Effects of dechlorane plus on the hepatic proteome of juvenile Chinese sturgeon (*Acipenser sinensis*)

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ABSTRACT

Dechlorane Plus (DP), an alternative to decabromodiphenyl ether (BDE-209), is a widely used polychlorinated flame retardant that is frequently detected in aquatic ecosystems. While the mechanisms of toxicity of BDE-209 have been well documented, less is known about the toxicity of DP. In this study, juvenile Chinese sturgeon (*Acipenser sinensis*) were treated with DP at doses of 1, 10, and 100 mg/kg wet weight for 14 days via a single intraperitoneal injection (i.p.). After 14 days, liver proteomes of juvenile Chinese sturgeon were analyzed using two-dimensional electrophoresis (2-DE) coupled matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF-MS). A total of 39 protein spots were significantly altered in abundance (>2-fold) and of these proteins, 27 were successfully identified. Proteins related to the stress response that included heat shock cognate protein 70 and T-complex protein 1 were significantly increased and decreased in abundance, respectively. Moreover, Ras-related protein Rab-6B and GDP dissociation inhibitor 2, proteins that are involved in small G-protein signal cascades, were decreased in abundance 2- to 5-fold. Annexin A4, which is associated with Ca²⁺ signaling pathways, was also markedly decreased by 2-fold in the liver. Pathway analysis of differentially regulated proteins revealed that DP interfered with metabolism and was associated with proteins related to apoptosis and cell differentiation. Based upon protein responses, we suggest that DP has effects on the generalized stress response, small G-protein signal cascades, Ca²⁺ signaling pathway, and metabolic process, and may induce apoptosis in the liver. This study offers novel mechanistic insight into the protein responses induced in the liver with DP, an increasingly used and understudied flame retardant.

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1. Introduction

Dechlorane Plus (DP) is used as a replacement of Mirex, penta- and octa-BDE products which were banned from widespread use due to their toxicity, persistence, and bioaccumulation (Hoh et al., 2006). DP and its analogs are high production volume chlorinated flame retardants that are used in coating electrical wires and cables, computer connectors, and plastic roofing material (Betts, 2006). Relatively high concentrations of DP in environmental media and biota, as well as their persistence, bioaccumulation, and long-range

transportation, suggests that DP and its analogs might be persistent organic pollutants (POPs) (Sverko et al., 2011). After the chemical was first identified in wildlife in the North American Great Lakes in 2006, DP has been detected on a more global scale (Möller et al., 2010, 2012; Qi et al., 2010). For example, concentrations of DP in surface water ranged from 0.0013 to 2.4 ng/L, while in suspended sediment from an E-waste recycling site in South China, concentrations of DP were as high as 78.8 µg/g dry weight (Xian et al., 2011; Zhao et al., 2011). It has been reported in other studies that the levels of DP and its isomers in aquatic and terrestrial biota such as zooplankton, shellfish, fish and birds can vary from 0.02 to 2200 ng/g lipid (Feo et al., 2012). Additionally, DP has been detected in human hair (Zheng et al., 2010), serum (Ren et al., 2009) and breast milk samples (Siddique et al., 2012). Thus it appears as though DP can be relatively ubiquitous in aquatic systems.

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However, despite growing evidence that DP is detectable across multiple taxa and tissues, the health risks of DP exposure to aquatic organism and humans are not fully characterized.

The bioaccumulation of DP in tissues has been reported in aquatic organisms including zooplankton, shellfish and fish (Feo et al., 2012). For example, in teleost fishes such as juvenile rainbow trout (*Oncorhynchus mykiss*), the biomagnification factor (BMF) for isoforms of DP is 5.2 and 1.9 for syn-DP and anti-DP, respectively; thus it appears as though the syn form of DP is more readily bioavailable (Tomy et al., 2008). In a freshwater food web from a reservoir in the vicinity of electronic waste recycling workshops in South China, there were trophic magnification factors (TMFs) of 11.3 and 6.6 for syn-DP and anti-DP, respectively (Wu et al., 2010). In addition to bio-magnification, there is evidence that DP can preferentially accumulate in tissues in aquatic organisms. For example, higher concentrations of DP were observed in liver and brain of Northern snakehead and Mud carp compared to muscle (Zhang et al., 2011). A recent study in Chinese sturgeon indicated high bioaccumulation potential of DP in heart, liver and eggs as well as high maternal transfer efficiencies of DP based on its tissue distribution (Peng et al., 2012). Therefore, the data support the hypothesis that DP can bioaccumulate readily in aquatic food webs and bioconcentrate in fish tissues.

The uptake and bioconcentration of pollutants can be associated with adverse biological effects in aquatic organisms. Studies have demonstrated that exposure to polybrominated diphenyl ethers (PBDEs) are associated with reproductive and developmental effects, neurobehavioral toxicity, thyroid hormone disruption, immunotoxicity and that DP exposures are potentially related to cancers (Siddiqi et al., 2003). While there have been a number of studies reporting on the toxicology of PBDEs, few data are available for DP. Studies on the toxicity of DP that measured higher level biological endpoints, as well as clinical or anatomical pathology, have demonstrated that the toxic effects of DP may be relatively low (Brock et al., 2010; Crump et al., 2011). However, research on DP at the molecular level suggests that DP can induce adverse effects and there may be subtle endpoints that can be used to assess biological impacts in organisms (Li et al., 2013; Wu et al., 2012). For example, sub-chronic exposures to DP in rats (90 days period, 1–100 mg/kg/d) showed no adverse effect based upon histopathology and survival; however mRNA levels of *sulfotransferase (SULT) 1A1*, *1C2*, and *2A1* in the low dosage group (1 mg/kg/d) were significantly decreased and enzyme activity of CYP 2B1 was increased with DP exposure (Li et al., 2013). Recently, hepatic oxidative damage, perturbations in metabolism, and signal transduction in mice were reported to be induced by DP at the dose of 500–5000 mg/kg by daily gavage for 10 days (Wu et al., 2012). These studies indicate that DP may induce toxic effects in organisms at the level of the transcriptome and metabolome. However, additional research on the toxicity of DP at the protein level is required to better assess the overall impact of DP exposure and to more fully characterize the molecular responses underlying DP exposure.

To address this knowledge gap, the proteomic response in the liver of Chinese sturgeon (*Acipenser sinensis*) was measured after animals were treated with DP to learn more about the pathways affected by DP in this detoxifying tissue. Quantitative proteomics, a powerful tool for the global evaluation of protein expression, provides an effective method for characterizing toxicity pathways of chemical pollutants (Monsinjon and Knigge, 2007). To date, proteomics approaches have been successfully employed in several studies on the toxic effects of PBDEs in aquatic organisms (Chiu et al., 2012; Kling and Förlin, 2009; Kling et al., 2008). The model chosen for this study was the Chinese sturgeon because (1) it is listed as a grade I protected animal in China (since 1988) and because (2) this species is experiencing dramatic declines in population number due to overfishing, loss of natural habitat

for reproduction, and anthropogenic activities (Qiao et al., 2006). Moreover, the Chinese sturgeon is an excellent sentinel species for monitoring environmental organic contaminants because it is a long-lived predatory fish (Wei et al., 2002).

2. Materials and methods

2.1. Chemicals

Dechloranes Plus (CAS no. 13560-89-9; M.W. 653.7; purity >95%) was purchased from Wellington Laboratories Inc. (Guelph, Ontario, Canada). Due to its extremely lipophilic character, DP was dissolved in corn-oil for intraperitoneal injections according to Wu et al. (2012).

2.2. Exposure experiment

Juvenile Chinese sturgeon individuals were obtained from Fisheries College of Huazhong Agricultural University. These juveniles are offspring of artificially propagated individuals, and the parents are released into the Yangtze River under the supervision of the local government, as described previously (Wan et al., 2006). Fish (about 1.0 kg in weight) were randomly stocked in 1200 L glass tanks that included replicated controls and three treatment groups. The fish were maintained in aerated de-chlorinated tap water (using an activated carbon filter) at a constant temperature ($15 \pm 2^\circ\text{C}$) with a photoperiod of 16 h:8 h (light:dark) in order to mimic their optimal temperature range in the natural environment. Fish were acclimated for one week prior to experimental injections. DP was dissolved in corn oil in order to prepare the stock solution. The control group was injected with corn oil only. Individuals in the treatment groups were injected intraperitoneally once with 1, 10, 100 mg/kg fresh wet weight DP. Over the experimental period of 14 days, fish were fed with tubificid worms twice a day. The DP exposure doses employed were chosen on the basis of a report of Wu et al. (2012) and toxicity data provided by OxyChem and U.S. EPA.

2.3. Sampling

Three fish were sampled at each dose after the 14-day exposure. Deep anesthesia was induced by a 0.05% solution of MS-222 (Sigma, USA). The liver samples from juvenile Chinese sturgeon were collected within 30 min of exsanguinations by tailing and immediately dipped into liquid nitrogen and stored at -80°C . The experimental procedures were based on the standards of the Chinese Council on Animal Care.

2.4. Proteomic analysis

2-DE coupled MALDI-TOF-TOF was performed to quantify the proteomic response of juvenile Chinese sturgeon after injection of DP in order to better elucidate toxicity responses of DP at the protein level.

2.4.1. Protein extraction and separation

Protein extraction and 2-DE were performed according to Fang et al. (2010). Please refer to the Supporting Information for more specific details.

2.4.2. Protein identification and data analysis

After in-gel tryptic protein digestion, the resulting peptide mixtures were subjected to MALDI-TOF/TOF-MS analysis according to Meng et al. (2009) using a Bruker UltraFlex MALDI-TOF/TOF instrument. The mass signals generated from the MS mode

2.5. Western blot analysis

Proteins from livers of juvenile Chinese sturgeon were extracted and separated by SDS-PAGE and transferred to PVDF membranes (Millipore). The blots were incubated for 1 h at room temperature in TBST containing 5% skim milk. The primary antibodies that were used were anti-Hsp70 mouse monoclonal antibody

(diluted 1:5000, Abcam), anti-Annexin IV rabbit polyclonal antibody (diluted 1:800, Abcam) and anti- β -actin rabbit polyclonal antibody (diluted 1:1000, CST). The blots were labeled with horseradish peroxidase-conjugated secondary antibody to mouse IgG and rabbit IgG and visualized by ECL reagents (Pierce). Images were captured by a ChemiDoc-It[®] 415 Imager (UVP, USA) and analyzed by VisionWorksLS Image Acquisition and Analysis Software. Three biological replicates were measured from the control group and from each of the three doses.

2.6. Statistical analysis

For proteomics data, a one-way ANOVA was used to analyze spot intensities among different groups. Protein spots of interest were those demonstrating differential expression ($p < 0.05$) and at least a 2.0-fold difference in abundance. These proteins were selected for identification by mass spectrometry. For Western blots, quantitative data are expressed as means $\pm$ S.E. Statistical analysis of variance (ANOVA) was performed using SPSS (version 17.0). A Levene's test of homogeneity of variance and Dunnett's test were used to compare data between treatments. A probability of $p < 0.05$ was selected to indicate statistical significance.

Fig. 1. Representative 2-DE gels of hepatic proteins from juvenile Chinese sturgeon for control and DP treatments. Proteins were separated on 18 cm pH 3–11 NL IPG strips before being loaded on SDS-PAGE (12% acrylamide) gels and visualized by silver staining. The molecular weights (M_w) and pI scales are indicated. Each gel is representative of three independent biological replicates. Numbers are allocated by the Image Master 2D Platinum 7.0 software and represent the spots with a significant variation in intensity ($p < 0.05$; ratio > 2).

Table 1

A detailed list of protein spots identified by MALDI–TOF/TOF–MS from the liver of juvenile Chinese sturgeon following DP exposure.

Spot ID ^a	GI accession no.	Protein name ^b	M _w /PI	score	Matched peptides	Fold change ^c		
						1mg/kg Vs ctl	10mg/kg Vs ctl	100mg/kg Vs ctl
Metabolism								
17	112180601	Gapdh protein	35,989/8.20	147	2	−1.02	2.62	−2.21
21+	82245450	Triosephosphate isomerase B	27,096/6.45	63	6	3.14	−1.06	1.04
48	41393155	Isocitrate dehydrogenase [NADP] cytoplasmic	48,802/7.62	112	2	−1.09	2.42	0.91
53	169403947	Glyceraldehyde-3-phosphate dehydrogenase	35,989/8.20	84	1	0.66	2.00	−0.90
56	47085883	Malate dehydrogenase, mitochondrial	35,797/8.40	57	1	−0.76	2.80	1.50
501+	48762657	Alpha-enolase	47,372/6.16	132	16	1.20	−0.64	2.68
100	27881963	sb:cb825 protein	55,113/6.32	56	1	0.71	−3.14	0.57
84	41054541	Beta-ureidopropionase	43,699/6.51	51	1	−2.72	0.88	−1.18
224	50539730	Alanine-glyoxylate aminotransferase a	43,105/8.47	74	1	−3.02	−2.16	−5.61
499	60279651	Betaine-homocysteine S-methyltransferase 1	44,641/6.61	64	1	N/F ^d	N/F	N/F
Signal transduction								
86+	77681452	Ras-related protein Rab-6B	24,322/5.09	62	11	−5.91	−5.22	−4.09
118	37362224	GDP dissociation inhibitor 2	51,011/5.60	115	2	1.37	−2.48	1.51
150+	326674505	Predicted: diacylglycerol kinase delta-like, partial	46,163/6.53	73	7	0.88	−1.12	2.42
517+	62955633	BAI1-associated protein 2-like 1b	54,747/8.09	58	8	−1.36	−2.72	−8.34
Calcium ion binding								
44	32401412	Annexin A4	35,953/6.07	40	1	−2.47	−2.86	0.84
177+	326673841	Predicted: hypothetical protein LOC100536704, partial	264,427/5.1	66	21	2.12	0.66	1.82
522+	41053718	Hippocalcin-like protein 1	22,422/5.11	58	7	N/F	2.87	4.11
Protein folding								
28	317108145	Heat shock cognate 70 kDa protein	71,391/5.33	66	1	0.59	3.72	1.58
333	47086803	T-complex protein 1 subunit epsilon	59,927/5.39	39	1	−6.46	−3.60	−2.03
Cytoskeleton/structural proteins								
91+	50539690	Intraflagellar transport protein 172 homolog	199,024/5.60	65	18	1.35	−14.89	3.75
117+	82207947	Keratin, type I cytoskeletal 18[Acipenser baerii]	48,456/5.10	177	32	−3.00	−4.40	1.04
388+	28279111	Bactin1 protein	42,068/5.30	70	6	−0.37	2.08	0.90
Other function								
3+	348528476	Predicted: low quality protein: CAP-Gly domain-containing linker protein 1-like [Oreochromis niloticus]	255,321/5.14	79	44	N/F	0.94	2.23
29+	62955139	Exosome complex exonuclease RRP42	32,618/5.13	62	6	−1.18	2.84	1.08
43+	115529409	U3 small nucleolar RNA-associated protein 18 homolog	57,743/5.82	58	7	−0.86	2.27	−0.82
65+	326666641	Predicted: golgin subfamily B member 1	38,7997/4.75	61	31	−4.00	−1.31	0.88
146+	41055554	Ubiquitin-associated protein 1	32,781/4.93	67	8	1.64	−1.19	2.03

^a Proteins identified by PMF noted by "+" following spot ID.^b All proteins from *Danio rerio* database except specially noted by "[]" in the end of the protein name.^c The average fold changes as compared to the controls. Bold character indicates significant fold change values, and the down-regulations are noted by "–".^d N/F, not found in the gel of related exposure sample.

3. Results

3.1. Hepatic proteome profiles

Representative 2-DE gels of hepatic proteins from control and DP-treated groups are shown in Fig. 1. Quantitative spot comparisons were performed with image analysis software and approximately 740 spots were detected on each gel. Among these proteins, 39 protein spots were found to be altered in abundance (>2-fold) in one or more DP-treated groups compared to that of controls. According to the ratio value, there were 12, 24, and 15 significantly altered spots at the 1, 10, and 100 mg/DP/kg wet weight, respectively. Seven spots (spots 10, 86, 125, 224, 333, 517, 520; shown in Fig. 1 and Fig. S1) displayed consistent directional changes (increasing/decreasing) in response to the DP dose response. Altered protein spots were submitted for identification using MALDI-TOF-TOF analysis and searches for protein homology the NCBI nr database. There were 27 proteins that were successfully identified (Table 1).

3.2. Differentially expressed proteins and pathway analysis

In general, the identified proteins were involved in metabolism, signal transduction, calcium ion binding, protein folding,

structure stabilizing, as well as other functions (Table 1). Following a more general description of proteins based upon gene ontology, pathway analysis was performed to further integrate protein data and to determine if there were common cell processes affected by altered proteins (Fig. 2). Based upon protein responses and interactions (e.g. expression, binding, regulation), the processes of cell structure (actin organization, microtubule assembly), transcription regulation (protein folding, mRNA degradation, RNA processing and metabolism) and cell metabolism (glucose metabolism and the tricarboxylic acid cycle) were increased while the process of organelle transport (intra-golgi transport, endoplasmic reticulum and golgi transport) was decreased based on protein responses. Proteins that were altered in abundance by DP were also related to processes such as apoptosis, cell differentiation, and cell death. Interestingly, GAPDH and heat shock cognate protein 70 (also known as HSPA8) were significant hubs within the network, being involved in multiple cell processes and having many interactions with the other proteins regulated by DP. Below we describe in more detail the different proteins associated with these cell processes.

The abundance of many metabolism-associated proteins was increased by DP, indicating that the metabolic process was a main target of DP. Most of these proteins were associated

Fig. 2. Pathway analysis for proteins differentially expressed with dechlorane plus in the liver of Chinese sturgeon. Red indicates that the cell process/protein is increased up-regulated while green indicates the cell process/protein is down-regulated. Abbreviations are as follows; alanine-glyoxylate aminotransferase, AGXT; alpha-enolase, ENO1; annexin A4, ANXA4; bactin1 protein, ACTB; BAI1-associated protein 2-like 1b, BAIAP2L1; betaine-homocysteine S-methyltransferase 1, BHMT; beta-ureidopropionase, UPB1; exosome complex exonuclease RRP42, EXOSC7; glyceraldehyde-3-phosphate dehydrogenase protein, GAPDH; GDP dissociation inhibitor 2, GDI2; heat shock cognate 70 kDa protein, HSPA8; hippocampal-like protein 1, HPCAL1; intraflagellar transport protein 172 homolog, IFT172; isocitrate dehydrogenase [NADP] cytoplasmic, IDH1; keratin, type I cytoskeletal 18, KRT18; malate dehydrogenase, mitochondrial, MDH2; predicted: diacylglycerol kinase delta-like, partial, DGKD; predicted: golgin subfamily B member 1, GOLGB1; predicted: hypothetical protein LOC100536704, partial, CDHR2; predicted: CAP-Gly domain-containing linker; protein 1-like, CLIP1; ras-related protein Rab-6B, RAB6B; Sb:cb825 protein, PDIA3; T-complex protein 1 subunit epsilon, CCT5; triosephosphate isomerase B, TPI1; U3 small nucleolar RNA-associated protein 18 homolog, UTP18; ubiquitin-associated protein 1, UBAP1.

Fig. 3. Validation of Hsp70 and annexin A4 by Western blot. Western blots were performed in the liver lysates from juvenile Chinese sturgeon exposed to DP and control tissues. Equal amounts of the protein were electrophoresed in each gel lane, and representative immunoblots are shown. β -actin was used as a loading control. The ratios of respective proteins to β -actin were calculated by performing densitometric analysis using VisionWorksLS software, and the data are represented as means $\pm$ S.E.; * $p < 0.05$.

with carbohydrate metabolism and included glyceraldehyde-3-phosphate dehydrogenase (GAPDH), triosephosphate isomerase B, isocitrate dehydrogenase [NADP] cytoplasmic (IDH), malate dehydrogenase, mitochondrial, and alpha-enolase (Table 1). Three proteins associated with amino acid metabolic process were markedly down-regulated, including beta-ureidopropionase, alanine-glyoxylate aminotransferase a, and betaine-homocysteine S-methyltransferase 1 (Table 1). In the pathway analysis, the process of serine glycine metabolism was decreased, consistent with the suggestion that amino-acid related processes are suppressed by DP.

Four significantly altered protein spots were involved in signal transduction. Ras-related protein Rab-6B (RAB6B) and BAI1-associated protein 2-like 1b were significantly down-regulated in liver samples exposed to DP (Table 1). Expression of GDP dissociation inhibitor 2 (GDI2) was inhibited in 10 mg/kg group and the protein predicted: diacylglycerol kinase delta-like, partial (DGKD) was induced in 100 mg/kg group.

Annexin A4 (ANXA4), predicted: hypothetical protein LOC100536704 (partial) (CDHR2) and hippocalcin-like protein 1 are proteins associated with calcium ion binding; each of these proteins were also altered in abundance by DP. ANXA4 was down-regulated in the 1 mg/kg and 10 mg/kg groups while exposure to DP increased the abundance of CDHR2 in the 1 mg/kg and 100 mg/kg treatment groups (Table 1). Hippocalcin-like protein 1 exhibited a 2–4 fold increase in 10 mg/kg and 100 mg/kg DP-treated groups (Table 1).

Heat shock cognate protein 70 (HSC70) and T-complex protein 1 subunit epsilon (CCT5), proteins involved in stress responses, showed opposite directional changes in abundance levels. HSC70 was up-regulated after exposure to 10 mg/kg DP while CCT5 was down-regulated in all three DP-treated groups (Table 1). Structural proteins (intraflagellar transport protein 172 homolog, keratin type I cytoskeletal 18, and bactin1 protein) were also significantly altered in abundance

by DP, and proteins changes ranged from 2 to 14 fold (Table 1).

3.3. Validation by Western blot

To further confirm changes in the abundance of proteins identified in the proteomic analysis and to further investigate the toxicity pathways of DP, two proteins (HSP70 and ANXA4) were analyzed using Western blot (Fig. 3). Anti-HSP70 mouse monoclonal antibody was used to measure the expression level of HSC70. HSP70 was significantly up-regulated following DP exposure ($p < 0.05$), a result that was consistent with 2-DE observation. However, ANXA4 was not significantly altered in response to DP, which was not in agreement with the 2-DE results. Data generated from 2-DE determined that this protein was decreased approximately 2.5 fold at 1 and 10 mg/kg DP.

4. Discussions

To gain insight into the mechanisms of toxicity of DP, 2-DE coupled MALDI-TOF-TOF was used to study the hepatic proteome response of juvenile Chinese sturgeon injected intraperitoneally (i.p.) with DP. A total of 39 significantly altered proteins were detected and 27 of these proteins were identified by mass spectrometry. These proteins were primarily involved in metabolism, signal transduction, calcium ion binding, protein folding, and structure stabilizing. Previous studies have mainly focused on PBDEs (Alm et al., 2006; Chiu et al., 2012; De Wit et al., 2008; Kling and Förlin, 2009; Kling et al., 2008), and molecular data are limited for DP. However there are some data for PBDEs that can be compared to proteomic data collected in the liver of Chinese sturgeon. For example, the hepatic proteome of zebrafish exposed to tetrabromobisphenol A (TBBPA) was analyzed by differential in-gel electrophoresis (DIGE) and 12 proteins were found to be significantly altered. These proteins were also associated with stress

response, metabolism, and the stabilization of cell structure (De Wit et al., 2008). In addition, mussels exposed to PBDE-47 had proteomic responses that were related to xenobiotic stress, amino acid metabolism, oxidative stress and effects on the cytoskeleton (Apraiz et al., 2006). Thus, these cell processes and associated proteins may be useful toxicological signatures for the general class of flame retardants.

Pathway analysis suggested that differentially expressed proteins were involved in carbohydrate and amino acid metabolic process. In the present study, GAPDH was identified in two spots, most likely due to post translational modifications of the protein, resulting in different pI focusing and electrophoresis. In previous studies, oxidative modification of GAPDH was found to cause significant inhibition of GAPDH dehydrogenase activity in Alzheimer's disease brain (Butterfield et al., 2010). Sheng and Wang (2009) also detected several isoforms of GAPDH in T cell with higher molecular mass and more basic pI. Increases of GAPDH and IDH, two proteins that are involved in carbohydrate metabolism, have also been reported in zebrafish exposed to hexabromocyclododecane (HBCD) and TBBPA (Kling and Förlin, 2009). It was hypothesized in the study that an over-expression of these proteins may contribute to increased cytotoxicity and the production of cellular defense systems involved in xenobiotic metabolism and oxidative stress. In addition, betaine homocysteine methyltransferase (BHMT) is an enzyme responsible for remethylation of homocysteines to form methionine. Suppression of this enzyme can result in increased generation of homocysteine and an increased activity of antioxidant enzymes (Kharbanda et al., 2005; Moat et al., 2000). In a previous study, De Wit et al. (2008) found that BHMT was down-regulated in the liver of zebrafish after TBBPA exposure, and concluded that this was a result of oxidative stress. Our results are consistent with these previous observations, and it is hypothesized that DP may affect energy production and induce oxidative stress in Chinese sturgeon.

Two stress-related proteins, HSC70 and CCT5 were significantly altered with DP treatment. The protein HSC70 is a constitutively expressed molecular chaperone which belongs to the heat shock protein 70 family. It plays an important role in facilitating protein folding and maintaining their structure and function (Liu et al., 2012). It is also involved in many clinical diseases such as cancer, cardiovascular, neurological, and hepatic diseases; thus it is a significant target for therapeutic treatments (Liu et al., 2012). HSC70 is therefore a ubiquitous protein that is multi-functional and is responsive to a multitude of internal and external signals. In the present study, HSC70 was found to be significantly up-regulated following DP treatment. Western blot analysis of HSP70 further supported our proteomic results. In previous proteomic studies with PDBEs, many HSP70 family members such as HSP70 protein 5, HSP70 protein 8, and HSP70 protein 9B were quantified in the livers of zebrafish (De Wit et al., 2008; Kling and Förlin, 2009). De Wit et al. (2008) reported that HSP70 protein 5 was markedly up-regulated in response to TBBPA, whereas, Kling and Förlin (2009) found that HSP70 protein 8 and HSP70 protein 9B were down-regulated in exposures using TBBPA and a mixture of HBCD and TBBPA, respectively. These results imply that different flame retardants might induce divergent downstream pathways related to HSP70 proteins. The protein CCT5 plays an important role in maintaining cellular homeostasis by assisting the folding of many proteins involved in cytoskeleton organization and cell cycle (Huang et al., 2012). In addition, CCT5 in the cell nucleus might play unexpected roles in biological processes including RNA processing, apoptosis, and cell metabolism (Huang et al., 2012). In this study, the expression of CCT5 was consistently decreased in individuals from all three DP treatments. In HBCD exposures, CCT5 subunit 6A was decreased (1.5 fold) in the liver cells of zebrafish (Kling and Förlin, 2009). Our results are consistent with previous studies, which suggest that

DP may affect protein folding and biological processes related to CCT5. Our pathway analysis suggested that proteins related to cell cytoskeleton were affected as well and this could be due, in part to disruptions in CCT5 expression. The abnormal expression of HSC70 and CCT5 by DP implies that DP may induce a series of biological responses, with some leading to changes in cell metabolism and apoptosis.

Proteomic analysis also suggested that DP may affect small G-protein signaling cascades, as there was a decrease of RAB6B as well as GDI2 and an increase of DGKD. RAB6B is a small G-protein (GTPase) of the Ras oncogene family. Small G-proteins regulate a wide variety of cell functions including gene expression, intracellular vesicle trafficking, and the cell cycle (Matozaki et al., 2000). GDI inhibits GDP dissociation and keeps the small G-protein in the inactive form (Matozaki et al., 2000). Dysfunction in the regulation of Rab GTPases and GDIs can lead to a variety of cancers and neurological diseases (Harding and Theodorescu, 2010; Hutagalung and Novick, 2011) and proteins related to this signaling pathway can be affected by flame retardants. For example, up-regulation of G-protein subunit α was observed in the tubificid (*Monopylephorus limosus*) exposed to BDE-183 for 8 weeks. Moreover, mitogen-activated protein kinase 12 (MAPK12), which is a downstream signal protein of Ras, was significantly decreased in response to BDE-47 and BDE-183 (Chiu et al., 2012). In the present study, the consistent down-regulation of RAB6B, a protein which mediates gene expression and affects cellular proliferation, may suggest that this protein is sensitive to DP exposure. In addition, DGKD, a type II DGK, was found to be increased in response to DP. DGK may indirectly regulate protein kinase C (PKC) and small GTPases levels or their activated state through phosphorylation of diacylglycerol (DAG). It is hypothesized that this may affect downstream biological processes such as cell proliferation, cell differentiation, and cytoskeletal rearrangements (Topham and Prescott, 1999; van Blitterswijk and Houssa, 2000); processes that were identified in our pathway analysis. Our findings and that of previous studies indicate that small G-protein signaling cascades may be impaired by DP and other flame retardants.

Proteomic data and pathway analysis indicated that calcium ion signaling pathways may also be affected by DP, as Ca^{2+} was a small molecule that contained numerous interactions with differentially expressed proteins in the protein network. Therefore, we hypothesize that DP may result in adverse effects on aquatic organisms via impaired Ca^{2+} signaling. One of the impacted proteins with an integral role in Ca^{2+} signaling is ANXA4, and this protein was down-regulated in individuals from two of the three doses of DP. ANXA4 is a member of the annexin protein family which can bind to membrane phospholipids in a Ca^{2+} -dependent manner, providing a bridge between Ca^{2+} signaling and membrane functions (Gerke et al., 2005). However, in addition to regulating Ca^{2+} signaling and membrane functions, ANXA4 is also a modulator of chloride (Gerke and Moss, 2002). Noteworthy is that previous studies suggest that one of the underlying molecular mechanisms of the adverse effects of polychlorinated biphenyls (PCBs) and PBDEs have been perturbations in intracellular signaling, including Ca^{2+} homeostasis and PKC translocation (Kodavanti and Ward, 2005). Our results suggest that DP may influence Ca^{2+} homeostasis by regulating the expression of ANXA4, and we hypothesize that Ca^{2+} signaling may be affected by DP in a similar way to that of PCBs and PBDEs. Lastly, we point out that in the present study, 2-DE revealed that the expression of ANXA4 was decreased; however Western blot results showed that the abundance of ANXA4 was not significantly affected after DP treatment. The discrepancy between the two methods may be due to a low affinity antibody for ANXA4 as Chinese sturgeon are quite evolutionary divergent than other teleost fishes and mammal (Ma et al., 2011). Despite the lack of technical congruence, previous

Fig. 4. A model for dechlorane plus action in the liver of Chinese sturgeon. Proteins altered in abundance by DP were involved in Small G protein signal transduction, Ca^{2+} signal transduction, protein folding, and metabolism. Red indicates an induction in protein abundance and green indicates a reduction in protein abundance. Yellow indicates the up/down-regulation in protein abundance at different exposure concentration of DP. Abbreviations are as follows: alanine-glyoxylate aminotransferase (a) AGXTA; annexin A4, ANXA4; predicted: hypothetical protein LOC100536704, partial, CDRH2; alpha-enolase, ENO1; glyceraldehydes -3-phosphate dehydrogenase, GADPH; GDP dissociation inhibitor 2, GDI2; heat shock cognate 70 kDa protein, HSC70; isocitrate dehydrogenase [NADP] cytoplasmic, IDH1; malate dehydrogenase, mitochondria, MDH2; Sb:cb825 protein, PDIA3; ras-related protein Rab-6B, RAB6B; T-complex protein 1 subunit, TCP-1; triosephosphate isomerase B, TPI1B; beta-ureidopropionase, UPB1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

literature in flame retardants and pathway analysis of all differentially expressed proteins suggests that DP affects the regulation of Ca^{2+} signaling.

Regulation of Ca^{2+} signaling can also occur via different cellular mechanisms independent of annexins. For example, calcium can regulate Ras activation, which activates MAPK kinase (MEK) (Cullen and Lockyer, 2002). The activated MAPK translocates to the nucleus and stimulates the activity of transcription factors through phosphorylation, which in turn regulates cell proliferation and apoptosis (Matozaki et al., 2000). Moreover, Fan et al. (2010) showed that PBDE mixtures and congeners activate the MAPK pathway, which may be involved in the initiation of events that lead to adverse effects that are associated with these persistent chemicals. Proteomic results from the present study indicated that small G-protein signaling cascades may be regulated by DP. Therefore, we hypothesize that DP may affect gene expression by activating MAPK pathway.

Based on our data and principles of cell signaling, we generate a model for how DP may act in the liver of aquatic organism (Fig. 4). Initially, DP may impair the movements of Ca^{2+} from the extracellular compartment to the intracellular compartment by affecting intermediate ANXA4 function, followed by effects on the Ras signal cascade and protein folding process. Gene expression and protein synthesis may be impacted by upstream signals from GTPase pathways and mis-folded proteins accumulated by abnormal protein folding process. At the same time, the xenobiotic system may be evoked to degrade DP. These signaling events may affect the processes of cell proliferation and apoptosis, and may lead to adverse effect. We point out that this is only a model of DP action based upon proteomics data, and generates a general framework for future

studies. Similar to other flame retardants, it is expected that DP affects multiple signaling cascades within the teleost liver and these molecular events must be further validated experimentally.

In conclusion, 39 protein spots were significantly altered in abundance with different doses of DP and of these, 27 proteins were successfully identified using MS. Differentially expressed proteins and pathway analysis indicated that DP exposure may induce oxidative stress, cell proliferation and apoptosis. Meanwhile, these responses may be mediated through the stress response, small G-protein signaling cascades, calcium ion binding and carbohydrate metabolism. The underlying mechanisms of DP appear comparable to PBDEs, impacting calcium homeostasis and activation the Ras signal cascade. Future studies should continue to validate these proteins as potential biomarkers for the exposure to DP in fish.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2014.01.003>.

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