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PCB Congener Specific Oxidative Stress Response by Microarray Analysis using Human Liver Cell Line

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Abstract

In this study we have examined the effect of exposure to different congeners of PCBs and their role in oxidative stress response. A metabolically competent human liver cell line (HepG2) was exposed with two prototype congeners of PCBs: coplanar PCB-77 and non-coplanar PCB-153. After the predetermined times of exposure (0-24 hours) at 70μM concentration, the HepG2 cells showed significant apoptotic changes by fluorescent microscopy after 12 hours of exposure. Gene set enrichment analysis (GSEA) identified oxidative stress as the predominant enrichment. Further, paraquat assay showed that PCB congeners lead to oxidative stress to different extents, PCB-77 being more toxic. This study, with emphasis on all recommended microarray quality control steps, showed that apoptosis was one of the most significant cellular processes as a result of oxidative stress, but each of these congeners has a unique signature gene expression, which was further validated by Taqman real time PCR and immunoblotting. The pathways involved leading to the common apoptotic effect were completely different. Further *in-silico* analysis showed that PCB-153 most likely acted through the TNF receptor, leading to oxidative stress involving metallothionein gene families, and causing apoptosis mainly by the Fas receptor signaling pathway. In contrast, PCB-77 acted through the aryl hydrocarbon receptor. It induces oxidative stress through the involvement of cytochrome P450 (CYP1A1) leading to apoptosis through AHR/ARNT pathway.

Keywords

Apoptosis; Aryl hydrocarbon receptor; Cytochrome P450; PCB 77; PCB 153; Oxidative stress

1. Introduction

Polychlorobiphenyls (PCBs) are a class of lipophilic inert compounds, theoretically classified into 209 congeners according to the position of the chlorine atoms in the biphenyl ring, which also decides the 'planarity' of the PCBs. Though production of PCBs was banned from the USA in 1977, persistent PCB congeners in our biosphere is known to cause

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reproductive (Loch-Caruso, 2002), neurological (Schantz et al., 2003), endocrinal (Portugal et al., 2002) and other defects. PCBs also adversely affect fetal and infant developments (Winneke et al., 2002), causing neuro-developmental disorders as well as autism (Kimura-Kuroda et al., 2007) and are also immunotoxic (Lyche et al., 2004). The carcinogenic effects of PCBs (Faroon et al., 2001a; Faroon et al., 2001b; Laden et al., 2002) are however controversial (Golden et al., 2003). PCBs are known to cause apoptosis in neuronal tissue (Sanchez-Alonso et al., 2003) but little is known about the mechanism leading to apoptosis in any tissue. Moreover, the cellular processes activated due to PCB exposure are also unknown. It is known, however, that unlike the coplanar PCBs (like PCB-77), non-coplanar PCBs (like PCB-153) do not act through aryl hydrocarbon receptors (AhR) (Vondracek et al., 2005). PCB-153 and PCB-77 were chosen as representative of the two classes of the PCBs and several studies have shown that these cause many deleterious effects on different organ systems in rodents (Berberian et al., 1995; Glauert et al., 2005; Hemming et al., 1993; Tharappel et al., 2002). It is also known that oxidative stress leads to autism (Chauhan et al., 2004; James et al., 2004), cancer (Nair, 2006; Tas et al., 2005; Valko et al., 2006; Yoshida and Ogawa, 2000) and heart diseases through endothelial damage (Ramadass et al., 2003). Hence it was worthwhile to study the effect of oxidative stress induced by different classes of PCBs in our model cell line, HepG2. Moreover, oxidative stress response genes have been implicated in development of DNA based biomarkers (Bartsch and Nair, 2000) and this expression profiling study can further be developed into mRNA expression based biomarkers. This study gives us a molecular insight of PCB-153 and PCB-77 induced changes in gene expression with special reference to oxidative stress in the human liver cell line (HepG2), which in turn lead to the apoptosis. A time-series of microarray studies was performed as gene expression changes over time. Further, oxidative stress pathways induced by different congeners of PCBs are also explored through *in-silico* analysis.

2. Material and Methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM, Cat#11995-065), Fetal Bovine Serum (FBS, Cat#12318-028), Penicillin-Streptomycin (PS, Cat# 15140-122) and Trizol (Cat# 15596026) were obtained from Invitrogen, CA. Non-coplanar PCB-153 (2,2',4,4',5,5'-hexachlorobiphenyl; Cat# PRC-047) and coplanar PCB-77 (3,3',4,4'-tetrachlorobiphenyl; Cat# PRC-036) were obtained from Ultra Scientific, RI. 50mM stock solutions of each type of PCBs were prepared in dimethyl sulfoxide (DMSO, Cat# C6164) from Sigma-Aldrich, MO. CellLytic-M (Cat# C2978) was also obtained from Sigma-Aldrich, MO. High Capacity cDNA Archive kit (Cat# 4322171) and FAM labelled primers were obtained from Applied Biosystems, CA.

2.2. Cell Culture and Treatment

Human liver cell line (HepG2) (Cat# HB-065) was obtained from American Type Culture Collection (ATCC), VA. Cells were grown in DMEM medium supplemented with 10% FBS (heat inactivated) and 1X Penicillin-Streptomycin in 25cm² flasks. The cells were grown at 37°C in 5% CO₂ incubator. After 48 hours (at 80% confluence), the cells were washed with 1x PBS, medium was refreshed and 70µM of PCB-153 or PCB-77 was added. The cells were treated with graded concentrations (0 to 100µM) of PCB-153 or PCB-77 (in triplicates) and analyzed for cell death by Trypan Blue (1:1 with PBS) uptake over 24 hours to determine the appropriate concentration and time-points for the following experiments (data not shown) (Duttaroy et al., 1998). For all following experiments a concentration of 70µM PCB-77 and PCB-153 was used with a maximum exposure time of 18 hours (PCB-77) and 24 hours (PCB-153) which are the exposure time points where 50% cell death was observed.

(LC₅₀ time point). All PCBs were dissolved in DMSO and added to each plate in such a way that the final concentration of DMSO was $\leq 0.1\%$.

2.3. Microarray

2.3.1. Experimental Design—All experiments were done in triplicates. Equal amount of liver cells (10^6 cells/ml) were inoculated and the medium was refreshed and cells rested before experiments. The exposures for the microarray experiments were 0, 30 minutes, 6 hours and LC₅₀ (median lethal concentration) time point (which is 18 hours for PCB-153 and 24 hours for PCB-77 both at 70 μ M concentration) as mentioned in section 2.2. The 0 hour served as the control.

2.3.2. Sample preparation for Microarray—The cells were grown for the stipulated time (0, 30 minutes, 6 hours and LC₅₀ time) and the medium was removed. The cells were washed thrice with PBS and harvested with 2.5ml of Trizol by scrapping and transferred to Eppendorf tubes (1ml in each tube) and homogenized by pipetting and incubated for 5 minutes at 30°C. RNA was extracted by standard Trizol:Chloroform protocol of Invitrogen. Purification of RNA was done using Qiagen RNeasy Mini Kit (Cat# 74106) from Qiagen, CA according to manufacturer's protocol. 1 μ l of RNA was used for agarose gel electrophoresis to test the purity and the amount of RNA was quantified spectrophotometrically by GeneSpec-3 software at 260nm and 280nm. First strand cDNA, second strand cDNA and Biotin-Labelled cRNA were generated according to Affymetrix protocol. The cRNA was purified with Qiagen RNeasy Mini Kit followed by quantification and agarose gel electrophoresis as before. To obtain optimal assay sensitivity, the cRNA was fragmented with 5X fragmentation buffer containing 200mM Tris-acetate, 500mM Potassium-acetate (KOAc) and 150mM Magnesium-acetate (MgOAc).

2.3.3. Microarray Hybridization, Washing and Staining—Hybridization cocktail was prepared with 15 μ g of fragmented cRNA, control oligonucleotide B2, eukaryotic hybridization controls (bioB, bioC, bioD, cre), herring sperm DNA, BSA and 2X hybridization buffer according to Affymetrix protocol. The "Standard" protocol for Affymetrix arrays was used and HG-133 plus 2 arrays were filled with 200 μ l of the cocktail and incubated in 45°C hybridization oven at 60 rpm for 16 hours. Washing was done in the fluidics station (model 450) along with double staining (streptavidin phycoerythrin (SAPE) and biotinylated antibody to streptavidin). EukGE-WS2 protocol for fluidics station was used for all staining and washing purposes. The cartridge was removed from the fluidics station and scanned with GeneChip Scanner 3000.

2.3.4. Quality control assurance—Proper quality control measures were taken throughout the procedure. Three replicates per experiment along with three controls have been performed to reduce the experimental variability. The total RNA concentration was more than 0.5 μ g/ μ l with at least four fold amplifications during labelled cRNA synthesis (Zhao and Hoffman, 2004). After scanning, first, the image was checked for alignment to grid and image contamination. Above all, the scan report generated by Gene Chip Operating Software (GCOS) had a scaling factor between 0.5 to 5, total percent of 'P-calls' between 30% to 50%, external controls cre>BioD>BioC>BioB and internal control GAPDH was 1 ± 0.1 (pivot table). This pivot table was then further evaluated by Hierarchical Clustering Explorer (HCE) (Zhao et al., 2003) and after this final quality control the data were analyzed with Genespring 7.2. During unsupervised clustering in HCE, row by row normalization was done by mean $\pm$ SD and Euclidian distance was calculated with average linkage. Several steps were chosen for quality control of the microarray analysis as otherwise it may produce erroneous.

2.3.5. Microarray Analysis—The data were normalized by Genespring software by per chip basis to the 50th percentile followed by per gene basis by normalizing all the chips (both control and treated) against all the control chips. The genes were then filtered on flag first (“P” flags) followed by a filter on confidence (t-test) at $p=0.05$. ANOVA was done to find the significantly changed genes compared to the control. Finally a filter on fold change was added (≥ 3 fold). The results were cross checked with dChip software where a model based normalization was performed (Li and Wong, 2001). The significant gene list (with all common genes) identified with Genespring and dChip with Affymetrix probe set ID at different time points were imported into the dChip and clustered based on similarity in expression.

2.4. Gene set enrichment Analysis

Gene set enrichment Analysis software (v2) was obtained from the Broad Institute. Weighted enrichment statistic was done using Signal2Noise metric for ranking genes with 1000 permutations with normalized unfiltered data (as recommended by GSEA authors) from Genespring.

2.5. Identification of Cell Processes and Pathway construction

The filtered data from Genespring were put into GenMAPP and human standard database updated till May 2006 was used along with the contributed MAPPs till august 2006. Using MAPP finder, the significant cellular processes were identified. Cross checking for cell processes was done using Ingenuity Pathways. For drawing the final pathways, all significant genes from different time points were pooled together and the preliminary network with direct connections was created automatically in Ingenuity Pathways. It was further crosschecked and manually curated with PathwayAssist software for the specific cellular process for apoptosis mediated cell death and oxidative stress.

2.6. Software Development

Genespring and dChip have a limitation of finding common sets of genes for more than three time points (using Venn diagram). Also it is impossible to compare the common genes between the gene lists obtained in the Genespring and dChip software. Hence there was a necessity to not only write a program to find out the common genes but to extend its capability to find genes which are repeated more than three times. To find out the common genes between the lists, the native function of the MS Excel for Microsoft Windows was enhanced with the use of a VBA addin “Find Common Genes” which is available from the author on request. It can be opened by MS Excel from any folder and can be started by pressing “Ctrl, Shift and F” simultaneously. The only prerequisite is that the column, based on which the duplicates or triplicates needed to be found, are to be sorted in either ascending or descending order. The macro program was written based on programming code released in the Microsoft article number Q142591 with lots of additions. It has been tested with MS Excel XP and MS Excel 2003 under Microsoft Windows 2000 and Windows XP only.

2.7. Validation by Taqman Real-Time PCR

For true validation, the HepG2 cells were grown again in triplicates exactly as was done for microarray analysis. RNA was extracted and purified as before. The quality and quantity of the RNA was checked spectrophotometrically using Nanodrop1000-Spectrophotometer. For cDNA synthesis, the reagents were added to make a master mix according to Applied Biosystems protocol. Taqman Gene Expression assays for specific primers were obtained from <http://myscience.appliedbiosystems.com>.

2.8. Immunoblotting studies

The cells were grown in triplicates again for respective time points and lysed with 1ml of cell lysis buffer (CellLytic-M) mixed with the recommended Protease inhibitor cocktail according to Sigma protocol. The protein was measured by BCA protein assay using microplate reader (Molecular Devices) at 562nm wavelength. 10µg of whole cell extracts were resolved in Novex 4-20% Tris-Glycine SDS polyacrylamide Gel at 90V. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P 0.45µm Cat# IPVH08100 from Millipore, MA) overnight at 22V with constant stirring at 4°C. The proteins of interest were identified with corresponding primary antibodies (1:1000 in TTBS) at 37°C for 2 hours followed by horseradish peroxidase (HRP) conjugate specific secondary antibody (1:1000 in 5% fat free milk) for 1 hour and detected by ECL plus chemiluminescence detection system. β -actin antibody was used as gel loading control. Immunoblotted data were analyzed using ImageJ software. The blots were normalized with loading control and non-linear regression (second order polynomial) was used to visualize the expression data of duplicate immunoblots (figure not shown). As microarray and real time PCR relies on mRNA and proteins are the ultimate effectors, immunoblotting was used for confirmation of the microarray data as well as validation of the pathways.

2.9. Identification of oxidative stress induced apoptosis by paraquat (PQ) assay

Paraquat (PQ) is a broad-spectrum herbicide and one of the widely studied redox cycling compounds involved in the generation of superoxide anion, giving rise to reactive oxygen species (ROS). It has been used by several authors for qualitative measurement of oxidative stress inducing potential of any compound in *in-vitro* experiments (Kim et al., 2004). We used HepG2 cells for the cellular assay system and paraquat exposure (PQ) as a pro-oxidant model agent to evaluate whether PCB 153 and PCB 77 provide aggravating effects towards PQ-induced oxidative stress and cell death (Sousa et al., 2009). For that purpose, we have combined paraquat-induced oxidative stress with 70µM of PCB-153 or PCB-77 and demonstrated a synergistic effect of PCBs on the cellular viability. HepG2 cells were treated concurrently with PCB-153 or PCB-77 (70µM) and with or without PQ (30µM) with a parity of the microarray studies. Apoptosis was confirmed with fluorescent microscopy and cell death was assayed with Trypan Blue staining. An independent exposure of PQ and the respective PCBs to the HepG2 were also done to see their individual effects over time. Cells without PQ and PCBs served as control.

2.10. Fluorescent Microscopy

Cells were grown in monolayer in LAB-TEK four well slides and washed with PBS to remove the media. The cells were fixed with 100% cold methanol for 20 minutes and washed with ice cold PBS. Staining was done with 3µM Hoechst dye 33342 for 15 minutes followed by a wash with PBS. The cells were mounted with glycerol (90% glycerol:10% PBS). The fluorescent nuclei were seen under fluorescent microscope at 400X microscopic magnification and 3X magnification by the camera i.e. a total magnification of 1200X.

3. Results

3.1. Microarray

Unsupervised hierarchical clustering was performed for the samples to show the temporal effect of PCB treatment and to check the quality of microarray experiments with Hierarchical Clustering Explorer (Zhao et al., 2003). Results show that samples clustered according to treatment groups, indicating that length of exposure to PCBs was the dominant variable (Fig.S1). For clustering, the Euclidian distance with average linkage was used.

Gene set enrichment analysis (GSEA) shows that one of the most important enriched group of genes belonged to the oxidative stress. Some of the highest enrichments along with their congener specificity were ESR_FIBROBLAST_UP and OXSTRESS_BREASTCA_UP common to both PCB-77 and PCB-153; STRESS_GENOTOXIC_SPECIFIC_UP and STRESS_P53_SPECIFIC_UP specific for PCB-153; STRESS_TPA_SPECIFIC_UP and STRESS_IONIZING_SPECIFIC_UP specific for PCB-77 (Fig.1). It is interesting to note here that some effects were unique to each type of PCB although there are some common stress effects for both congeners.

Statistically significant (t-test, P-value <0.05) filtered genes with present flags among 54,675 probes present in the HG U133 plus 2 oligonucleotide microarray are 225 genes at 30 minutes, 134 at 1.5 hours, 405 at 6.0 hours and 315 at 18 hours for PCB153 and have two fold or more expression changes. Similarly for PCB-77, 156 genes were identified at 30 minutes, 194 at 6 hours and 231 at 24 hours. Principal Component Analysis (PCA) with the filtered genes shows the first three patterns of variation among gene expression are time dependent authenticating the time dependent clustering effects (Fig.S2). Principal Component-1 (PC-1) shows that there is a group of genes whose expression increased at 30 minutes and 6 hours but remain plateaued at 18 hours after PCB-153 treatment. PC-2 shows that there is a group of genes whose expression increased at 30 minutes and 6 hours but decreased at 18 hours after PCB-153 treatment. PC-3 shows that there is a group of genes whose expression increased at 30 minutes but decreased at 6 hours and remain plateaued at 18 hours after PCB-153 treatment. Similar (not identical) pattern were also observed with PCB 77. As the number of time points was four which has a degree of freedom (n-1) three, only three Principal components were statistically significant and reported here.

The common genes between these filtered sets and the enrichment of oxidative stress response genes with three fold or more changes were further manually curated and clustered. The clusters show the patterns of gene expression of the selected gene sets (Figs. 2 and 3) and have similarity to PCA analysis. A few of these genes such as metallothionein (MT1K), thioredoxin interacting protein (TXNIP) was validated using Taqman RTPCR (Fig. 4). It was clearly evident that metallothionein family of genes were specifically induced by PCB-153 and not by PCB-77 (data not shown) instead cytochrome P450 family and NQO1 (Fig. 5) was by far more involved in case of PCB-77. Analysis of differentially expressed genes following exposure of HepG2 cells to PCB 153 and PCB 77 using GenMapp v2.0 software shows the increasing number of cell processes up-regulated or down-regulated over time. A z-score of 2.0 and p value of 0.05 was used to filter out the insignificant cell processes. Cells treated with PCB-153 clearly shows lipid metabolism and apoptosis as highly prominent processes whereas cells treated with PCB-77 clearly shows steroid metabolism and DNA damage as prominent processes most likely as the end result of oxidative stresses (Fig.S3) with a significance of z-score of >2 (P-value<0.05).

'Minimum Information about a Microarray Experiment' (MIAME) compliant data has been submitted to Gene Expression Omnibus (GEO) database. All the microarrays used in this paper can be accessed from the GEO accession number: GSE6878. Individually PCB-153 microarrays can be accessed from the GEO accession number: GSE6494 and PCB-77 from GSE6869.

3.2. Oxidative stress assessed by paraquat studies

Results shown in Fig. 6 indicate that 5-33% cell death measured by Trypan blue uptake occurs with PCB-153 or PQ alone at 6 and 18 hours but around 60% in combination. The toxicity of PCB153 plus PQ at these time points is significantly higher than the toxicity of either compound alone and is more than additive, indicating synergistic effects. Moreover, PCB-153 alone is basically not toxic at 1.5 hour exposure, but significantly increases the

toxicity of PQ. No significant augmentation of toxicity is visible after 24 hour of exposure to both compounds. For comparison, 24-60% cell death occurs with PCB-77 or PQ alone at 18 and 24 hours, but over 62 and 87% respectively in combination. In this case no combination effect occurs at 1.5 or 6 hours and at 18 and 24 hours the combined effect is significantly higher than either effect alone, but less than additive. This observation suggests that PCB-77 and PCB-153 augment the oxidative stress of PQ, indirectly confirming that PCB-77 and PCB-153 are inducing oxidative stress. The oxidative stress most likely triggers cell death by enhanced apoptosis corroborating with our gene expression data as shown below.

3.3. Immunoblotting

Apoptosis was identified as a significant cell process; some of the apoptosis related proteins were checked by immunoblotting such as BAK, BAX, BCL2, BCL2L1, Caspase 8 and Caspase 9. In cells treated with PCB-153, BAK had a slight downward trend, BCL2 was highly lowered, Bax, Caspase 8 and Caspase 9 which were responsible for inducing apoptosis was found to be significantly elevated (Fig. 7A). In PCB-77, Caspase 3 and Bax were elevated but BCL2 and Caspase 9 remained flat proving a lesser degree of mitochondrial control of apoptosis (Fig. 7B). This gene also had significantly high expression pattern in the microarray. However, their fold change was around two fold. Hence they were not mentioned in the table which only showed three fold or more up regulated genes.

3.4. A pathway leading to apoptosis

The automatically generated network of cell death, cytochrome P450 mediated metabolism and oxidative stress is given in Supplementary figures S4 and S5 along with relative importance of the different cell processes identified by Ingenuity Pathways (Fig.S6). From that complex network, the curated pathway showed that PCB-153 most likely induces apoptosis through tumor necrosis factor receptor superfamily member 6 (TNFRSF6) (FAS which are well known for inducing apoptosis). This in turn activated Caspase-8 (CASP8) Caspase-9 (CASP9) (Fig. 8A). Both lead to apoptosis, membrane blebbing and DNA fragmentation. These TNF superfamily receptors also activate Bcl2-like 1 (Bcl2L1), which acts pro6 apoptotic here. Bcl2L1 along with BH3 interacting domain death agonist (BID) inhibits B-cell leukemia/lymphoma 2 (Bcl2) which is anti-apoptotic thereby its effect is removed. TNFRSF6 also activated FBJ osteosarcoma oncogene (FOS) and JUN which in turn activated metallothionein family of proteins (MT1G and MT1X) as well as cytochrome P450 (CYP1A1). FOS also stimulates transforming growth factor, beta 1 (TGFβ1) and thioredoxin interacting protein (TXNIP). All of these in turn lead to oxidative stress. Similarly, PCB-77 (Fig. 8B) most likely induces apoptosis through Aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (ARNT) death receptor as previously known. As AHR and ARNT stimulates the Cytochrome P450 subfamily 1A1 (CYP1A1), its expression increases 84 fold in qRT-PCR. Some components of AKT1 signaling pathway and NFκB pathway are also activated. However, other mitochondrial apoptosis related proteins such as Bcl2 as well as other caspases such as Caspase 8 and Caspase 9 are not active. Hence, it is inferred that PCB-77 induced apoptosis is mainly through the nuclear pathway (Fig. 8B). ARNT also activates FOS which in turn activates GLI-Kruppel family member (GLI2), tumor necrosis factor (TNF), twist homolog 2 (TWIST2) as well as myelocytomatosis viral oncogene (MYC) and N-myc downstream regulated gene 1 (NDRG1). NADPH dehydrogenase quinone 1 (NDRG1) is also activated by FOS and CYP1A1. All these genes ultimately lead to oxidative stress. It is important to mention here that some validations have been done with real time PCR and immunoblotting for the genes present in these predicted pathways.

3.5. Fluorescent microscopy results

Fluorescent microscopy shows the chromatin condensation which appears as dense granules stained with Hoechst dye (Fig. 9). PCB-153 (Fig. 9A-F) and PCB-77 (Fig. 9G-L) induced chromatin condensation and membrane blebbing are marked with arrows. DNA fragmentation assay also showed the laddering pattern which has been published elsewhere (Ghosh et al., 2007).

4. Discussion

The current work addresses an important aspect of the congener specific action of PCBs from gene expression profiles to cellular function. The important outcome of the work is that it deals with the congener specificity of PCBs and the different groups of genes are induced at the early hours but ultimately leading to oxidative stress and cell death through apoptosis. However, as the gene expression profiles are different, it can certainly be hypothesized that the cells which do not die even in this high concentration may ultimately lead to other diverse adverse effects of PCBs. It is observed in the gene expression profile study that only a few hours of high exposure is required for inducing wide variety of genes and before the human body has excreted the principal amount of PCBs, the trigger for damage is already pulled. It should be pointed out that it is those cells which live after the induction with high concentration for quite short time is more damaging to certain organs. On the contrary, the death of cells leads to the main effects in other organs with low repairing capabilities, such as brain. Hence the acute effects of PCBs are because of cell death which is studied in this paper and chronically due to survival of the dysfunctional cells (Ghosh et al., 2007) which together has lead to the diverse effects of PCBs. PCB-153 was chosen as less is known about its mode of action compared to the dioxin like coplanar PCBs like PCB-77. However several studies have shown that it has a lot of deleterious effects on the different organ systems (Berberian et al., 1995; Hauser et al., 2005; Laden et al., 2002; Machala et al., 2003; Rusiecki et al., 2005). It has also been shown that PCB-77 works through Aryl hydrocarbon receptors (AhR) unlike the PCB-153 whose receptor is unknown (Vezina et al., 2004). From the gene expression profiles it can be hypothesized that tumor necrosis factor superfamily may be one of the major receptors in action. Hierarchical clustering using Hierarchical Clustering Explorer (HCE) (Zhao et al., 2003) software generated dendrograms shows time as the most important variable (Fig.S1). For the PCB-153 experiments, microarrays at 0, 30 minutes, and 18 hours had 3 replicates each while microarrays at 6 hours had two replicates due to improper hybridization. However the two other replicates were extremely well in quality control. Taqman real time PCR was used to validate some of the genes mostly with the high fold changes. However, a few like Caspase 8 and NQO1 having a lower fold change was also validated with Taqman real time PCR and it has comparable results with the microarray data. In fact, recently microarray quality control consortium has concluded that if the quality control steps are all maintained strictly the microarray data is as good as (and may be less noisier) than the real time PCR data (Canales et al., 2006; Shi et al., 2006). Hence, for this study only a few of the genes were validated from the top and the bottom of the list of significant genes. Some of the apoptosis related proteins have more dynamic changes than in the gene expression such as Caspase 8 and Caspase 9. Recent studies have revealed that stress responsive signal transduction pathways are strictly regulated by the intracellular redox state, which precisely balances between the levels of oxidizing and reducing equivalents, such as reactive oxygen species (ROS) and endogenous antioxidants. The generation of ROS fluctuates in response to alterations of both external and internal environment and, in turn, triggers specific signaling cascades, which determine cell survival or cell death (Hogberg et al., 2009). The oxidative stress found in environmental stress response (ESR_FIBROBLAST_UP) and in breast cancer patients (OXSTRESS_BREASTCA_UP) were common to both congeners. This may also suggest

the fact that PCB-77 and PCB-153 have the potential to form cancer (Chuang et al., 2002). However, as carcinogenesis is a multidimensional process it may be suppressed by other cellular processes. In our study the DNA damaging effects of PCB-153 were more pronounced as evident by gene set enrichment analysis (STRESS_GENOTOXIC_SPECIFIC_UP) as well as in DNA fragmentation assay (data not shown) and fluorescent microscopic studies (Fig. 9). Moreover, there is an enrichment of the STRESS_P53_SPECIFIC_UP in PCB-153 treated cells, similar to earlier published reports in (Amundson et al., 2005), proving that oxidative stress induced p53 specific DNA damage is present in these cells. These are however completely absent in the PCB-77 treated cells. In contrast, in PCB-77 treated cells the oxidative stress was similar to TPA induced stress STRESS_TPA_SPECIFIC_UP as well as stress induced by ionizing radiation STRESS_IONIZING_SPECIFIC_UP. In conclusion, our current proposed model is that PCB-153 and PCB-77 induce oxidative stress in the HepG2 cell line but through involvement of different gene sets and hence different sets of proteins. Due to the activation of a particular set of genes, apart from other effects, PCB-153 triggers the mitochondrial apoptotic pathway (Fig. 8A), whereas PCB-77 triggers the receptor mediated nuclear apoptotic pathway (Fig. 8B).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure-1.

Gene Set enrichment analysis plots showing the highest enriched genesets over all time points. The data were grouped into “Control” (time 0) and “Treated” (all other time points). The red portion in the left half of each graph shows the positive correlation with the PCB treated expression patterns. The height above the dotted line shows the strength of correlation. Panel A, B, C, & D shows the enrichment plots of PCB-153 for ESR_FIBROBLAST_UP, OXSTRESS_BREASTCA_UP, STRESS_GENOTOXIC_SPECIFIC_UP & STRESS_P53_SPECIFIC_UP respectively as mentioned in the Broad Institute molecular Signature database. Similarly, Panel E, F, G, & H shows the enrichment plots of PCB-77 for ESR_FIBROBLAST_UP,

OXSTRESS BREASTCA UP, STRESS TPA SPECIFIC UP & STRESS IONIZING SPECIFIC UP respectively as mentioned in the Broad Institute's Molecular Signature Database. Detailed description of the gene sets are mentioned in the text.

Figure-2.

PCB-153 (70µM) induced enriched genes having at least three fold changes in gene expression in at least in one time point. For the time points 'x' means more than 1.5 fold change. The maximal fold-change is mentioned in the figure.

Figure-3.

PCB-77 (70µM) induced enriched genes having at least three fold changes in gene expression in at least in one time point. For the time points 'x' means more than 1.5 fold change. The maximal fold-change is mentioned in the figure.

Figure-4.

Validation of the PCB-153 (70 μ M) induced expression microarray data with real time PCR. At all time points qRT-PCR data matches closely with the microarray data. In many cases, real time data however shows higher fold differences as it is more accurate. (mean $\pm$ SD, n=3)

Figure-6.

Comparison of cell death induction by PCB-153 and PCB-77 on HegG2 cells, alone or in combination of paraquat (PQ). Two-way ANOVA showed a significance level of P-value < 0.0001 for individual treatments as well as combined treatment with PCB and PQ compared to control at 1.5 to 24 hour time points and for combined treatment compared to individual treatments at the time points marked with *.

Figure-7.

This figure shows the changes in protein expression at different time points. Panel-A shows that in PCB-153 (70μM) treated cells, Bcl2L1 (Bcl-xs), Caspase-8, Caspase-9 (Pro & Active form) which are responsible for inducing apoptosis were found to be significantly elevated. Bcl2 and Bak were found to be down regulated which matches with microarray data. Panel B shows that in PCB-77 (70μM) treated cells, Caspase 3 and Bax were elevated but BCL2 and Caspase 9 remained flat proving a lesser degree of mitochondrial control of apoptosis. B-actin was used as control.

Figure-8.

A composite figure from all time points of microarray data showing apoptotic pathways in HepG2 cells when exposed to PCB-153 (70 μ M) and PCB-77 (70 μ M). —| negative direct regulation; → positive direct regulation; — binding; → change in expression; → protein modification;> indirect effect through chemicals, ion transport, opening of channels etc.). Figure drawn with PathwayAssist 3 software from Ariadne Genomics. A: PCB-153 most likely acts through membrane receptor leading to oxidative stress followed by apoptosis via mitochondrial pathway. B: PCB-77 most likely acts through membrane and nuclear receptor leading to oxidative stress followed by apoptosis via nuclear pathway.

Figure-9.

Fluorescent stained apoptotic studies with PCB-153 (70 μ M) and PCB-77 (70 μ M). A: Control; B: 4 hours with PCB-153; C: 8 hours with PCB-153; D: 12 hours with PCB-153; E: 18 hours with PCB-153; and F: 24 hours with PCB-153; G: Control; H: 4 hours with PCB-77; I: 8 hours with PCB-77; J: 12 hours with PCB-77; K: 18 hours with PCB-77; and L: 24 hours with PCB-77. (white arrow – chromatin condensation; black arrow – vacuole formation; A, B, G and H are healthy cells)