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Proteomic Analysis of Hepatic Protein Profiles in Rare Minnow (*Gobiocypris rarus*) Exposed to Perfluorooctanoic Acid

Yanhong Wei,[†,§] Leo. L. Chan,[‡] Dazhi Wang,[‡] Hongxia Zhang,[†] Jianshe Wang,[†] and Jiayin Dai*[,†]

Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, P.R. China, State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, P.R. China, and Graduate School of the Chinese Academy of Sciences, Beijing 100080, P.R. China

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Perfluorooctanoic acid (PFOA) is a ubiquitous contaminant that has been shown to lead to hepatoxicity and is implicated in the incidence of liver tumors in mammals. A number of previous studies have described the toxic effects of PFOA based on conventional toxicological indices and transcriptional data. However, little evidence on protein levels is available. To further our understanding of mechanisms of action and identify the potential protein biomarkers for PFOA exposure, two-dimensional electrophoresis coupled with mass spectrometry has been used to identify proteins differentially expressed in the livers of rare minnow (*Gobiocypris rarus*) following PFOA exposure of 3, 10, and 30 mg/L. After comparison of the protein profiles from treated and control groups, 34 and 48 protein spots were found altered in abundance (>2-fold) from males and females, respectively. Matrix-assisted laser desorption/ionization (MALDI) tandem time-of-flight mass spectrometry (TOF/TOF) analysis allowed the unambiguous identification of 25 spots, corresponding to 22 different proteins. These proteins were involved in intracellular fatty acid transport, oxidative stress, macromolecule catabolism, the cell cycle, maintenance of intracellular Ca^{2+} homeostasis, and mitochondrial function. In addition, marked gender differences in response to PFOA have been well-described from the comparison of the male and female protein profiles. Transcriptional analysis of nine mRNAs encoding proteins altered by PFOA in the proteome analysis was determined by real-time PCR. The consistent and discrepant results between mRNA and protein levels suggested that complicated regulatory mechanisms of gene expression were implicated in the response to PFOA exposure.

Keywords: perfluorooctanoic acid • proteome • toxicology • liver • rare minnow

Introduction

Perfluorooctanoic acid (PFOA) is a man-made perfluorinated eight-carbon organic chemical. It is used in the production of fluoropolymers which are used in the manufacture of a variety of industrial and commercial products (e.g., textiles, houseware, paper coatings, electronics).[1] The stability of PFOA renders it practically nonphotolytic, nonhydrolytic, and nonbiodegradable, and so it is persistent in the environment.[2] It has been detected worldwide in a variety of environmental matrices,[3–5] wildlife,[6,7] and humans.[8–10]

Several studies have demonstrated that the liver is a primary target organ for both the short-term and chronic toxic effects of PFOA.[11] PFOA has been shown to elicit liver enlargement, induce peroxisome proliferation, and interfere with fatty acid metabolism and lipid transport in the livers of not only mammals,[12] but also aquatic organisms.[2,13] Moreover, PFOA has been implicated in the incidence of liver tumors.[14] However, these findings are mainly derived from conventional toxicological indices and/or transcriptional data reflected by altered expressed genes. Since the changes in protein levels, in many organisms, are not accompanied by altered levels of transcription,[15,16] previous research on PFOA could not describe well the toxic effects nor elucidate the modes of action more accurately and comprehensively due to the scarcity of translational data.

Proteomic analysis, providing global protein information, is of great virtue for toxicological studies.[17,18] The approach allows the association to be made between the alteration of expressed proteins elicited by chemicals with their specific toxicity. These proteins can subsequently be used as potential biomarkers for monitoring studies and help to draw conclusions regarding the molecular mechanisms of this case of toxicity.[19] Therefore, proteomic analysis offers several practical benefits in the assessment of the toxicity of a variety of chemicals,[20,21] although the application of proteomics to environmental toxicology is still in its infancy due to a number of caveats, such as the limited number of organisms fully covered in the sequence databases.[22]

* To whom correspondence should be addressed. Prof. Jiayin Dai, Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, P.R. China. Tel.: +86 10 64807185. Fax: +86 10 64807099. E-mail: daijy@ioz.ac.cn.
† Institute of Zoology, Chinese Academy of Sciences.
‡ Xiamen University.
§ Graduate School of the Chinese Academy of Sciences.

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To further our understanding with respect to the toxic effects and modes of action of PFOA, we performed two-dimensional electrophoresis (2-DE) to delineate the expressed protein patterns in the liver of rare minnow (*Gobiocypris rarus*) following PFOA treatment. Considering possible gender differences, patterns from male and female rare minnows were analyzed separately. Eighty-two spots were found to be altered in abundance (>2-fold) and subsequently analyzed with sensitive and accurate MALDI-TOF-TOF mass spectrometry, coupled with database interrogation. Twenty-two proteins were successfully identified. Rare minnow is a suitable organism in aquatic toxicological tests which has been used in a variety of toxicological assessments of chemicals.[23,24] Our previous study has constructed the liver cDNA library of rare minnow,[25] and a good number of expressed sequence tags (ESTs) from this organism are available in public databases (www.ncbi.nlm.nih.gov/projects/dbEST), which facilitates parallel studies at the levels of transcription and translation. Real-time PCR was then used to assay the mRNA expression of nine selected altered proteins. On the basis the proteomic analysis together with the transcriptional data, this work will provide the basis for understanding the underlying mechanisms of toxicity of PFOA.

Materials and Methods

Fish and PFOA Exposure. Adult male and female rare minnows (~9 months old with an average body weight of 1.4 ± 0.4 g and an average total length of 47.7 ± 3.6 mm) obtained from a laboratory hatchery were held in 20-L glass tanks (~2 g of fish/L). Fish were acclimated and treated as previously described.[26] Briefly, after a 1-week acclimation period, randomly selected male and female rare minnows (~1:1 sex ratio, gender determined by observing the shape of the abdomen and the distance between the abdominal fin and the tail fin) were assigned to 4 treatment groups: PFOA exposure at 0, 3, 10, or 30 mg/L. These concentrations were selected based on the literature.[2] PFOA (98%) was purchased from Acros Organics (Geel, Belgium). Each treatment group contained six male and six female rare minnows in duplicate tanks. At the end of the 28-day exposure period, all fish were anesthetized on ice for sampling. Gonadal tissues were quickly dissected and fixed in 10% formalin for gender determination. The livers were removed and portioned in halves. After three rinses with phosphate-buffered saline buffer (pH 7.6), one-half of the liver from each fish was used for protein extraction and another was used for real-time PCR analysis. The liver samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

Protein Extraction. Frozen hepatic tissue samples were sonicated in 500 μL of ice-cold lysis buffer on ice for 5 min with a Digital Sonifier. The lysis buffer consisted of 40 mM Tris buffer (pH 7.5) containing 7 M urea, 2 M thiourea, 1% dithiothreitol (DTT), 4% 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 1 mM phenylmethylsulfonyl fluoride. Cellular debris was removed by centrifugation at 12 000*g* for 20 min at 4 °C, and the supernatants were collected. The trichloroacetic acid−acetone precipitation method was used to remove ionic interfering components from the protein extraction. Eight volumes of 100% ice-cold acetone and 1 vol of 100% trichloroacetic acid were added into 1 vol of supernatant. The samples were precipitated at −20 °C for 1 h and then centrifuged at 18 000*g* for 15 min at 4 °C. The supernatants were discarded, and the protein pellets were washed with 1 mL of ice-cold 20 mM DTT in acetone. After another centrifugation at 18 000*g* for 15 min at 4 °C, the protein pellets were dissolved in a urea−potassium carbonate−sodium dodecylsulfate (SDS) mixture (50 μL/mg pellets), containing 9.5 M urea, 5 mM K_2CO_3, 0.4% SDS, 0.5% DTT, and 6% CHAPS. The samples were centrifuged at 12 000*g* for 10 min at room temperature, and supernatants were collected for 2-DE analysis. Protein concentration was determined using the 2-D Quant Kit (GE Healthcare). To increase the protein concentrations to values where the identification by MS becomes feasible, equal amounts of six individual samples of the same gender in each treatment were pooled.

Two-Dimensional Electrophoresis. The prepared pooled protein samples (100 μg) were mixed with rehydration solution (8 M urea, 2% CHAPS, 0.5% immobilized pH gradient (IPG) buffer, and a trace of bromophenol blue) to a volume of 350 μL. Immobiline DryStrips (18 cm, linear pH gradient from pH 4–7, GE Healthcare) were allowed to rehydrate (12 h, 30 V) in the rehydration solution containing protein samples under low viscosity oil in strip holders. Then, isoelectric focusing (IEF) was performed at 100 V for 2 h, 200 V for 2 h, 500 V for 1 h, 1000 V for 1 h, 4000 V for 2 h, and 8000 V for 6 h on a Multiphor II system (GE Healthcare). The temperature was maintained at 20 °C. After completion of the IEF program, the strips were equilibrated in two steps: 15 min in an IPG equilibration buffer (50 mM Tris-HCl solution (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and a trace of bromophenol blue) plus 1% DTT, followed by 10 min in IPG equilibration buffer plus 1% iodoacetamide. For the second-dimension electrophoresis, the IPG strips were placed on top of a 12% polyacrylamide gel and the proteins were then separated according to their molecular weights (MW) using an electrophoresis system (Ettan Dalt, GE Healthcare). 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 bottom of the gels. The gels were then visualized by either silver or Coomassie Brilliant Blue G-250 staining. Three 2-DE gels were performed for each group. Unless otherwise stated, the gels shown were representative of the gels performed.

Image Acquisition and Analysis. Silver staining was selected for image analysis in this study on account of its more sensitivity for spots detection as compared to Coomassie Brilliant Blue G-250 staining. Gels stained with silver were imaged on an ImageScanner (GE Healthcare) in a transmission mode. To subtract the variations of background, calibration among different gels was carried out before gel image capture. Image Master 2D Platinum (GE Healthcare) software was then used for matching and analysis of protein spots. The spots detection was performed based on the parameters: (i) minimal area > 5 pixels, (ii) smooth factor > 2.0, and (iii) saliency > 1.0. Manual editing was performed to remove artifacts. After defining landmark annotations (represent the same protein form) on both the gels to be aligned and the master/reference gel, the gels alignments were carried out based on the landmark annotations. Taking into account variation due to protein loading and staining, percentage volume (%Vol) was used for spot quantification. Gels from male and female fish were analyzed separately. Each sample was analyzed based on triplicate gels in order to diminish the experimental errors. An average increase or decrease higher than 2-fold compared with the control group was used to identify differentially expressed proteins.

In-Gel Tryptic Protein Digestion. Considering the compatibility to MS analysis used in this study, we selected the Coomassie Brilliant Blue G-250 staining gels for in-gel tryptic

Table 1. Primers for Quantitative Real-Time PCR Analysis

gene[a]	accession no.	forward primer (from 5′ to 3′)	reverse primer (from 5′ to 3′)	amplicon size (bp)
GAPDH	EE397198	CGTGCTGCTGTCCAGTCCAA	GCCGCCTTCTGCCTTAACCT	138
M-FABP	EE395774	CACGCTTTCTTTCTTCCTCG	ATGATGGTTGTGGGTTTGGT	189
GPx	EE394559	TGCTTATTGAAAATGTGGCG	CTGATGTCCGAACTGGTTGC	134
Prx	EE397066	GCAACCTGCTCCTCAGTTCA	ACCTCACAGCCGATTTTACG	189
TCTP	EE396557	TGGCTTCTCATTTGCTGTGC	CCTGTGAATCAATGGGAACG	84
MSRb	EE396924	GCGTCTCCTCTATCGTTTCC	AGCTTCCTCTGCCAATCTGT	148
GST	EE397703	ACAGTGAAGTCGTGGGAAAT	GGAGAAGATGGGTAAAGGGT	100
PH	EE396780	TCGCAACAAACAGAAAGACA	TCGGACCCGTAACTGAGAAT	93
GAMT	EE393949	TGACTACAATGCAGCCGACA	TCTCAAGAACCCTTCCACCT	125

[a] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; M-FABP, muscle fatty acid binding protein; GPx, Glutathione peroxidase 1; Prx, peroxiredoxin; TCTP, translationally controlled tumor protein; MSRb, methionine sulfoxide reductase B; GST, glutathione *S*-transferase; PH, phenylalanine hydroxylase; GAMT, guanidinoacetate *N*-methyltransferase.

protein digestion. Differentially expressed protein spots were manually excised from Coomassie Brilliant Blue G-250-stained gels. The gel pieces were washed twice with 200 mM ammonium bicarbonate in 50% acetonitrile/water (20 min at 30 °C), then dehydrated using acetonitrile, and spun dry. Gels were subjected to digestion in 25 mM ammonium bicarbonate buffer containing 10 ng/μL modified trypsin (Promega,) at 37 °C for 16 h.

Protein Identification. One microliter of digest mixture was applied to a MALDI target and mixed on-target with 1 μL of 100 mM α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile and 0.1% trifluoracetic acid (TFA). MALDI-TOF MS and TOF/TOF tandem MS were performed on a MALDI-TOF-TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems). Data were acquired in 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, combination of peptide mass fingerprints (PMFs) and peptide fragmentation patterns were used for protein identification in an NCBI nonredundant (nr) database using the Mascot search engine (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/).

Quantitative Real-Time PCR. Nine differentially expressed proteins were examined to detect the corresponding mRNA levels by quantitative real-time PCR based on the sequences in rare minnow cDNA library. The real-time PCR was performed as previously described.[13] Gene names, accession numbers, forward and reverse primer sequences, and amplicon sizes are listed in Table 1. PCR amplification was conducted on a Stratagene Mx3000P qPCR system (Stratagene). Every sample was analyzed individually and processed in triplicate. On the basis of the results of the microarray data, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels were not significantly different between control and treated groups, and it was chosen as an internal control to normalize the data. After verifying that the amplification efficiencies of the selected genes and GAPDH were approximately equal, differences in expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.[27] The method was based on the equation: the relative expression ratio of a target gene = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{target} - Ct_{GAPDH})_{treatment} - (Ct_{target} - Ct_{GAPDH})_{control}$. One-way analysis of variance (ANOVA) was used for statistical analysis between the PFOA-exposed groups and the control. Differences were considered significant when *p*-values were less than 0.05.

Results and Discussion

Overview of Hepatic Protein Profiles of Rare Minnows Treated with PFOA. Protein samples from livers of rare minnows treated with three different concentrations of PFOA or untreated were subjected to 2-DE, and quantitative spot comparisons were made with image analysis software. Considering the possible gender differences in the response of organisms to PFOA, the male and female samples were analyzed separately. The reference gel images from the control and the 3 mg/L PFOA-treated groups with silver stain are shown in Figure 1. On the basis of the analysis with Image Master 2D Platinum software, approximately 500 proteins spots were detected on each gel under the filtering parameters mentioned above. Compared with the gels from the controls, 34 and 48 protein spots were found to be altered in abundance (>2-fold) from male and female PFOA-treated groups, respectively. Of these, 8 proteins from males and 35 proteins from females exhibited consistently altered trends among the three PFOA-treated groups. As for the altered spots in males, 13 (7 in 3, 4 in 10, and 7 in 30 mg/L) were up-regulated and 24 (13 in 3, 20 in 10, and 11 in 30 mg/L) were down-regulated following PFOA exposure. In gels from female PFOA-treated groups, a total of 21 protein spots (17 in 3, 16 in 10, and 17 in 30 mg/L) were up-regulated and 29 (23 in 3, 28 in 10, and 26 in 30 mg/L) were down-regulated.

All the altered protein spots were submitted for identification using MALDI-TOF-TOF analysis and searches in the NCBI nr database. Twenty-five spots (15 from males corresponding to 13 different proteins and 10 from females corresponding to 9 different proteins) were successfully identified with C.I.% values greater than 95% (Tables 2 and 3). The matched proteins in the database were mainly from zebrafish (*Danio rerio*), which was consistent with the higher similarity in expressed sequences between rare minnow and zebrafish shown in a previous study.[25] The remaining 57 protein spots could not be identified, mainly because their abundance was too low to produce a spectrum, or because the C.I.% of the database search score was not higher than 95% in order to yield unambiguous results. The MW, p*I*'s and fold change of the unidentified proteins are shown in Table 4. Although these altered proteins have not been identified at present, several of

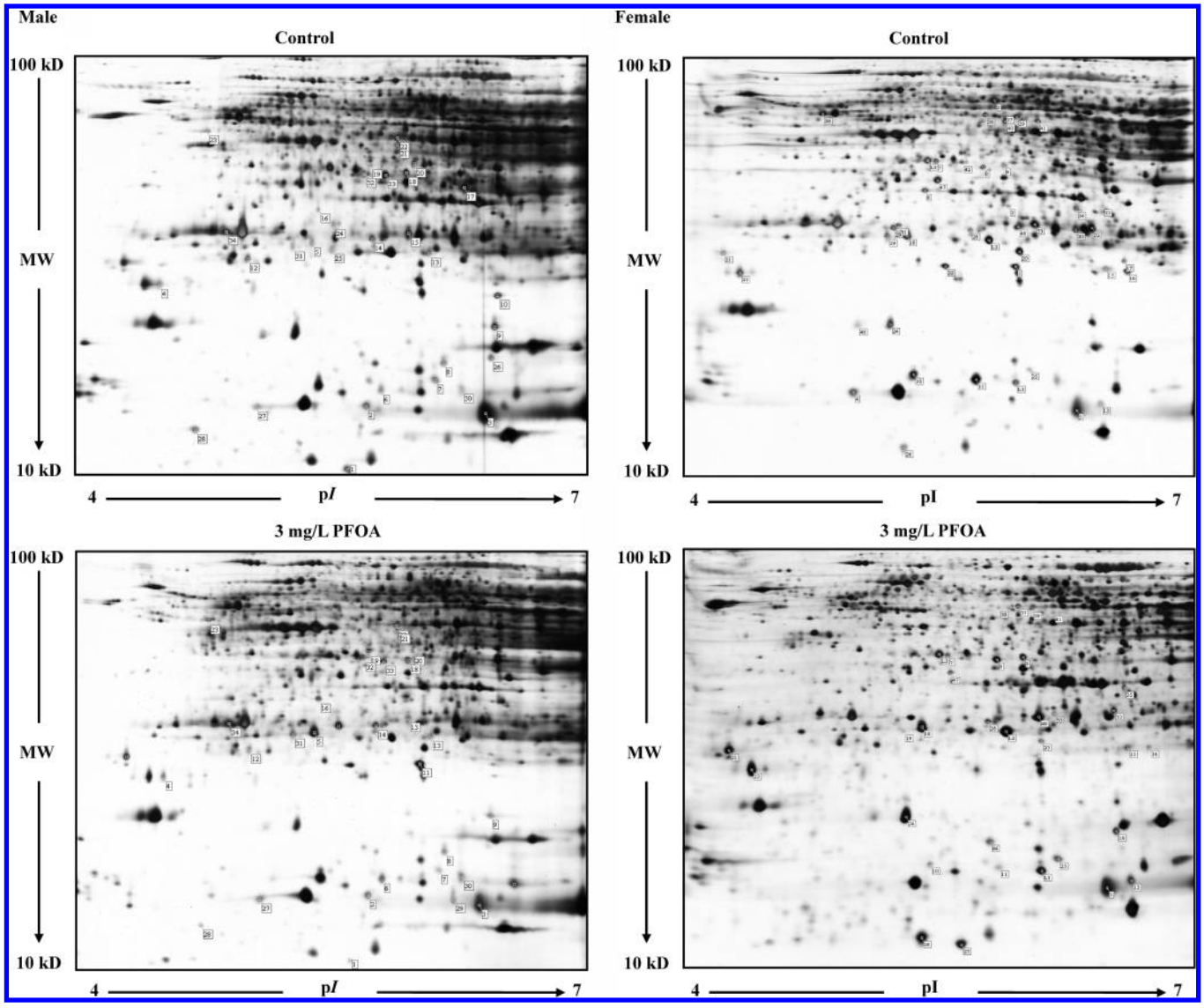

Figure 1. Representative 2-DE gels of hepatic proteins from male and female rare minnows of the control and PFOA-treated groups (the 3 mg/L treatment was selected). Whole hepatic soluble proteins from livers of male and female rare minnows were separated by 2-DE and visualized by silver staining. Protein spots that were altered by PFOA exposure are labeled by numbers. The molecular weights (MW) and p*I* scales are indicated. Each gel is representative of three independent replicates.

them display the greatest changes, and either disappear (spots m17 and m25 in gels from males and spots f1, f3, f4, f6, f17, f29, f30, f34, and f42 in gels from females) or appear (spots f14, f27, and f44 in gels from females) in PFOA-treated groups as compared with control gels. These proteins may be closely related to the response of rare minnows to PFOA exposure.

The Identified Differentially Expressed Proteins of Diverse Functional Categories. The identified differentially expressed proteins following PFOA exposure represented a heterogeneous group and took part in a variety of cellular biological processes, such as lipid transport, response to oxidative stress, metabolism of lipid, carbohydrate and amino acid, oxidative phosphorylation, cell cycle progression, protein repair, and cellular calcium ion homeostasis (Tables 2 and 3). Some of these genes have been demonstrated to have altered mRNA levels following the PFOA exposure using the microarray analysis,[13,28–30] whereas the majority of the identified proteins were not detected in the response of organisms to PFOA in previous studies.

1. Proteins Related to Intracellular Fatty Acid Transport. It has been suggested that the hepatotoxicity of PFOA is largely ascribed to the induction of peroxisome proliferation by perturbing lipid metabolism and transport.[31] In the present study, we identified four altered proteins involved in fatty acid transport and metabolism. Muscle fatty acid binding protein (M-FABP) (spot m2) was induced in male fish treated with 30 mg/L PFOA, and fatty acid binding protein 3, muscle and heart (M-H-FABP) (spot m27) was up-regulated in two male groups (10 and 30 mg/L), whereas liver fatty acid binding protein (L-FABP) (spot m3) exhibited an approximately 2-fold decrease in all three PFOA-treated male groups as compared to the controls (Table 2).

FABPs, which are small cytosolic proteins that facilitate the solubility and intracellular transport of fatty acids,[32] comprise nine types of proteins named after the first tissue of isolation.[33] An *in vitro* study[34] has shown that PFOA may interfere with the binding of fatty acids or other endogenous ligands to L-FABP and displacement of endogenous ligands from L-FABP

Table 2. A Detailed List of Protein Spots Identified by MALDI-TOF/TOF from the Livers of Male Rare Minnows Following PFOA Treatment (score C.I > 95%)

no. on gel	protein name	species	accession no.	protein score	total ion score	theoretical M_r (kDa)/p*I*	experimental M_r (kDa)/p*I*	fold change[b] (treated vs control) 3 mg/L	10 mg/L	30 mg/L	functional category[c]
						Intracellular Fatty Acid Transport					
3	Fatty acid binding protein 10, liver basic	*Danio rerio*	AAH76219	68	58	14.11/8.87	14.23/6.29	−2.04	−2.44	-	Bile acid binding/ fatty acid metabolic process/transport
2	Muscle fatty acid binding protein	*Salmo salar*	AAR91708	259	217	14.62/5.52	14.70/5.60	-	-	3.66	Fatty acid metabolic process/transport
27	Fatty acid binding protein 3, muscle and heart	*Danio rerio*	NP_694493	280	250	14.87/5.74	14.64/5.04	-	3.44	3.52	Phosphatidylcholine biosynthetic process
						Responses to Oxidative Stress					
8	Glutathione peroxidase 1	*Danio rerio*	AAH83461	111	70	16.57/5.93	16.61/6.05	-	−16.67	-	Response to oxidative stress
11	Zgc:92891 (Peroxiredoxin)	*Danio rerio*	NP_001002468	282	224	21.95/5.93	21.83/5.91	2.96	2.03	2.18	Response to oxidative stress
31	Zgc:92891 (Peroxiredoxin)	*Danio rerio*	NP_001002468	148	101	21.95/5.93	25.00/5.24	2.48	-	2.32	Response to oxidative stress
						Metabolism					
18	Zgc:101639 (Phytanoyl-CoA dioxygenase)	*Danio rerio*	AAH85396	115	102	33.40/5.39	35.59/5.85	-	−7.14	−2.04	Lipid metabolism
21	Galactokinase 1	*Danio rerio*	AAH83195	134	87	43.02/5.52	43.42/5.80	−2.38	-	2.48	Galactose metabolic process
22	Galactokinase 1	*Danio rerio*	AAH83195	134	87	43.02/5.52	43.70/5.80	-	−2.94	-	Galactose metabolic process
30	6-pyruvoyl tetrahydropterin synthase isoform	*Poecilia reticulata*	AAK59698	63	42	15.75/7.01	15.80/6.15	2.02	-	-	Tetrahydrobiopterin biosynthetic process
						Other Functions Related					
10	ATP synthase, H+ transporting, mitochondrial F0	*Danio rerio*	NP_956996	100	84	18.25/7.82	18.39/6.36	−25.00	−5.88	−4.17	Oxidative phosphorylation
19	Beta-Actin	*Pagrus major*	BAA89429	146	109	42.10/5.30	38.61/5.65	3.50	-	-	Cytoskeleton
28	Similar to vertebrate cyclin G associated kinase	*Danio rerio*	CAI21335	70	N/F[a]	96.56/6.42	13.16/4.71	−5.26	−16.67	−16.67	Protein phosphorylation/ regulation of progression through cell cycle
33	Ribosomal protein, large, P0	*Danio rerio*	NP_571655	311	261	34.90/6.16	35.42/5.73	-	−4.35	-	Translation
32	Unnamed protein product	*Tetraodon nigroviridis*	CAF96953	75	N/F[a]	38.84/9.13	35.42/5.62	-	−12.50	−12.50	

[a] N/F, not found. [b] The average fold changes as compared to the controls. Only the fold changes higher than or equal to 2-fold are shown, and the fold changes lower than 2-fold are noted by "-". Values >1 indicate up-regulations, and <1 indicate down-regulations, [c] Biological processes in GO terms.

may be one mechanism by which PFOA induces peroxisome proliferation. According to our microarray analysis of rare minnow livers treated with PFOA in our previous study,[13] the mRNA expression of L-FABP was suppressed in male fish under treatments of 10 and 30 mg/L PFOA. In our current study, we detected that L-FABP exhibited the highest abundance in gels from male controls, and obvious decreases in abundance were shown in gels from the PFOA-treated male fish (Figure 2). The results suggest generally consistent trends of alteration of L-FABP mRNA and protein levels induced by PFOA. The suppression of L-FABP may be associated with the interference of PFOA with the binding of ligands to L-FABP which has been demonstrated by *in vitro* study.[34] The present results demonstrated the suppressive effects of PFOA on L-FABP *in vivo* which may be implicated in the perturbation of fatty acid transport in the liver.

The other two types of FABPs, M-FABP and M-H-FABP, displayed converse directions of alteration compared with L-FABP. Because M-FABP and M-H-FABP are not the major FABP in liver since their major roles involve fatty acid transport in muscle and heart cells,[33] minor spots were detected in gels from male controls. Considering that these two proteins

Table 3. A Detailed List of Protein Spots Identified by MALDI-TOF/TOF from the Livers of Female Rare Minnows Following PFOA Treatment (score C.I > 95%)

no. on gel	protein name	species	accession no.	protein score	total ion score	theoretical MW (kDa)/p*I*	experimental MW (kDa)/p*I*	fold change[a] (treated vs control) 3 mg/L	10 mg/L	30 mg/L	functional category[b]
					Responses to Oxidative Stress						
25	Zgc:85965 (methionine sulfoxide reductase B)	*Danio rerio*	NP_998086	261	229	20.38/7.63	20.37/6.01	5.17	5.56	4.20	Protein repair/ response to oxidative stress
32	Glutathione *S*-transferase	*Pimephales promelas*	AAF78081	95	80	22.02/7.64	26.85/6.39	−2.67	−4.04	−2.52	Response to oxidative stress/toxin catabolic process
					Metabolism						
38	Phenylalanine hydroxylase	*Danio rerio*	AAP82284	115	63	51.85/5.86	51.93/5.75	−6.77	−14.70	−14.70	Amino acid metabolism
39	Phenylalanine hydroxylase	*Danio rerio*	NP_956845	113	57	51.84/5.60	51.24/5.93	−3.25	−8.30	−7.30	Amino acid metabolism
41	Similar to proteasome (prosome, macropain) 26S subunit, ATPase 2	*Danio rerio*	AAH53187	114	63	49.07/5.73	49.36/6.05	−6.27	−16.80	−6.25	Proteasomal ubiquitin-dependent protein catabolic process
48	Guanidinoacetate *N*-methyltransferase	*Danio rerio*	AAQ13341	106	84	26.97/5.88	27.05/5.94	2.39	2.07	2.27	Creatine biosynthesis/ muscle contraction
					Other Functions Related						
23	Putative translationally controlled tumor protein	*Lateolabrax japonicus*	AAP43627	86	71	19.41/4.55	24.66/4.32	4.46	4.38	3.37	Cell cycle progression and apoptosis
47	Regucalcin	*Danio rerio*	AAQ94576	76	58	33.19/5.39	33.29/5.47	−3.18	−4.50	−2.79	Cellular calcium ion homeostasis/ positive regulation of ATPase activity/ regulation of calcium -mediated signaling
16	Unnamed protein product	*Tetraodon nigroviridis*	CAF91796	62	217	73.60/9.05	24.70/6.61	−4.17	−4.55	−3.33	
33	Hypothetical protein LOC393297	*Danio rerio*	NP_956621	67	50	29.25/5.20	27.33/6.04	−8.95	−57.30	−57.30	

[a] The average fold changes as compared to the controls. Values >1 indicate up-regulations, and <1 indicate down-regulations. [b] Biological processes in GO terms

circulating in the blood may represent on the gels due to tissue heterogeneity of liver organ, we washed the separated liver tissues three times prior to protein extraction. Since the liver of rare minnow is dispersive, the effect of the vascularization of the liver sample and the level of blood proteins were neglectable in current study. On the basis of the sequences in the rare minnow cDNA library, we determined the mRNA levels of M-H-FABP in males using real-time PCR. No changes were detected except in males treated with 10 mg/L PFOA (spot 27 in Figure 2). However, M-FABP and M-H-FABP proteins were up-regulated in one or two male treated groups. Although there is little evidence available, so far, for the interference of PFOA with the binding of fatty acid and M-FABP and M-H-FABP, the induction by PFOA may contribute to the up-regulation of these two proteins based on similarity in structure of PFOA and fatty acid. However, the cause of the discrepancy of alteration of different types of FABPs elicited by PFOA still calls for further investigation. Nevertheless, the results in our present study

Table 4. Proteins Altered in the Livers of Rare Minnows Following PFOA Treatment (except the identified proteins listed in Tables *2* and *3*)

			fold change[a] (treated vs control)		
no. on gel	M_r (KDa)	p*I*	3 mg/L	10 mg/L	30 mg/L
		Male			
1	11.04	5.53	−25.00	−10.00	−2.78
4	20.24	4.52	-	−16.67	2.47
5	25.49	5.34	6.89	−14.29	-
6	15.76	5.71	-	2.88	2.00
7	16.03	6.01	−2.70	-	-
9	17.48	6.34	−3.03	-	-
12	23.63	4.99	-	−20.00	-
13	24.20	5.97	-	−20.00	-
14	25.94	5.66	-	−3.70	−2.78
15	26.39	5.86	−2.94	−2.63	−2.04
16	29.73	5.38	4.01	-	-
17	32.92	6.17	−100.00	−100.00	−100.00
20	37.74	5.89	−2.56	−20.00	−20.00
23	47.88	4.78	-	2.01	-
24	27.74	5.46	−12.50	−12.50	-
25	24.62	5.44	−16.67	−16.67	−16.67
26	16.67	6.32	−16.67	−16.67	-
29	14.54	6.10	19.85	-	-
34	26.68	4.89	-	-	−3.23
		Female			
1	24.95	5.93	−33.33	−33.33	−33.33
2	58.93	5.80	−50.00	−50.00	−50.00
3	29.57	5.88	−4.35	−4.35	−4.35
4	19.48	4.98	−25.00	−25.00	−25.00
5	18.82	6.30	-	−100.00	3.71
6	31.73	5.40	−16.67	−16.67	−16.67
7	37.81	5.46	-	−2.78	−2.08
8	36.26	5.74	4.16	2.59	2.77
9	36.75	5.86	4.52	2.20	-
10	20.18	5.33	−6.67	−3.85	-
11	19.99	5.69	−10.00	−6.67	−4.17
12	17.99	6.38	-	114.55	-
13	19.05	6.45	3.43	2.02	4.23
14	21.15	6.36	29.40	14.65	23.45
15	24.84	6.48	-	−11.11	−11.11
17	25.20	6.59	−7.14	−7.14	−7.14
18	26.47	5.30	2.05	-	−50.00
19	26.45	5.18	−2.44	−14.29	−14.29
20	25.69	5.95	−4.55	−4.55	−3.45
21	25.61	4.22	7.31	5.04	5.12
22	24.97	5.52	−25.00	−11.11	−25.00
24	22.28	5.19	3.92	3.02	3.88
26	17.48	5.28	6.96	4.11	3.88
27	17.27	5.50	62.10	12.75	20.60
28	26.80	5.66	2.06	-	4.31
29	26.90	5.21	−16.67	−16.67	−16.67
30	26.82	6.29	−33.33	−33.33	−33.33
31	27.16	5.24	2.84	4.13	2.64
34	29.18	6.30	−16.67	−16.67	−16.67
35	29.57	6.46	2.88	3.91	11.67
36	52.09	4.80	-	−33.33	−2.94
37	52.66	5.86	3.71	−3.70	-
40	49.36	5.87	−33.33	−33.33	−33.33
42	37.36	5.62	−8.33	−8.33	−8.33
43	18.27	5.85	-	-	20.40
44	21.06	5.66	18.20	8.90	11.60
46	22.24	5.00	-	14.10	34.80

[a] The average fold changes as compared to the controls. Only the fold changes higher than or equal to 2-fold are shown, and the fold changes lower than 2-fold are noted by "-". Values >1 indicate up-regulations, and <1 indicate down-regulations.

suggest that the FABPs may be regarded as candidate biomarkers for PFOA exposure.

2. Proteins Associated with Responses to Oxidative Stress. Four proteins associated with antioxidative stress were

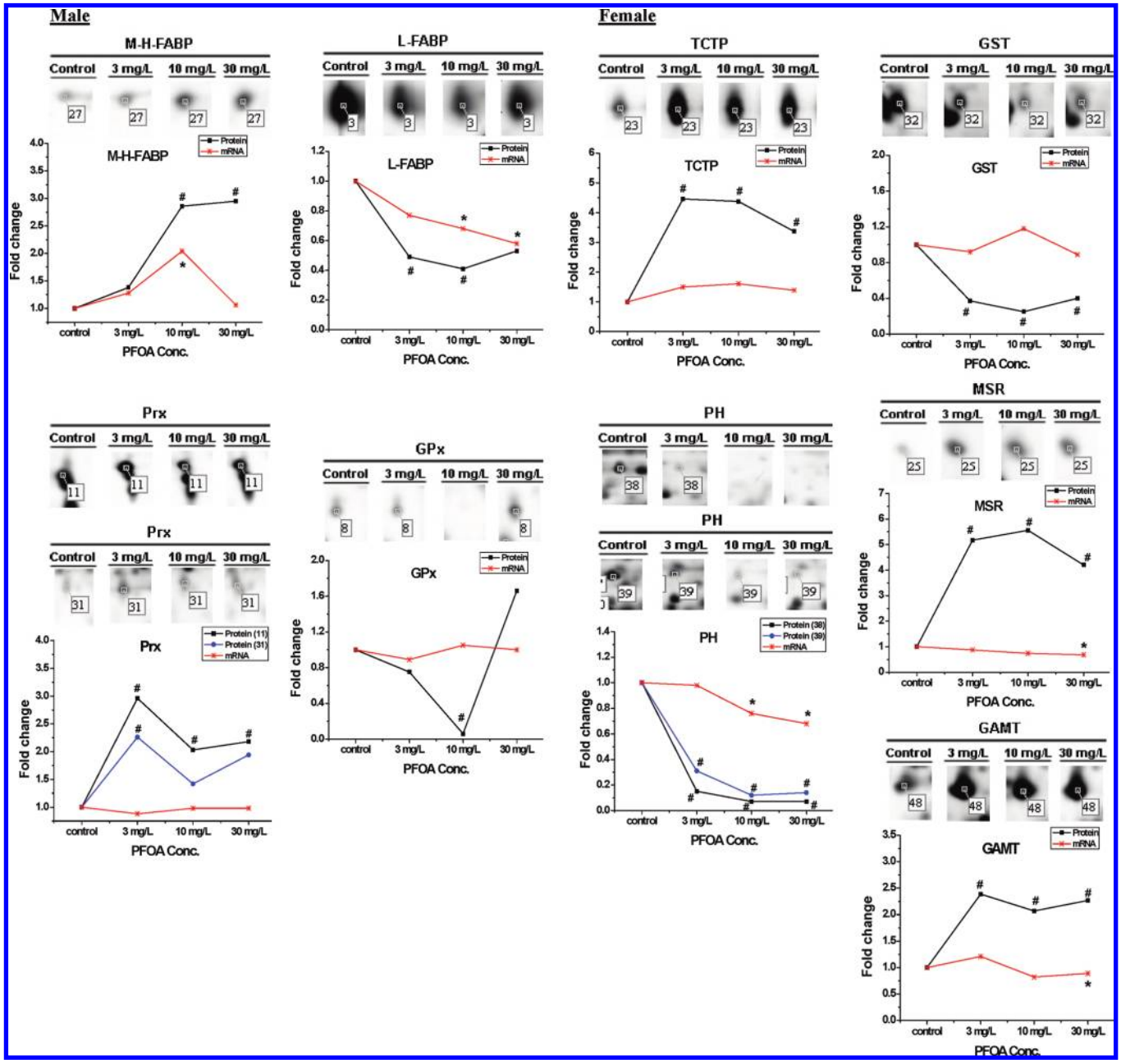

Figure 2. The alteration of protein and mRNA expression of selected altered proteins in the livers of male and female rare minnows following PFOA treatments. The magnified images of protein spots from the 2-DE gels are shown in the upper part of each panel. The line charts below show the protein levels based on 2-DE and mRNA levels using real-time PCR analysis. The values represent the average fold changes. The values of protein abundance are the average %Vol of spots in three replicated gels. Significant changes of mRNA levels as compared to the controls ($p < 0.05$) are indicated by asterisks (*), and pound sign (#) refers to an average increase or decrease higher than 2-fold in protein levels compared to the controls.

altered in the protein profiles of male or female rare minnow following PFOA exposure. Glutathione peroxidase (GPx), a key antioxidant enzyme which catalyzes the reduction of H_2O_2,[35] diminished in male livers exposed to 10 mg/L PFOA (spot m8). The detoxification enzyme to protect organisms against the electrophilic compounds, glutathione *S*-transferase (GST),[36] was also down-regulated in three female treated groups (spot f32). Conversely, another two protective enzymes involved in oxidative stress, peroxiredoxin (Prx) (spots m11 and m31) and methionine sulfoxide reductase B (MSRb) (spot m25), exhibited up-regulation in male or female treated groups. Prx is a ubiquitously expressed thiol-specific antioxidant enzyme that catalyzes the degradation of H_2O_2 and other reactive oxygen species. It has been shown that the Prx family consists of six isoforms.[37] From the hepatic protein profile of males, two spots possessing different MW and p*I*'s shared an identical annotation of Prx. They displayed consistent trends of up-regulation in male fish exposed to 3 or 30 mg/L PFOA. The two spots may be different isoforms of Prx that could not be discriminated based on annotation from the database search. Nevertheless, the results confirmed the induction of these two proteins in males following PFOA exposure. MSR acts as a repair enzyme that plays an important role in the recovery of function of methionine residue-containing proteins impaired by oxidative modification. It contains two subtypes, MSRa and MSRb.[38] An identified protein (spot m25) containing the domain of MSRb

had a 4∼6-fold increase in protein levels in female treated groups (Tables 2 and 3).

On the basis of the alterations of the enzyme proteins with respect to protection from oxidative stress, we observed the induction of antioxidant protein Prx and repair protein MSRb as well as the suppression of another two antioxidant enzymes GPx and GST. Previous studies have shown that PFOA could elicit the elevation of enzymatic activities of a variety of antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), and glutathione reductase, in an acute mode of exposure within 8 days *in vivo*[39]or *in vitro*,[40] but enzymatic activities of CAT in rats of different ages recovered to basal value at the 28 day exposure to PFOA.[39] Similarly, CAT and SOD activities showed significant decreases in rats exposed to perfluorododecanoic acid (a chemical with similar structure to PFOA) of 5 and 10 mg/(kg day) for 14 days.[41] The direct inhibition of excessive production of superoxide radicals may account for the diminished antioxidant proteins.[42] However, some kind of antioxidant proteins still plays a role in responses to damage by up-regulation of expression and enhancing the catalyzing activities. Prx and MSRb may be two members of this group. Moreover, the regulations of these gene expressions were supposed to be derived from the post-transcriptional levels since mRNA expression were generally equal to the controls (Figure 2). In addition to the enhancement in translational level induced by PFOA exposure, oxidation of amino acid residues may be another important reason for the increase in abundance of spots related to antioxidant proteins. It is not unusual to find oxidized isoforms of Prx on 2D gels, with the same MW but a slightly lower p*I* to the native form, in response to oxidative stress.[43] As the mRNA expression levels of Prx do not appear to increase in response to PFOA exposure, it is possible that the increase in protein abundance of the Prx spots is due to cysteine oxidation. Likewise, the increase in protein abundance of MSRb could also be a result of oxidation.

3. Proteins Relevant to Metabolism. Two spots (spots m21 and m22) from male gels were identified as galactokinase 1. Spot m21 was decreased in the 3 mg/L group and increased in the 30 mg/L group, whereas spot m22 was decreased in the 10 mg/L group. These two spots had the same p*I*'s and gained the same protein score and total ion score, but were different in MW. We predicted that the change of MW may be attributed to the modification of the protein or to the part of peptides of an identical protein that may be derived from the tryptic protein digestion. A protein containing the domain of phytanoyl-CoA dioxygenase (spot m18) was down-regulated in male 10 and 30 mg/L groups. In the male 3 mg/L groups, spot m30, identified as 6-pyruvoyl tetrahydropterin synthase isoform, was about twice as high as the control, while the identical protein in another higher-dosage groups were unchanged. As for enzymes involved in the metabolism of amino acids, the phenylalanine hydroxylase identified from two spots in female gels (spots f38 and f39) was consistently down-regulated (<−3-fold) in the three treated groups. Protein modification or different polypeptides from an identical protein was likely to be the reason for alteration in MW and p*I*'s. In addition, the proteins involved in protein biosynthesis and biological degradation were suppressed by PFOA exposure. Spot f33, identified as a large ribosomal protein P0, was down-regulated following 3 and 10 mg/L PFOA exposure in females. Spot f41, identified as proteasome (prosome, macropain) 26S, was dramatically decreased to a level 6-fold lower in the three female PFOA-treated groups as compared to the controls (Tables 2 and 3). Therefore, the results of proteins involved in cellular macromolecule catabolism suggest that PFOA exposure inevitably influenced the metabolism processes in the liver directly or indirectly. However, the mechanisms of the effects need further studies.

4. Proteins Correlated with Other Functions. In gels from female groups, we identified a spot (spot f23 in Table 2) as translationally controlled tumor protein (TCTP) that was up-regulated in three treated groups as compared to the controls (3∼5-fold) (Table 3). TCTP has been implicated in a wide range of cellular processes, such as cell growth, cell cycle progression, and malignant transformation. In addition, TCTP was shown to display an extracellular function as a histamine releasing factor and to have the ability to protect cells against various stress conditions and apoptosis.[44] The up-regulation in protein level in all female treated groups may predict a role of TCTP in responses to PFOA exposure. Moreover, as the mRNA expressions determined by real-time PCR were unchanged following PFOA exposure (Figure 2), regulation was mainly based on the post-transcriptional level. Regucalcin, a pivotal regulatory protein to maintain the intracellular Ca^{2+} homeostasis,[45] was identified from female protein profiles (spot f47 in Table 3). It exhibited decreases in abundance in the three treated groups (<−2.5-fold). This result suggested that disruption of cellular Ca^{2+} homeostasis and the corresponding adverse effects may be involved in the response to PFOA exposure. Additionally, a component of mitochondrial oxidative phosphorylation, ATP synthase, H^+ transporting, mitochondrial F0 (spot m10 in Table 2), was suppressed in all three male PFOA-treated groups, which confirmed the results from the microarray analysis in rare minnows[13] and the dysfunction of mitochondria in rats by PFOA.[46]

The Gender Difference in Altered Proteins. The gels from males and females were matched to define the corresponding spots based on their location in the gels. The matched spots altered by PFOA exposure in either male or female groups are shown in Supporting Information. Marked gender differences in alteration of protein levels were demonstrated from 12 pairs of spots, although some of them were not identified. The gender differences in response, such as pharmacokinetics and mRNA expression to PFOA exposure, have been well-described in previous studies.[13,47] In our current study, we further confirmed the gender differences in the response of organisms to PFOA on the basis of protein levels.

The Relationship between Transcription and Translation. Consistent and discrepant results between mRNA and proteins for nine genes were obtained (Figure 2). The protein levels of these genes were changed in abundance by PFOA exposure and the corresponding ESTs, which had identical annotations with the proteins, are available in the rare minnow cDNA library we constructed.[25] M-H-FABP expression in males showed the same up-regulated trends in mRNA and protein levels with the exception of the mRNA level in the 30 mg/L group. L-FABP in males and phenylalanine hydroxylase in females displayed a generally consistent down-regulated trend in mRNA and protein levels. The other 6 genes exhibited an increase or decrease in protein levels, whereas the mRNA expressions were virtually unchanged following PFOA exposure. As was shown in recent papers performing parallel proteomic/gene expression studies on the effects of dioxin[48] on rats or interferons on the SHK-1 cell line from Atlantic salmon,[49] the relationship between transcription of mRNA and the abundance of protein is not always a direct one as there are many

regulatory mechanisms that can affect these processes. Our results suggest that the gene expression in response to PFOA involves diverse regulatory mechanisms from transcription of mRNA to the formation of functional proteins.

Concluding Remarks

In this study, we used proteomic analysis to describe the hepatic protein profiles of rare minnows following PFOA exposure. A number of proteins were altered in abundance with different doses of PFOA and some of them were successfully identified. These proteins were involved in multiple functions and took part in a variety of biological processes. The results not only further our knowledge of the effects of PFOA on organisms, but also provide the basis for predicting the underlying mechanisms of toxicity as well as the identification of protein biomarkers for PFOA exposure. The marked gender differences in protein profiles and discrepancy of mRNA and protein expression complicate the responses of organisms to PFOA which should be highlighted in future studies. However, since a nonmodel fish about which little protein data are available was used, only a part of the altered proteins were identified based on PMF and peptide fragmentation analysis in the present study. Perhaps the protein sequencing adds to our knowledge of function and predicting responses to PFOA in future studies. However, the role of the proteins identified in this study will be the main focus of future studies on mechanisms of PFOA toxicity. Furthermore, whether (or what kind of) protein modification is involved in the responses to PFOA requires further investigation.

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Supporting Information Available: A table of comparison of the match spots altered by PFOA treatment between male and female rare minnow can be found in the supporting material. This material is available free of charge via the Internet at http://pubs.acs.org.

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