CHANGES IN PROTEOMIC PROFILES OF CEREBELLUM FOLLOWING DEVELOPMENTAL EXPOSURE TO AROCLOR 1254 OR DE-71

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Abstract

Chronic low level exposure to polychlorinated biphenyls (PCBs) has been shown to adversely affect human health, including learning and memory. Polybromiated diphenyl ethers (PBDEs) are structurally similar to PCBs and have been shown to have neurotoxic effects *in vitro* and *in vivo* at concentrations/doses similar to those of PCBs. Although the cellular and molecular bases for these actions is not fully understood, several studies have shown that in addition to their effects on thyroid hormones, disruption of Ca^{2+}-mediated signal transduction plays a significant role in the developmental neurotoxicity of these persistent chemicals. Changes in a variety of signal transduction pathways can lead to gene regulation and protein expression, which affect the growth and function of the nervous system. Our previous studies on PCBs showed changes in gene expression profiles related to signal transduction and neuronal growth. In this study, we have examined the protein expression profiles in cerebellum following developmental exposure to Aroclor 1254 (a commercial PCB mixture) or DE-71 (a commercial PBDE mixture). Pregnant rats (Long Evans) were dosed perinatally with 0.6 mg/kg/day of Aroclor 1254 or 30.2 mg/kg/day of DE-71 from gestation day 6 through postnatal day (PND) 21, and the cerebellum from PND14 animals were analyzed. Proteins differentially expressed following PCB or PBDE exposure were detected by two-dimensional differential gel electrophoresis (2D-DIGE) using fluorescent cyanine dye labeling and identified by tandem mass spectrometry (MS). Two proteins were found to be differentially expressed in the cerebellum following PCB exposure and four were differentially expressed following DE-71 exposure. Protein identification revealed that these proteins are related to mitochondrial energy metabolism, calcium signaling, and growth of the nervous system. These results suggest that changes in energy metabolism and intracellular signaling may be involved in the developmental neurotoxicity of persistent chemicals.

Introduction

PBDEs are used as flame retardant additives in electrical equipment, plastics, and building materials. PBDEs share chemical structure and physicochemical properties with other persistent pollutants such as polychlorinated biphenyls (PCBs; Figure 1). Their global production is in the range of 80 million pounds annually[1], and they are becoming ubiquitous contaminants because of the high production, persistence in the environment and lipophilic characteristics, leading to persistence in biological systems. PBDEs have been detected in human blood, adipose tissue and breast milk. Long-term exposure to these contaminants may pose a health risk, especially to children. While the presence of other persistent organic pollutants, such as PCBs and dioxins, have decreased in environmental and human samples[2], levels of PBDEs in the environment have been increasing in the past 20-30 years. Despite of their widespread occurrence in the environment, only limited information is available on the toxicology of PBDEs. Recent studies showed that PBDE exposure can result in alterations in spontaneous behavior and reduced learning and memory in mice[3,4]; these effects are similar to those seen after exposure to DDT or PCBs[5], although the mode of action of PDBEs remains unclear.

Previously, we demonstrated that PCBs and PBDEs affect intracellular signaling pathways including [^{3}H]arachidonic acid ([^{3}H]AA) release, calcium homeostasis, and translocation of protein kinase C (PKC)[6,7]. All of these pathways are known to associate with the development of the nervous system as well as learning and memory[8]. A culminating event for many signal transduction pathways is gene regulation and protein expression. In

this study, the protein expression profiles in cerebellum following developmental exposure to Aroclor 1254 (a commercial PCB mixture) or DE-71 (a commercial PBDE mixture) were examined using DIGE-based proteomics.

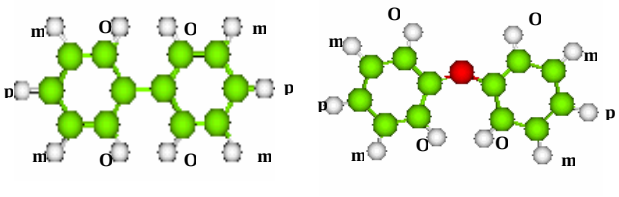

FIG. 1: Structural features of biphenyls and diphenyl ethers

Materials and Methods

Dosing of animals: Long-Evans rats received from Charles Rivers (Portage, OR) on gestational day (GD) 3 (the day of insemination being GD 0) were housed in AAALAC approved animal facilities. Food (Purina lab chow) and water were provided *ad libitum*. All the experiments were approved in advance by the NHEERL/USEPA Animal Care Committee.

A commercial PCB mixture, Aroclor 1254 (Lot # 124-191; purity >99%) was purchased from AccuStandard, Inc. (New Haven, CT). DE-71 (Lot # 7550OK20A) was a gift from Great Lakes Chemicals Corporation (West Lafayette, IN). In each cohort, at least 15 dams per dosage group were given Aroclor 1254 (0 or 6 mg/kg/day) or DE-71 (30.2 mg/kg/day) in corn oil (2 ml/kg) by oral gavage starting on GD 6 and lasting through postnatal day (PND 21). The dams were weighed daily before dosing. Beginning at GD22, rats were checked twice daily (AM and PM) for births, and the date the birth was first discovered was designated PND 0. The litter sizes varied between 7-15 pups/litter. On PND4, litters were culled to 8 pups/litter (four males and four females). The results on behavioral, functional, neurochemical, and morphological end points have been published elsewhere. For proteomic analysis, cerebella were dissected from PND 14 rats, quick frozen on dry ice, and stored at –80^{o}C.

Protein extraction. Proteins from cerebella were extracted in lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris containing protease inhibitors and phosphatase inhibitor, NaVO4) as described elsewhere[9]. The lysates were prepared for electrophoresis using the GE-Healthcare clean up kit and protein concentration was determined using 2D-Quant kit (GE Healthcare, Piscataway, NJ).

Cy-dye labeling, 2-D gel electrophoresis, and imaging: A 120 µg aliquot of total protein from each sample was labeled with 200 pmol of Cy dyes followed by active rehydration at 30V for 16 hours in 13 cm (3–10) immobilized pH gradient (IPG) strips (GE Healthcare) and isoelectric focusing using an Ettan IPGphor II (GE Healthcare) for a total of 26 kVh (step to 500 V for 1 h, step to 1000 V for 1 h, step to 8000 V to a total of 26KVh). After the isoelectric focusing, disulfide bridges were reduced by placing the strips for 10 min in 20 ml equilibration buffer (6M urea, 50mM tris, pH 8.8, 30% glycerol, 2% SDS) supplemented with 5 mg/mL DTT. Focused strips were incubated for 10 min in fresh equilibration buffer supplemented with 45 mg/mL iodoacetamide to oxidize sulfhydryl groups and the IPG strips were placed on 12% homogeneous polyacrylamide gels (4% stacking). Gels were prepared using low fluorescence glass plates (13 cm plates, GE Healthcare) pretreated with bindsilane (GE Healthcare). SDS-PAGE gels were run at 9mA for 16 hours in a HOEFER SE-600 system and individual images of Cy2-, Cy3-, and Cy5-labeled proteins were obtained via a Typhoon 9410 scanner (GE Healthcare) with excitation/emission wavelengths of 480/530 nm for Cy2, 520/590 nm for Cy3, and 620/680 nm for Cy5. After imaging, the gels were stained with colloidal Coomassie stain (BioRad, Hercules, CA).

***DIGE analysis*:** DeCyder 2-D software (GE Healthcare) was used for simultaneous comparison of abundance changes across gels and for pair-wise comparisons of individual Cy3- and Cy5- labeled samples. Pair-wise comparisons of each PND14 control and corresponding A1254 C sample to the pooled internal standard present on each gel was performed with the DeCyder differential in-gel analysis (DIA) module. Two standard deviations (2 S.D.) from the mean volume ratios (95th percentile confidence) were used as threshold to determine levels of significance for a given set of samples [9, 10]. The values for 2 standard deviations of the mean volume ratios for each set comparison were 2.44 for gel set 1 (n=3), and 2.57 for gel set 2 (n=3).

Protein identification: Protein spots displaying statistically significant changes in expression were excised from the immobilized gels with the Ettan Spot Picker (GE Healthcare) and the proteins in the spots were identified by tandem mass fingerprinting using MS/MS data[11.] In-gel protein digestion was carried out with modified trypsin, and MALDI-MS/MS data were acquired using a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Inc. (ABI), Framingham, MA). MS and MS/MS peak spectra were acquired and the 15 most intense peaks with a signal-to-noise ratio greater than 20 were selected automatically for MS/MS analysis. The peptide mass fingerprinting and sequence tag data from the TOF/TOF were evaluated with GPS Explorer scores (ABI). The MS and MS/MS spectra were submitted to non redundant databases (NCBI, MSDB) to identify proteins via the Mascot search engine[11.]

Statistics. The data were analyzed using student's t-test. The level of significance was set at $p < 0.05$.

Results and Discussion

In the present work, we have used DIGE-based proteomics to identify proteins that are differentially expressed in the cerebellum of rats treated with two developmental neurotoxicants. Using this approach, we were able to quantitate expression changes. 2-DIGE of cerebella revealed 1600 ± 10 spots. Among these proteins, only two proteins were found to have statistically significant differential expression following PCB exposure (Figure 2). Similarly, four proteins were found to be differentially expressed following DE-71 exposure (Figure 3). Using mass spectrometry we were able to identify these proteins as members of pathways regulating several homeostatic mechanisms. These proteins are related to energy metabolism in mitochondria and growth of the nervous system (Dihydrolipoamide dehydrogenase, aldolase, and cytochrome C).

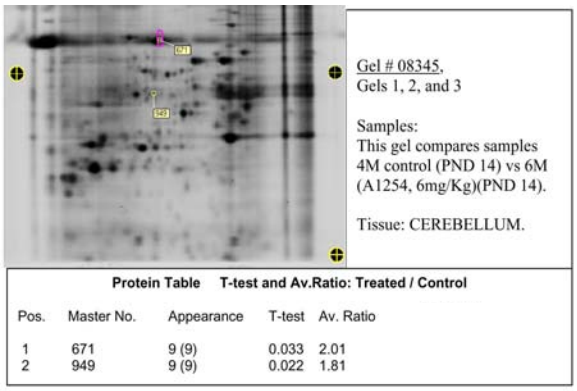

Figure 2. Proteins in cerebellum with significant changes following developmental exposure to Aroclor 1254. Two proteins were affected by Aroclor 1254.

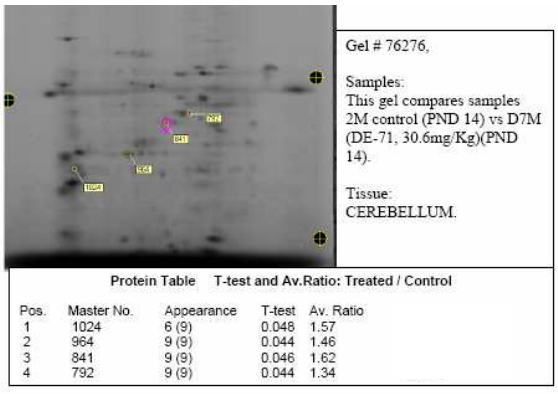

Figure 3. Proteins in cerebellum with significant changes following developmental exposure to DE-71. Four proteins were altered by DE-71.

Table 1. Mass Spec identification of proteins in cerebella following developmental exposure to Aroclor 1254 or DE-71.

Sample (Band/ Spot on Gel	Protein Name	Database Accession ID	MW (Da)	MS & MS/MS score	Peptide sequence	Function
Aroclor 1254 treatment						
01-671	Liver regeneration-Related protein LRRG03 (Transferrin)	Q7TNX0_RAT	76345.6	893	565	Iron homeostasis, Iron transport
02-949	Dihydrolipoamide dehydrogenase	Q6P6R2_RAT	54004.1	249	175	Electron transport
02-949	Rat cytochrome C Pseudogene, clone	CAB23487	11588.9	81	71	Cytochrome C pseudogene
DE-71 treatment						
03-792	Ratsg1	Q8VHV7_RAT	40580.1	187	139	Nucleotide binding
03-792	Heterogenous nuclear Ribonucleoprotein H2	Q6AY09_RAT	49262.3	184	133	Nucleoside binding
03-792	Hnrp1 protein	Q499R8_RAT	20567.3	152	113	
04-841	BC078896 NID	AAH78896	47098.2	551	406	Enolase protein
05-964	Aldolase C,fructose biphosphate	Q54AI4_RAT	39259.2	1200	920	Glycolysis
06-1024	AF463524 NID	AAN76992	37063.2	285	209	Ribonucleoprotein
06-1024	Albumin	Q5U3X3_RAT	68714.1	47	37	Macromolecule binding

Our previous results with Aroclor 1254 and DE-71 indicated effects on calcium signaling and development of the nervous system[7]. Results from the proteomics studies are in agreement with these observations and further confirm that changes in energy metabolism and intracellular signaling may be involved in the developmental neurotoxicity of persistent chemicals.

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