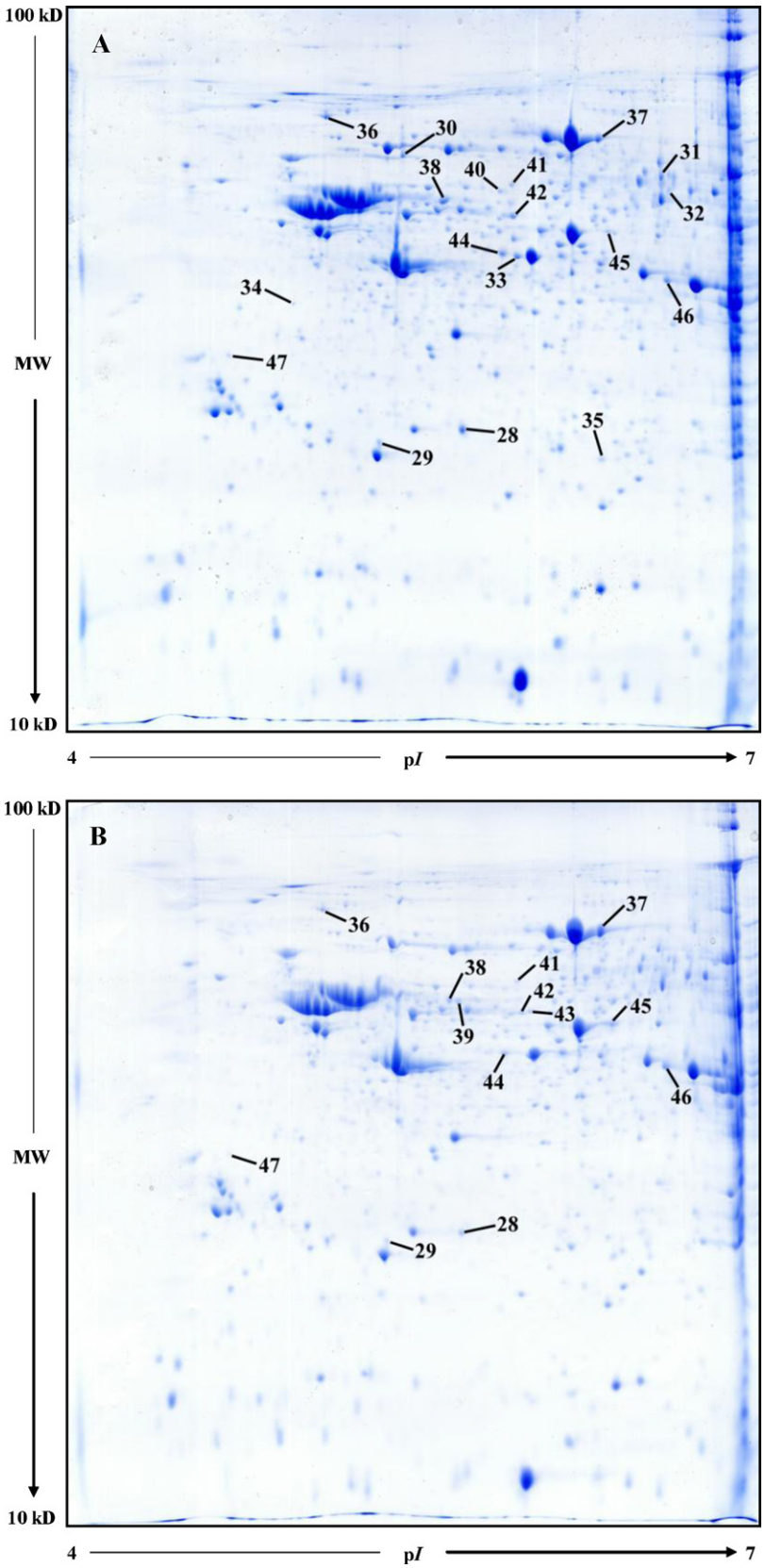

Fig. 2. Representative 2-DE gels of brain proteins in the medaka *Oryzias melastigma* after mercury exposure. (A) Control and (B) 1000 μg/L. The soluble proteins from medaka brain were separated by 2-DE and visualized using the colloidal Coomassie G-250 staining. The protein spots altered by mercury exposure are labeled with numbers. The molecular weights (MWs) and p*I* scales are indicated. Each gel is representative of three independent replicates.

Table 2
A detailed list of protein spots identified using MALDI-TOF/TOF MS from the brain of medaka *Oryzias melastigma* following $HgCl_2$ exposure.

Spot id.	Protein identity	Accession number	Protein score	Protein score C.I. %	Peptide count	MW/p*I*	Organism	Fold change
Cell structure								
30	β-Actin	gi|157278351	1140	100	19	42.05/5.29	*Oryzias latipes*	D[a]
33	Keratin 18	gi|82191536	281	100	9	49.21/5.60	*Carassius auratus*	D[a]
39	Krt5 protein	gi|39645432	180	100	11	57.79/5.27	*Danio rerio*	I[b]
43	Type II basic cytokeratin	gi|18858947	383	100	19	54.30/5.42	*Danio rerio*	I[b]
Oxidative stress response								
38	Aldehyde dehydrogenase 1 family, member A2	gi|190337771	160	100	7	57.11/5.89	*Danio rerio*	−2.61
42	Aldehyde dehydrogenase, mitochondrial	gi|209154764	282	100	9	57.27/5.93	*Salmo salar*	−1.87
Signal transduction								
35	Transforming protein RhoA	gi|259089462	397	100	8	22.27/5.83	*Oncorhynchus mykiss*	D[a]
47	Annexin A13	gi|209153068	153	100	6	35.84/4.43	*Salmo salar*	−2.15
Protein modification								
36	HSP-90	gi|37623887	770	100	22	83.56/4.95	*Dicentrarchus labrax*	−1.77
41	Chaperonin containing TCP1, subunit 8 (theta)	gi|37362194	348	100	14	60.00/5.22	*Danio rerio*	−1.75
Metabolism								
29	Apolipoprotein A1	gi|257132944	83	99.93	2	18.00/9.19	*Chelon labrosus*	−2.49
31	Pyruvate kinase	gi|224587654	950	100	19	57.95/5.97	*Salmo salar*	D[a]
32	Dihydropyrim-idinase-related protein 5	gi|223648356	247	100	5	61.65/6.17	*Salmo salar*	D[a]
40	Dihydropyrim-idinase-like 2	gi|66472782	488	100	14	62.83/6.05	*Danio rerio*	D[a]
44	Glutamine synthetase	gi|185135730	712	100	11	42.52/5.93	*Oncorhynchus mykiss*	−1.96
45	Enolase 1, (alpha)	gi|37590349	608	100	16	47.39/6.16	*Danio rerio*	2.72
46	Creatine kinase, brain b	gi|27545193	846	100	13	43.14/5.49	*Danio rerio*	1.83
Other related functions								
28	Carbonic anhydrase 1	gi|148886610	171	100	1	28.67/5.58	*Chionodraco hamatus*	−2.10
34	ATPase, H^+ transporting, V0 subunit D isoform 1	gi|41054531	850	100	17	40.62/4.85	*Danio rerio*	D[a]
37	Transferrin	gi|171544935	364	100	13	76.45/6.14	*Oryzias latipes*	2.83

MW: molecular weight; p*I*: isoelectric point. *Note*: Variations were calculated as treated/control spot volume ratio and if the result was below 1, it is reported as – control/treated ratio.

[a] Indicates a protein which disappeared in the treated group.

[b] Represents a protein newly induced in the treatment.

response, ionoregulation and transporting). However, the altered proteins in our study distinctively differed from those proteins in fish brains exposed to organic mercury (Berg et al., 2010; Keyvanshokooh et al., 2009), indicating that the toxicity of inorganic mercury is different from that of organic mercury. So it is quite important to elucidate the toxicity mechanism of inorganic mercury in organisms, since most efforts have been paid to organic mercury toxicity.

Peroxiredoxins are known to play a physiologically important role in the enzymatic removal of ROS (Radyuk et al., 2001) and to protect the cells of an organism against oxidative stress. In our experiments, a significant increase of peroxiredoxin 4 and an induction of peroxiredoxin 6 in the treated liver indicated a biological response aimed at reducing the effects of mercury, and thus avoiding oxidative stress. Nevertheless, due to its great ability to react with and deplete free sulfhydrilic groups (e.g. GSH) (Hansen et al., 2006), mercury treatment should lead to a highly reactive oxidative stress that may contribute to making it impossible for the medaka to survive in the toxic environment. Depletion of GSH is demonstrated as an effect of mercury exposure in cells (Gatti et al., 2004; Kim et al., 2005). In fact, such a possibility was confirmed in our results by the remarkable downregulation of superoxide dismutase [Cu–Zn] (SOD) and glutathione S-transferase (GST) in the treated liver, as well as a depressed expression of two aldehyde dehydrogenases (ALDHs) in the mercury-exposed brain. SOD catalyzes the dismutation of the superoxide anion to water and hydrogen peroxide, which is further detoxified by catalase. GST is the most important phase II enzyme in the metabolic detoxification process (Frova, 2006). ALDHs are known to participate in the detoxification process of exogenous or endogenous aldehydes (e.g. benzaldehyde and acetaldehyde), which are highly reactive and cytotoxic and are involved in various physiological processes such as enzyme activation, protein modification and DNA damage (Lindahl, 1992; O'Brien et al., 2005). Therefore, these enzymes play an important role in oxidative damage defense. Overall, oxidative stress plays a central role in mercury-mediated toxicity in the cells of organisms, which is in accordance with several studies (Berg et al., 2010; Berntssen et al., 2003; Monteiro et al., 2010; Ung et al., 2010).

The α-tubulin proteins are linked to heavy metal tolerance (Mattingly et al., 2001; Mireji et al., 2010), which was exemplified in our study where α-tubulin 1 was significantly enhanced in the treated liver. Keratins are important intermediate filament proteins, and their primary function is to protect the cells from stress damage that may result in cell death (Lau and Chiu, 2007; Looi et al., 2009; Omary et al., 2002). For instance, keratin 8/18 is considered to carry out essential functions in protecting hepatocytes from cadmium stress (Lau and Chiu, 2007). The noticeable upregulation in our study of several keratins in the treated liver and brain was supposed to fight against mercury-induced stress damage. Actins are highly conserved proteins that are involved in various important cellular processes including cell motility, cell signaling, and the establishment and maintenance of cell junctions and cell shape. The change of actin isoforms (α- and β-Actin) in abundance might have been related to mercury-induced oxidative stress in this study, since actins can be a direct target for oxidative modification (Fratelli et al., 2002; Lassing et al., 2007). Lamins are a family of nucleoskeletonal proteins, which play roles in nuclear activities including gene expression and DNA replication (Broers et al., 2006; Schirmer and Foisner, 2007). The increased expression of lamin type B in our study highlighted the fact that the mercury treatment interfered with the nuclear activities in the liver. Taken together, mercury attack caused cytoskeletonal reorganization and/or disruptions in the fish cells, and it might be ascribed to metal-induced oxidative stress.

Mercury exerts a significant effect on signal transduction in fish. Annexins belong to a large family of glycoproteins that bind both Ca^{2+} and negatively charged phospholipids (Gerke and Moss, 1997), and are considered as an important component of calcium signaling pathways. The downregulation of annexin in the treated liver and brain in our study might have indicated a disturbance in cellular calcium homeostasis due to mercury toxicity, which is in agreement with previous studies (Berg et al., 2010; Keyvanshokooh et al., 2009). In the treated liver, the abundance of 14-3-3E1 protein and 14-3-3 protein was significantly reduced, suggesting that mercury attack caused apoptosis in the cells, since the 14-3-3 protein can bind the phosphorylated Bad protein and so prevent apoptosis (Muslin et al., 1996). Meanwhile in the treated brain in our study, mercury totally inhibited the expression of the transforming protein RhoA, which belongs to the Rho family of small GTPases. Zhu et al. (2008) find that RhoA prevents apoptosis during zebrafish embryogenesis through activation of the Mek/ErK pathway. Overall, the mercury-induced dysregulation in signal transduction might lead to cellular damage in the medaka via disturbing cellular calcium homeostasis and inducing cell death.

Our study showed that mercury might interfere with protein modification in fish. Cytosolic nonspecific dipeptidase is involved in proteolysis. Consequently, mercury influenced the protein degradation in organisms, and this is consistent with a study where an induction of severe proteolysis is observed in the tissues of the freshwater fish *Cyprinus carpio* exposed to mercury (Suresh et al., 1991). Proteasome alpha 1 subunit is a component of the proteasome, which is sometimes responsible for the degradation of abnormal proteins. We proposed that the increased expression of cytosolic nonspecific dipeptidase and proteasome alpha 1 subunit which occurred in the treated liver in our study might have collaborated to affect the turnover of abnormal proteins (e.g. misfolded and damaged proteins) when the cells were stressed by mercury. Additionally, mercury significantly decreased the abundance of HSP-90 and chaperonin containing TCP1, subunit 8 (theta) in the brain. HSP-90 belongs to the heat shock protein family (HSPs), which are well known to protect the structure and function of cells from stress (e.g. metal attack) and play an important role in maintaining cellular homeostasis (Sanders, 1993). The deletion of HSP-90 is lethal for eukaryotic cells (Csermely et al., 1998; Voss et al., 2000), and in our study, the suppressed expression of HSP-90 suggested that the medaka brain was vulnerable to mercury attack. Chaperonin containing TCP1 (CCT) is a member of the group II chaperonins, and has an important function in maintaining cellular homeostasis by assisting the folding of many important proteins (Spiess et al., 2004; Won et al., 1998). CCT subunit levels are reported to up-regulate in several mammalian cell lines during recovery from chemical stress (Yokota et al., 2000), suggesting that they respond to protein damage and play a role in recovery of the cells from stress. Thus, the affected protein modification could be evidence of an accumulation of abnormal proteins (e.g. oxidized and misfolded proteins) in the liver and brain due to the protein damage caused by mercury toxicity.

Metabolism is also disturbed by mercury treatment. In the liver, eight affected proteins are involved in metabolism. Homogentisate 1,2-dioxygenase is involved in the catabolism of aromatic rings, more specifically in the breakdown of the amino acids tyrosine and phenylalanine (Titus et al., 2000). Its deficiency directly leads to a type of metabolic disease called alkaptonuria, which is a rare autosomal recessive metabolic disorder of tyrosine catabolism (La Du et al., 2008). Alanyl-tRNA synthetase, cytoplasmic participates in alanine and aspartate metabolism and aminoacyl-tRNA biosynthesis. Adenosylhomocysteinase converts S-adenosylhomocysteine (SAH) to homocysteine, and correlates with homocysteine metabolism. SAH is a competitive inhibitor of S-adenosylmethionine (SAM)-dependent methyl transferase reactions (Chiang et al., 1996). Mercury may affect biomethy-

lation in the cells, which is also confirmed by the decreased expression of methionine adenosyltransferase-like, which catalyzes the biosynthesis of SAM (the principal methyl donor) from methionine and ATP (Mato and Lu, 2007). Dihydrolipoamide S-acetyltransferase and pyruvate dehydrogenase E1 component subunit alpha belong to the multienzyme pyruvate dehydrogenase complex, which is responsible for pyruvate decarboxylation and thus links glycolysis to the citric acid cycle. Their decreased expression might undermine the ability of the cells to meet their energy requirements and could therefore result in cellular damage and death, which is probably attributable to oxidative stress caused by mercury-induced ROS (Tabatabaie et al., 1996). Mercury can influence fatty acid metabolism via upregulation of brain-type fatty acid binding protein. S-formylglutathione hydrolase is a highly conserved thioesterase in prokaryotes and eukaryotes, and forms part of the formaldehyde detoxification pathway, as well as functioning as xenobiotic-hydrolysing carboxyesterase (Cummins et al., 2006). Its inhibited expression suggests that mercury causes the organism to become sensitive to the toxic environment. In the brain, seven affected proteins are concerned with metabolism. Apolipoprotein A1 is a member of a family of proteins known as apolipoproteins, and this protein plays a protective role in inflammation and oxidative stress (Tabet et al., 2010; Wang et al., 2010). Pyruvate kinase (PK) is the key glycolytic enzyme. In humans, PK deficiency is the most prevalent glycolytic enzyme defect, being responsible for hereditary hemolytic anemia (Zanella et al., 2005). Dihydropyrimidinase-related protein 5 and dihydropyrimidinase-like 2 belong to dihydropyrimidinase-like proteins, which are involved in neuronal connection and communication. It is interesting to note that decreased expression of dihydropyrimidinase-like protein is observed in neurodegenerative diseases including Alzheimer's disease and schizophrenia (Johnston-Wilson et al., 2000; Lubec et al., 1999). Glutamine synthetase (GS) catalyzes the synthesis of glutamine from glutamate and ammonia. This reaction maintains the optimal level of glutamate and ammonia in neurons and modulates excitotoxicity. GS activity is shown to decrease in patients with the Alzheimer's or Huntington's diseases (Carter, 1982; Smith et al., 1991), and also it is depressed under mercury treatment (Kwon and Park, 2003), which is in line with our study. Enolase is a cytosolic enzyme involved in carbohydrate metabolism, cell differentiation, and normal growth, and a decline of enolase activity results in abnormal growth and reduced metabolism in brains (Tholey et al., 1982). Creatine kinase is an essential enzyme for brain energy buffering to maintain cellular energy homeostasis. Its activity is inhibited by mercury treatment (Araujo et al., 1996; Glaser et al., 2010). Then, the increased levels of enolase 1 (alpha) and creatine kinase, brain b may be an adaptive feedback to inhibitory activity in the brain under mercury stress. Overall, mercury attack causes a dysfunction in metabolism (e.g. energy metabolism), some of this being concerned with metabolic disorders or neurodegenerative diseases, while concomitantly rendering the cells unable to survive in the toxic environment.

Mercury affected immunity of the medaka, since the expression of complement component C3-1 was significantly decreased in the liver. Complement component C3-1 is an important component of the complement system, which is one of the first lines of immune defense (Lange et al., 2006). In the brain, mercury significantly decreased the abundance of carbonic anhydrase 1 and totally inhibited the expression of ATPase, H^+ transporting, V0 subunit D isoform 1. Carbonic anhydrase catalyzes the reversible hydration/dehydration reactions of carbon dioxide, and plays important roles in ion uptake (Chang and Hwang, 2004) and acid–base balance (Georgalis et al., 2006). ATPase, H^+ transporting is responsible for proton transport. Thus, mercury may lead to ionoregulatory change in the brain, and it may partly contribute to the mercury-induced

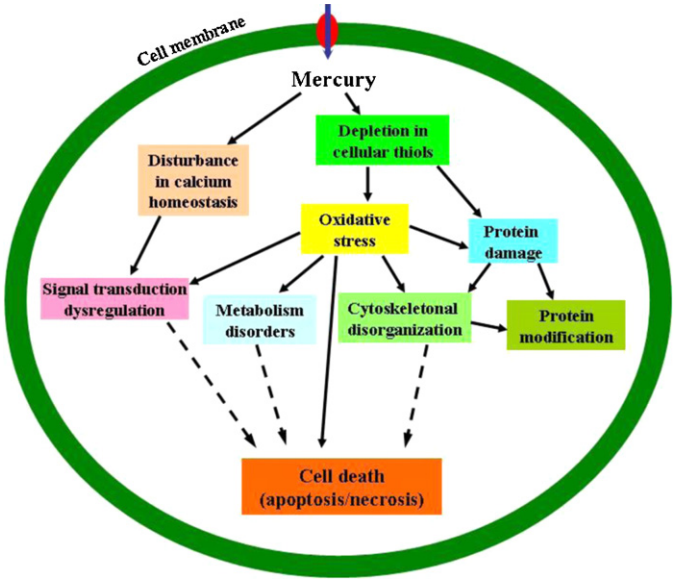

Fig. 3. Hypothetical model of the toxicity mechanism in the medaka *Oryzias melastigma* upon mercury exposure.

astrocytic swelling referred to in a previous work (Aschner et al., 1998). Also, mercury remarkably enhanced the expression of transferrin in the treated brain. Transferrin is one of the major serum proteins in eukaryotes and plays a crucial role in iron metabolism by binding and transporting iron, thus making it unavailable for catalysis of superoxide radical formation via Fenton reactions (Neves et al., 2009). Accordingly, the increased expression of transferrin might be targeted to reduce the production of superoxide radicals via Fenton reactions in the treated brain.

In conclusion, proteomics has been proved to be a valuable tool in investigating the biochemical mechanism involved in inorganic mercury toxicity in aquatic organisms. Our results showed that the presence of inorganic mercury strongly enhanced metal accumulation in both the liver and brain, and consequently induced oxidative stress, cytoskeletonal reorganization and/or disruption, and a dysfunction in metabolism and protein modification, as well as a concomitant interference with signal transduction and other related functions (e.g. immune response, ionoregulation and transporting), highlighting the fact that inorganic mercury toxicity seemed to be complex and diverse (Fig. 3). Meanwhile, oxidative stress played a central role in mercury-mediated toxicity (e.g. hepatotoxicity and neurotoxicity) in our study (Fig. 3). It should be emphasized that even though the biological processes affected by mercury overlapped in terms of the general functional categories (e.g. oxidative stress, cell structure, signal transduction, protein modification and metabolism), none of the individual proteins in the liver was the same as those in the brain. Therefore, a different mechanism should be involved in initiating mercury hepatotoxicity and neurotoxicity in the cells, and it might be illuminated to some extent by tissue-specific mercury accumulation in the present study. In future, efforts should be devoted to exploring the mechanism of mercury hepatotoxicity in organisms, based on the role of liver function in mercury transformation and cycling, as well as its central role in the control and synthesis of critical blood constituents that affect the whole body physiology (Ung et al., 2010).

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