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Assessment of the roles of antioxidant enzymes and glutathione in 3,3',4,4',5-Pentachlorobiphenyl (PCB 126)-induced oxidative stress in the brain tissues of rats after subchronic exposure

Ezdiyar A. Hassoun and SeAnna Periandri-Steinberg

College of Pharmacy, University of Toledo, Toledo, OH 43606, U.S.A.

Abstract

The abilities of various doses of 3,3',4,4',5-pentachlorobiphenyl (PCB126) to induce changes in antioxidant enzyme activities and glutathione levels in the brain tissues of rats were examined in rats after subchronic exposure. Groups of rats were administered 10,30, 100, 300, 550 or 1000 ng PCB 126/kg/day, p.o., for 13 weeks and the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as (GSH) levels were determined in the brain tissue homogenates. Treatment resulted in significant and dose-dependent increases in the activities of the three tested enzymes. While maximal increase GSH-Px activity was achieved with a dose of 100-175 mg/kg/day, CAT and SOD activities continued to increase in response to maximal dose used for this study. GSH levels on the other hand, were suppressed significantly in a dose-dependent fashion. Data suggest that previously observed increase in oxidative stress production by PCB-126 in the brain tissues of rats is associated with dose-dependent rise in antioxidant enzyme activities and GSH depletion. However, the increases in the antioxidant enzyme activities can not provide full protection against oxidative damage induced by the same doses. In addition, GSH depletion plays a critical role in the previously observed oxidative stress in response to this compound.

Keywords

3, 3', 4, 4', 5-Pentachlorobiphenyl; PCB 126; superoxide dismutase; glutathione peroxidase; catalase; brain; rats; oxidative stress; subchronic

Introduction

The polychlorinated biphenyls (PCB) were first manufactured in 1929 and used widely as commercial mixtures in many industrial products. Although the industrial application of the PCB was discontinued in the late 1970s, their environmental existence raises long-term toxicological concerns. The compounds were found to produce many biochemical and toxic effects in lab animals and humans, including developmental and reproductive toxicity, hepatotoxicity and carcinogenesis (Ahlborg et al., 94; Fishbein, 1974; Kimbrough, 1994; Safe 1984, 1994). Exposures of adult and developing animals to the commercial mixtures or the individual compounds were found to result in various neurotoxic and neurobehavioral effects (Agrawal et al., 1981; Chou et al., 1979; Eriksson and Fredriksson, 1996; Eriksson et al., 1991, 2008; Gardiner, 2001; Kodvanti and Tilson, 1997; Kodavanti et al., 1998; Seegal, 1996; Seegal et al., 1990,¹⁹⁹¹,1994; Tilson et al., 1979, 1990; Widholm et al., 2001). Neurological disorders and neurobehavioral development, including hypotonia and hyperflexia, delay in

psychomotor development and poor visual recognition were observed among children and infants exposed to background levels of the compounds (Fein et al., 1984; Jacobson and Jacobson 1996; Jacobson et al., 1984, 1985; Patandin et al., 1999, Schantz et al., 2003; Stewart et al., 2000). Neurotoxicity and neurobehavioral changes, and alterations in brain dopamine levels were also found in adult humans exposed to the commercial mixtures of PCB. Schantz et al. (2001) assessed the impact of PCB and other fish-borne contaminants on the intellectual functioning in older adults and showed fish eaters with high blood PCB level have lower scores on several measures of memory and learning.

Structure-toxicity studies suggested two different classes of the PCB, coplanar and non-coplanar congeners. The coplanar congeners, including 3, 3',4, 4',5-pentachlorobiphenyl (PCB 126) are aryl hydrocarbon (Ah) receptor agonists. Coplanar congeners induce responses similar to those induced by 2, 3,7, 8-tetrachlorodibenzo-p-dioxin (TCDD), the prototype compound for the receptor, and are therefore known as the dioxin-like congeners (Safe, 1990, 1994). The relative potencies of different TCDD congeners, refer to as the toxic equivalency factors (TEF) are related to their binding affinities to the Ah receptor and the induction of various biochemical effects, with TCDD being considered the most toxic congener (Ahlborg et al., 1994; Safe, 1990; Van den Berg et al., 1998). The TEF for the coplanar congener, PCB 126 was estimated to be 0.1 (Safe, 1994; Van den Berg et al., 1998).

Non -coplanar congeners of the PCB have an ortho-chlorine substitution on the biphenyl rings, do not display significant binding affinity to the Ah-receptor, and are therefore known as the non dioxin-like congeners. *In vitro* experiments on neuronal cells demonstrated the abilities of the non-coplanar congeners to induce changes in calcium homeostasis and dopamine levels and suggested the non association of the PCB neurotoxicity with binding to the Ah-receptor. (Kodavanti and Tilson, 2000; Kodavanti et al. 1996; Mundy et al., 1999; Seegal et al., 1990; Shafer et al., 1996). Similarly, *in vivo* studies showed that repeated exposure to the PCB mixture, Aroclor 1254 can induce changes in Ca^{+2} buffering of the brain which are related to the presence of the non-coplanar ortho PCB congeners, and at levels equivalent to the concentrations that produced significant effects on neuronal cultures *in vitro* (Seegal et al., 1991; Kodavanti and Tilson, 2000).

While the fore-mentioned studies suggested the association of the neurotoxicity with the non coplanar congeners, other studies demonstrated developmental neurotoxic effects of the coplanar congeners as well. *In utero* exposure of developing mice to the co-planar PCB, 3,3', 4,4'-tetrachlorobiphenyl was found to produce neurobehavioral and neuropathological changes associated with altered levels of dopamine and changes in the density of dopamine receptors (Agrawal et al., 1981). A high frequency of motor dysfunction was observed in weaning mice whose dams were exposed to 3,4,3',4'- tetrachlorobiphenyl during a certain period of gestation (Chou et al., 1979). Seegal et al. (2005) demonstrated the estrogenic activity of coplanar PCB congeners, including PCB-126, and the association of that activity with the compounds'-induced elevation in dopamine in the prefrontal cortex of developing rats. Further, exposure to coplanar PCB, including PCB- 126 during a defined critical stage of neonatal brain development was found to affect learning and memory abilities, as well as the density of the nicotinic and muscarinic receptors in mice (Eriksson 2008; Eriksson and Fredriksson, 91; Eriksson et al., 1991).

Oxidative stress indicated by the production of reactive oxygen species (ROS), lipid peroxidation and DNA damage occurred in a dose-response fashion in brain tissues of rats after chronic exposure to PCB-126 (Hassoun et al., 2000, 2002). Lyng and Seegal (2008) demonstrated the production of oxidative stress by a mixture of PCB in an organotypic co-culture system of developing rat striatum and ventral mesencephalon. Oxidative stress was also found to be associated with the inhibitory effects of Aroclor 1254 on membrane bound

ATPase in select brain regions of rats (Sredivi et al., 2007). Association between oxidative stress and the induction of apoptotic death by 2, 2', 5, 5'-tetrachlorobiphenyl was also reported in human neuronal SK-N-MC cells (Lee et al., 2004).

The antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), as well as glutathione (GSH) are part of the antioxidant defense mechanism of the cell, and play important roles in the protection against the induction of oxidative stress, in response to different xenobiotics (Davies, 1995; Josephy, et al., 97). SOD, CAT, GSH activities and GSH level were found to be suppressed in select brain region of rats exposed to Aroclor 1254 for 30 days, and the effects could be reversed by co- administration of vitamin C (Muthuvel et al., 2006; Venkataraman et al., 2007). This study was designed to investigate the possible modulation of the antioxidant enzyme activities and GSH level in the brain tissues of rats, in response to chronic exposure to the co-planar congener PCB 126. In addition, the study assessed the contribution of those mechanisms to the previously observed oxidative stress induced by the compound in brain.

Materials and Methods

Chemicals—3,3',4,4',5-Pentachlorobiphenyl (PCB 126), was provided by the National Cancer Institute (NCI) chemical repository (Kansas City, MO) and was 99.5% pure. All chemicals used for various assays in this study were obtained from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade or of the highest grade available.

Animals and treatments—These studies were carried out using tissues provided to our lab by the National Toxicology Program (<http://ntp-server.niehs.nih.gov>) as part of a series of 2-year rat bioassays to examine the relative potencies for carcinogenicity of individual and mixtures of dioxin-like compounds. Harlan Sprague-Dawley female rats weighing 170-190 g were used for this study and were 8 weeks of age at the time of first exposure. Animals were given irradiated NTP-2000 pelleted feed supplied by Zeigler Brothers, Inc. (Gardners, PA) and were provided tap water *ad libitum*. PCB 126 was administered by gavage to various groups of rats (6 rats/group), daily for 13 weeks (5 days/week) at doses of 10, 30, 100, 175, 300, 550 or 1000 ng/kg/day. The doses were based on the current WHO-TEF values (Van den Berg et al., 1998), and the 13 weeks period represents the shortest period for chronic studies. Control groups were given the vehicle used to dissolve the chemicals (1% acetone in corn oil) and the volume rate of administration was kept at 2.5 ml/kg body weight. The animals were euthanized at the end of the exposure period using carbon dioxide asphyxiation, brains were collected and immediately frozen in liquid nitrogen, and were then stored at -80°C until they were used for various biochemical assays.

Tissue samples preparation for various assays—The brain of each animal was divided into two portions, weighed and placed in either sucrose buffer containing 0.32 M sucrose, 1 mM EDTA, and 10 mM Tris-HCL, or in 5% sulfosalicylic acid (Jiang et al., 1998). The samples were homogenized using a Potter-Elvehjem homogenizer (Wheaton Science Products, Millville, NJ, USA), fitted with Teflon pestle to produce 10 and 20% (w/vol) homogenates in sucrose buffer and 5% sulfosalicylic acid, respectively. Sucrose buffer homogenates were centrifuged at 8000g for 30 min and the supernatants were used for the determination of SOD, CAT and GSH-Px activities. Sulfosalicylic acid homogenates were centrifuged at 1200g for 5 min and the supernatants were used for the determination of GSH.

Determination of SOD activity—SOD activity in brain tissues was determined according to the method of Marklund and Marklund (1974), which is based on the inhibition of pyrogallol autooxidation by SOD. An aliquot of the supernatant (200 µl) was mixed with 750 µl Tris-cacodylic buffer, containing 50 mM Tris-HCL, 50 mM cacodylic acid and 1 mM EDTA, pH

8.2. Pyrogallol solution (250 μ l of 2 mM) was then added to each sample and absorbance of mixtures was recorded at 420 nm, immediately and then every 30 sec for 3 min, using a Spectronic 20 spectrophotometer. Changes in the rates of absorbances were calculated and converted into units of SOD activity per mg protein, where one unit is equivalent to the quantity of SOD that is needed to produce 50% inhibition of pyrogallol autooxidation (Marklund and Marklund, 1974).

Determination of CAT activity—CAT activity was determined according to the method of Cohen et. al. (1970), with modification. The method was based on enzyme-catalyzed decomposition of H_2O_2 by potassium permanganate ($KMnO_4$) and had three components that were assayed simultaneously, the blank, standard and sample. The sample, blank and the standard tubes contained 100 μ l of supernatant, sucrose buffer and deionized water, respectively. One ml of H_2O_2 (6mM) was added to the sample and blank tubes and 1 ml of deionized water was added to the standard tube. The tubes were vortexed and placed on ice for 3 min and then 200 μ l of 6N H_2SO_4 was added to each tube to stop the reaction. $KMnO_4$ (2 mM) was then added to each tube at a volume of 1.4 ml, and absorbance was immediately recorded at 480 nm, using a Spectronic-20 spectrophotometer. Absorbance were converted into units of CAT/mg protein, where one unit is equal to $k/0.00693$. The first order reaction rate constant (k) is equivalent to $\log (S_0/S_2) \times (2.3/t)$. The log conversion factor is 2.3 and t was equivalent to the incubation time (3 min). S_0 and S_2 were determined by subtracting the blank absorbance from the standard absorbance, and the sample absorbance from the standard absorbance, respectively (Jiang et al., 1998).

Determination of glutathione peroxidase (GSH-Px) activity—GSH-Px activity was determined according to the method of Lawrence and Burk (1976) with modification. An aliquot of supernatant (100 μ l) was mixed with 700 μ l of the reaction mixture containing 1 mM EDTA, 1 mM NaN_3 , 0.2 mM NADPH and 1 mM reduced GSH in phosphate buffer saline, pH 7.2, and 100 μ l of glutathione reductase solution, containing 10 units. The tubes were incubated for 5 min at room temperature and then, 100 μ l of 0.2 mM H_2O_2 was added to each tube to initiate the reaction. The absorbance was recorded at 340 nm, immediately and then every 30 sec over a period of 3 min, using a Spectronic-20 spectrophotometer. Changes in the rate of absorbance was converted into nmol of NADPH oxidized/min/mg protein, using an extinction coefficient of $6.22 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$ (Jiang et al., 1998)

Determinaion of glutathione (GSH)—GSH equivalents were determined by the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)-GSSG reductase recycling assay of Anderson (1985), which was based on the reduction of oxidized glutathione (GSSG) to GSH by the action of glutathione reductase in the presence of NADPH, and the stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB) from DTNB. The assay required the stock buffer, containing 143 mM sodium phosphate and 6.3 mM tertra sodium EDTA, pH 7.5, the daily buffer, containing 0.248 mg/ml NADPH in stock buffer and GSSG reductase solution containing 266 units of the enzyme/ml of stock buffer. The assay mixture contained 10 μ l of supernatant, 700 μ l daily buffer, 100 μ l DTNB solution, 190 μ l deionized water. The mixtures were incubated at 30°C for 15 min, followed by the addition of 10 μ l of GSSG-reductase solution. Absorbance of the mixture was recorded at 412 nm immediately and then every 30 sec for 3 min, using a Spectronic20 spectrophotometer. A standard curve for GSH was obtained with GSH concentrations ranging from 1-4 nmole.

Determination of protein—Protein concentrations of the tissue homogenates according to the standard method of Lowry et. al. (1951), using bovine serum albumin as the standard.

Data analyses and statistical methods—Data were analyzed using SAS statistical package and the Microsoft Excel data analysis tool package. Each data point represents the mean of 6 animals + SD. A one-way Analysis of Variance (ANOVA) followed by Duncan's multiple range post hoc test to determine the significance in the differences among different groups, and a significance level of $p < 0.05$ was employed.

Data presented in the figures for each biomarker, including the control and various treatment groups were pooled and correlated with each other. Pearson's correlation coefficients were calculated for pairs of biomarkers, using the Microsoft Excel data analysis tool.

Results

The effects of various doses of PCB 126 on SOD activity in brain tissues are presented in Figures 1. Significant increases of approximately 1.7-4 fold in enzyme activity were observed with doses ranging between 30-1000 ng/kg/day compared with control. With the exception of 175 and 300 ng/kg/day doses where the induced rise was not significantly different from each other, all of the observed increases were dose-dependent, with maximal elevation achieved at a dose of 550 ng/kg/day.

Figure 2 presents the effects of various doses of PCB-126 on CAT activity in the brain tissues. PCB-126 administration to the rats at doses ranging from 30-1000 ng/kg/day resulted in significant increases in CAT activity of the brain tissues. With the exception of the 175 and 300 ng/kg/day doses that resulted in similar elevations in CAT activity, the observed rises were dose-dependent and were approximately 1.2-2 fold compared with control.

The effects of various doses of PCB 126 on GSH-Px activity in the brain tissues are presented in Figure 3. Treatment levels ranging between 30-1000 ng/kg/day resulted in significant elevations in GSH-Px activity compared with control. The observed increases were dose-dependent and maximal elevation achieved at doses of 100-175 ng/kg/day. The overall increases in GSH-Px activity as induced by different doses of the compound were estimated to be 1.3-2.4 fold compared with control.

Figure 4 presents the effects of various doses of PCB-126 on glutathione level in the brain tissues of rats. Exposure of rats to the compound at doses ranging from 30-1000 ng/kg/day resulted in significant suppression of GSH levels. The observed decreases were dose-dependent, with maximal effect achieved at a dose of 550 ng/kg/day. An overall suppression of approximately 1.7-3.3 fold was observed with various doses.

Table 2 indicates the calculated Pearson's correlation coefficients between various antioxidant enzymes and GSH levels, as modulated by PCB 126 in the brain tissues. Values approximating 1 indicate strong correlations, and negative values indicate inverse correlations between the two biomarkers. Strong correlations were demonstrated between SOD-CAT and SOD-GSH-Px. Strong inverse correlation was also found between GSH and GSH-Px.

Discussion

Previous studies in our lab demonstrated significant, and dose-dependent production of different biomarkers of oxidative stress, including, superoxide anion, lipid peroxidation and DNA damage in the brain tissues of rats after subchronic exposure to selected doses of PCB 126 (Hassoun et al., 2000). In order to assess the roles of the antioxidant enzyme activities and GSH level in the induction of those biomarkers, the same tissues were used for the assays conducted in this study. PCB 126 administration to rats resulted in dose-dependent increases in SOD, CAT and GSH-Px activities in the brain tissues. In an effort to adapt to oxidative stress induced by various xenobiotics, eukaryotes and prokaryotes are able to up-regulate their

antioxidant defense mechanisms in response to low concentrations of those xenobiotics (Davies, 1995). In addition, studies by Schlezinger and Stegeman (2001) demonstrated significant increases in hepatic antioxidant enzyme activities of the marine fish scup, in response to a low but not a high dose of PCB-126. Therefore, the observed rise in the enzyme activities are likely due to up-regulation of those enzymes in response to the low doses of the compound used for this study.

SOD is responsible for superoxide anion (SA) dismutation (Davies, 1995), and previous studies in our lab found significant production of this species in brain (Hassoun, 2000). Data indicated that the observed increases in SOD activity were not sufficient to provide full protection against SA over production in response to PCB-126. Although SOD results in SA dismutation and production of the more toxic ROS, H_2O_2 , the enzyme acts in concert with CAT and GSH-Px, where the latter two convert H_2O_2 to water molecules (Davies, 1995; Josephy et al., 1997). The results of this study indicated concerted increases in the activities of the three enzymes in response to PCB 126 doses of 30-1000 ng/kg/day. The concerted action of the enzymes is affirmed by the strong Pearson's correlation coefficients found between SOD-CAT and SOD-GSH-Px. However, the overall increases in SOD activity were 2-4 fold, while those of CAT and GSH-Px were 1.2-2 and 1.3-2.4 fold, respectively. Thus, the increases in SOD are greater than those of CAT and GSH-Px. These observations may indicate H_2O_2 overproduction in response to the significant rise in SOD activity, and the combined contribution of CAT and GSH-Px to the process of H_2O_2 inactivation.

Lipid peroxidation and DNA damage were previously shown to be induced in the same tissues used for this study (Hassoun et al., 2000). These observations may indicate the partial protective action of the enzymes against ROS over production in response to the compound and if the activities of those enzymes did not increase, significantly more SA and H_2O_2 would be produced, and more LP and DNA damage would have been induced in response to those ROS. These findings may also indicate that the previously observed increases in LP and DNA damage were not only due to overproduction of SA, but also to H_2O_2 over production. While maximal elevation in GSH-Px activity was achieved with doses of 100-175 ng/kg/day, CAT activity was continued to increase in response to the higher doses. This may indicate the better contribution of CAT, than GSH-Px to the process of H_2O_2 inactivation. The suggested contributions of H_2O_2 and CAT to the induction of oxidative stress by PCB 126 is supported by the studies of Ryu et al. (2003) demonstrating the role H_2O_2 in PCB-induced growth inhibition of sensitive yeast cells, as well as the significant contribution of CAT, but not GSH-Px to the protection of non sensitive cells against that effect.

GSH is an essential substrate for GSH-Px, and plays important roles in the protection of proteins against oxidation and cross-linking, and in scavenging of different free radicals (Davies, 1995; Josephy, 1997; Reed, 1994; Nakumura et al., 1997). A dose-response suppression of GSH levels shown in this study indicates depletion of the molecule in response to PCB 126. The role of GSH in GSH-Px action and depletion of the molecule in response to the increases in GSH-Px activity are clearly indicated by the strong but inverse correlation between the two biomarkers. Depletion of the GSH may also result from direct conjugation of the molecule with electrophiles or redox cycling, or indirectly through inhibition of its biosynthesis and regeneration (Reed, 1994). Although the nature of the electrophiles or redox cycling associated with PCB 126 exposure are not identified, studies by Seegal et al. (2005) found significant elevation of dopamine (DA) concentrations in the prefrontal cortex of the developing rats, in response to the compound. In addition, Lyng et al., (2008) demonstrated the role PCB-induced alterations in the vesicular storage of DA and the increased levels of unsequestered DA with the induction of oxidative stress and GSH depletion in neuronal tissues in culture. Furthermore, chronic exposure of rats to TCDD at doses that correspond to those used for PCB 126 in this study, when considering the TEF, was found to result in the elevation of DA and norepinephrine

in certain brain regions (Byers et al, 2006). When metabolized, DA and phenol-containing neurotransmitters generate free radicals that have prooxidant activity (Siraki and O'Brien, 2002). While those metabolites may contribute directly to GSH depletion in response to PCB 126, the possible indirect effect of PCB 126 on GSH biosynthesis needs to be further investigate.

The above discussion leads to the conclusion that antioxidant enzyme activities are significantly increased in response to different doses of PCB-126. However, the increases are not sufficient to provide full protection against PCB 126-induced oxidative stress. In addition, GSH depletion in response to PCB 126 exposure plays an important role in the PCB 126-induced production of oxidative stress in brain tissues. Since changes in the biomarkers of oxidative stress are known to be associated with various pathological and behavioral effects (Davies, 1995), the results of the present study, together with previous findings of Hassoun et al (2000) who suggested production of long term toxic and/or behavioral effects produced by coplanar PCB congeners. Previous studies on the induction of oxidative stress by TCDD demonstrated brain region selectivity after chronic exposure to doses that correspond to the PCB-126 doses used for this study (Hassoun et al, 2003). Therefore, the results of the present study may be used as basis for further investigation of possible induction of those effects by coplanar congeners of PCB in select brain regions of the animals, and also behavioral effects associated with those changes.

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Figure 1.

SOD activity in the brain tissues of rats after subchronic exposure to various doses of PCB 126. Each point indicates the mean $\pm$ S.D of 6 samples. Values that do not share any identical superscript are significantly different ($p < 0.05$).

Figure 2.

CAT activity in the brain tissues of rats after subchronic exposure to various doses of PCB 126. Each point indicate the mean $\pm$ S.D of 6 samples. Values that do not share any identical superscript are significantly different ($p < 0.05$).

Figure 3. GSH-Px activity in the brain tissues of rats after subchronic exposure to various doses of PCB 126. Each point indicates the mean $\pm$ S.D of 6 samples. Values that do not share any identical superscripts are significantly different ($p<0.05$).

Figure 4. GSH levels in the brain tissues of rats after subchronic exposure to various doses of PCB 126. Each point indicates the mean $\pm$ S.D of 6 samples. Values that do not share any identical superscript are significantly different ($p < 0.05$).

Table 1

The calculated Pearson's correlations coefficients

<u>Biomarkers</u>	<u>Pearson's correlation coefficient</u>
SOD-CAT	0.95
SOD-GSH-Px	0.80
GSH-GSH-Px	- 0.82