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N-acetylcysteine (NAC) diminishes the severity of PCB 126 – induced fatty liver in male rodents

Ian K. Lai^{*,†}, Kiran Dhakal^{*,†}, Gopi S Gadupudi^{*,†}, Miao Li^{*,†}, Gabriele Ludwig^{*,†}, Larry W. Robertson^{*,†,§}, and Alicia K. Olivier[‡]

^{*}Interdisciplinary Graduate Program in Human Toxicology, University of Iowa

[†]Department of Occupational and Environmental Health, College of Public Health, University of Iowa

[‡]Department of Pathology, University of Iowa

Abstract

Potent aryl hydrocarbon receptor agonists like PCB 126 (3,3',4,4',5-pentachlorobiphenyl) cause oxidative stress and liver pathology, including fatty liver. Our question was whether dietary supplementation with N-acetylcysteine (NAC), an antioxidant, can prevent these adverse changes. Male Sprague-Dawley rats were fed a standard AIN-93G diet (sufficient in cysteine) or a modified diet supplemented with 1.0% NAC. After one week, rats on each diet were exposed to 0, 1, or 5 $\mu\text{mol/kg}$ body weight PCB 126 by ip injection (6 rats per group) and euthanized two weeks later. PCB-treatment caused a dose-dependent reduction in growth, feed consumption, relative thymus weight, total glutathione and glutathione disulfide (GSSG), while relative liver weight, glutathione transferase activity and hepatic lipid content were dose-dependently increased with PCB dose. Histologic examination of liver tissue showed PCB 126-induced hepatocellular steatosis with dose dependent increase in lipid deposition and distribution. Dietary NAC resulted in a reduction in hepatocellular lipid in both PCB groups. This effect was confirmed by gravimetric analysis of extracted lipids. Expression of CD36, a scavenger receptor involved in regulating hepatic fatty acid uptake, was reduced with high dose PCB treatment but unaltered in PCB-treated rats on NAC-supplemented diet. These results demonstrate that NAC has a protective effect against

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[§]Corresponding Author: Larry W. Robertson, Ph.D., M.P.H., Professor, Department of Occupational and Environmental Health, The University of Iowa, College of Public Health, 100 Oakdale Campus #219 IREH, Iowa City, Iowa 52242-5000, Phone: 319-335-4346, Fax: 319-335-4225, larry-robertson@uiowa.edu.

Complete Contact information for all authors:

Ian K. Lai, Ph.D., Department of Occupational and Environmental Health, The University of Iowa, College of Public Health, 100 Oakdale Campus #121 IREH, Iowa City, Iowa 52242-5000, Phone: 408-507-1308; Fax: 319-335-4290, ian-lai@uiowa.edu

Miao Li, Department of Occupational and Environmental Health, The University of Iowa, College of Public Health, 100 Oakdale Campus #121 IREH, Iowa City, Iowa 52242-5000, Phone: 520-205-1368; Fax: 319-335-4290, miao-li@uiowa.edu

Kiran Dhakal, BVSc, MPH, Department of Occupational and Environmental Health, The University of Iowa, College of Public Health, 100 Oakdale Campus #121 IREH, Iowa City, Iowa 52242-5000, Phone: 573-529-2187; Fax: 319-335-4290, Kiran-dhalal@uiowa.edu

Gabriele Ludwig, Ph.D., Professor, Department of Occupational and Environmental Health, The University of Iowa, College of Public Health, 100 Oakdale Campus #2 IREH, Iowa City, Iowa 52242-5000, Phone: 319-335-4650 Fax: 319-335-4290, gabriele-ludwig@uiowa.edu

Larry W. Robertson, Ph.D., M.P.H., Professor, Department of Occupational and Environmental Health, The University of Iowa, College of Public Health, 100 Oakdale Campus #219 IREH, Iowa City, Iowa 52242-5000, Phone: 319-335-4346, Fax: 319-335-4225, larry-robertson@uiowa.edu

Alicia Olivier, DVM, PhD, *Diplomate*, ACVP, Assistant Professor, Department of Pathology, University of Iowa, 500 Hawkins Drive, 1163ML, Iowa City, IA 52242, Ph: (319) 384-4668, Fax (319) 335-8453, alicia-olivier@uiowa.edu

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hepatic lipid accumulation in rats exposed to PCB 126. The mechanism of this protective effect appears to be independent of NAC as a source of cysteine/precursor of glutathione.

Keywords

CD36; fatty liver; PCB 126; dietary supplementation; growth; CYP1A1

1. Introduction

The clinical benefits of N-acetylcysteine (NAC) are well documented (Parcell, 2002). These benefits accrue due to NAC's functionality as an antioxidant, a free radical scavenger, an exogenous source of cysteine/precursor of glutathione, and to other as yet unidentified mechanisms (Atkuri *et al.*, 2007; Dodd *et al.*, 2008).

Polychlorinated biphenyls (PCBs), originally manufactured commercially for industrial applications, were appreciated for their insulating and flame resistant properties (Safe, 1994). Industrial PCB mixtures of the 209 individual PCB congeners were widely used since the 1930s, which continued until the late 1970s in the USA, at which time their manufacture as commercial products was discontinued due to increasing environmental and health concerns. The lipophilicity and persistence of PCBs in the environment resulted in their bioaccumulation and biomagnification, effects of which are still felt to the present day (Hansen, 1987; Consonni *et al.*, 2012). The bioaccumulative and toxic effects of PCBs vary greatly depending on the chlorination patterns of the specific congeners. One approach to evaluating the toxicity of complex PCB mixtures is to identify the spectra of adverse effects and biochemical changes elicited by individual PCB congeners (Silberhorn *et al.*, 1990; Ludewig *et al.*, 2007).

Research with rodents demonstrated that, like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3,3',4,4',5-pentachlorobiphenyl (PCB 126) binds with high avidity to the aryl hydrocarbon receptor (Bandiera *et al.*, 1982), induces cytochromes P-450 (CYP), namely CYP1A1/2 (Parkinson *et al.*, 1983), and elicits these effects at much lower doses than other PCB congeners. Aside from the plethora of changes in gene expression, PCB 126 causes a wasting syndrome, severe thymic involution with loss of cortical lymphocytes, and liver enlargement with fatty change (Parkinson *et al.*, 1983; Lai *et al.*, 2010). Biochemical changes, aside from the efficacious induction of CYP1A proteins, include a reduction in hepatic glutathione (Lai *et al.*, 2010), loss of activity of the antioxidant enzyme selenium-dependent glutathione peroxidase (Schramm *et al.*, 1985), loss of hepatic selenium and zinc, and an increase of the pro-oxidant copper (Lai *et al.*, 2010).

The overexpression of hepatic CYP1A, especially in the presence of reducing equivalents and absence of an oxidizable substrate, is thought to be related to the toxic sequelae seen, in that during the catalytic cycle, CYP1A releases reactive oxygen species (ROS) as oxygen is only partially reduced (Schleizinger *et al.*, 2006). Another line of reasoning posits that the mitochondria are the source of ROS. This argument is buttressed by the observation that PCBs increased steady-state levels of superoxide that were found by confocal microscopy to be primarily located in the mitochondria (Zhu *et al.*, 2009).

Several attempts have been undertaken to ameliorate the adverse effects of halogenated biphenyls with dietary interventions, for example with fat substitutes like olestra (Jandacek *et al.*, 2010), minerals like selenium (Stemm *et al.*, 2008; Lai *et al.*, 2011) and manganese (Wang, submitted), various antioxidants (Robertson *et al.*, 1983; Tharappel *et al.*, 2008), or phytochemicals (Glauert *et al.*, 2008). Generally these dietary manipulations resulted in only marginal success. Zhu and coauthors (Zhu *et al.*, 2009), and Slim and co-authors (Slim *et al.*,

2000) found that NAC supplementation did reduce the toxicity of PCBs in human breast and prostate epithelial cells and in porcine vascular endothelial cells in culture. Therefore, our hypothesis is that dietary NAC supplementation will reduce the toxicity caused by PCB 126 *in vivo*.

2. Methods and Materials

2.1 Chemicals

All chemicals were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO) unless otherwise stated. PCB 126 (3,3',4,4',5-pentachlorobiphenyl) was prepared by an improved Suzuki-coupling method of 3,4,5-trichlorobromobenzene with 3,4-dichlorophenyl boronic acid utilizing a palladium-catalyzed cross-coupling reaction (Luthe *et al.*, 2009). The crude product was purified by aluminum oxide column and flash silica gel column chromatography and recrystallized from methanol. The final product purity was determined by GC-MS analysis to be > 99.8% and its identity confirmed by ¹³C NMR. *Caution: PCBs and their metabolites should be handled as hazardous compounds in accordance with NIH guidelines.*

2.2 Animals

This animal experiment was conducted with approval from the Institutional Animal Care and Use Committee of the University of Iowa. Male Sprague-Dawley rats weighing 75–100 grams from Harlan Sprague-Dawley (Indianapolis, IN) were housed in individual wire cages in a controlled environment maintained at 22 ° C with a 12 h light-dark cycle and water ad libitum. Animals were randomly divided into two dietary groups, and were fed ad libitum an AIN-93G diet or an AIN-93G based diet supplemented with 1.0% NAC (Table 1) purchased from Harland Teklad (Madison, WI). After one week, animals were given a single i.p. injection of vehicle (stripped corn oil; 5 mls/kg body weight; Acros Chemical Company, Pittsburgh, PA), or vehicle with 1 µmol/kg body weight (326 µg/kg body weight) or 5 µmol/kg body weight (1.63 mg/kg body weight) of PCB 126 (6 rats per dose). These doses were chosen based on a previous study in which a 1 µmol/kg dose of PCB 126 was shown to elicit mild fatty liver (Lai *et al.*, 2010). Animals were weighed and feed consumption determined two times per week. Two weeks following the PCB treatment rats were euthanized using carbon dioxide asphyxiation followed by cervical dislocation. The two week time period was shown to be sufficient for development of pathology in PCB 126-treated rats (Lai *et al.*, 2010). Livers and other organs were excised, weighed, and further processed as described below.

2.3 Hepatic subcellular fractions preparation

Liver tissues were excised immediately following euthanization, and homogenized in ice-cold 0.25 M sucrose solution, adjusted to pH 7.4. The homogenates were centrifuged at 10,000g for 20 min. The resulting supernatants were then centrifuged at 100,000g for 1 h. These supernatants, which contain the cytosolic fractions, were dispensed and aliquoted. The microsomal pellets were washed twice with cold sucrose solution and resuspended in that solution. Protein concentrations were determined by the method of Lowry et al. (1951).

2.4 Measurement of CYP1A1 activity

CYP1A1 activity was determined in hepatic microsomal fractions by the methods of Burke and Mayer (1974) with slight modifications, measuring the ethoxyresorufin deethylase (EROD) activity and using ethoxyresorufin as the substrate. The resulting fluorescent resorufin product from the monooxygenase reaction was detected using a Perkin-Elmer LS 55 spectrofluorometer at excitation wavelength of 550 nm and emission wavelength of 585 nm.

2.5 Glutathione analysis

Total glutathione levels in hepatic 100,000xg supernatants were determined by the methods of Griffith (1980) and Anderson (1985). Absorbance change at 412 nm over 5 minutes was measured in a Beckman DU-670 spectrophotometer. The rate of yellow color accumulation is the result of thionitrobenzoate formation from 5,5'-dithio-bis-(2-nitrobenzoic acid) proportional to the amount of total glutathione in the sample. Glutathione disulfide (GSSG) was measured independently by incubating the supernatants in the presence of 2-vinylpyridine, which conjugates reduced glutathione (GSH), followed by the determination of the remaining glutathione equivalents as described above. Glutathione levels are expressed as per mg protein.

2.6 Glutathione transferase (GST) activity

GST activity was determined in hepatic cytosolic fractions by the method of Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. The absorbance change at 340 nm caused by the conjugation of CDNB to reduced glutathione was followed in a Beckman DU-650 spectrophotometer for 5 min.

2.7 Histology and Special Stains

Liver sections were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Additionally sections were stained with Rhodamine for copper and periodic acid-Schiff (PAS) for glycogen (Sheehan and Hrapchak, 1987). Sections were immunostained for myeloperoxidase (MPO) with a rabbit polyclonal antibody (DAKO A0398) to detect neutrophils. Briefly, liver sections were cut at 4 μ m and antigen unmasking was performed in citrate buffer (pH 6.0) for 3 $\times$ 4 min in the microwave (1000 watts). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide and nonspecific background staining was blocked using background buster reagent (Innovex Biosciences, Richmond, CA). Slides were incubated with the primary antibody (1:1000) for 30 min at room temperature. The slides were then washed with buffer followed by application of DAKO rabbit Envision HRP System reagent for 30 min, washed again and then developed with DAKO DAB Plus for 5 min. Slides were counterstained with Surgipath hematoxylin, dehydrated and coverslipped.

2.8 Lipid Staining and Quantification

Formalin fixed liver sections were stained for lipid using osmium tetroxide (Luna, 1992). Samples were placed in a potassium dichromate (5%)/osmium tetroxide (2%) solution in water overnight. Samples were washed for 2 hours in running tap water and then processed normally and embedded in paraffin. Sections were cut at 4 μ m and baked in a 60°C oven overnight. Slides were cooled, deparaffinized and counterstained with nuclear fast red for 5 minutes. Slides were then dehydrated and coverslipped. Osmium stained slides were examined with a high resolution microscope (BX51, Olympus), digital images collected at 100X magnification (DP72, Olympus) and analyzed using Image J software (Image J, NIH). Images were converted to an RGB stack and thresholded in the red channel. The percentage of cellular staining was calculated by dividing the stained area by the total parenchymal area.

2.9 Lipid Extraction and Quantification

Frozen liver tissue (0.5 g) was homogenized in a solution consisting of 1.5 mL Millipore water and 2 mL methanol in a 15 mL glass tube with a Teflon lined screw cap. A solution of 2 mL methanol and 2 mL chloroform was added and the contents shaken. A second solution of 2 mL water and 2 mL chloroform was added, vortexed and centrifuged for 5 minutes at 1,800g to separate the phases. Following centrifugation, the lower layer was removed and 2

mL chloroform was added. Centrifugation and removal of lower layer was repeated twice. The lower layers were combined and washed once with 2 mL 1M KCl and once with 2 mL water. The extract was evaporated to nearly dryness under gentle stream of nitrogen, transferred to pre-weighed 2 mL glass vials and drying was continued in a fume hood overnight. The vials were then transferred to a desiccator and the extracted lipids were dried to a constant weight. This method is described in detail at the Kansas State University website at <http://www.k-state.edu/lipid/lipidomics/animal-lipid-extraction.htm>.

2.10 CD36 Gene Expression analysis

Total RNA of each rat liver sample was extracted using the RNeasy extraction kit from Qiagen Inc. (Valencia, CA). Briefly, 20–30 mg of liver tissue was homogenized and subjected to RNA extraction as described in the manufacturer's protocol. Absorbance of the isolated RNA was determined spectrophotometrically at 260 and 280 nm. RNA samples with purity ratios (A260/A280) between 1.8 and 2.0 were used for generating cDNA samples with the high-capacity cDNA reverse transcription kit from Applied Biosystems Inc. (Foster City, California) as described in their protocol. One µg of RNA template from all samples was used to generate an equivalent amount of cDNA in 20 µl of reverse transcription PCR (RT-PCR) reaction.

Consequently, the real-time qPCR analysis was performed at an optimized cDNA template concentration of 100 ng in a 20 µl reaction volume. The reaction was performed using SYBR Green Master Mix kit supplied by Applied Biosystems Inc. (Foster City, CA). An optimum primer concentration of 300 nM for both forward and reverse primers was used with no observed primer dimers. The primers used in the studies were synthesized by Integrated DNA Technologies Inc. (Coralville, IA) according to the sequence previously described (Fitzsimmons, 2010). The specificity of the primers was also verified using NCBI-primer BLAST tool. The sequences of the primers used were: CD36 (gene of interest): F- 5' TGCTGCACGAGGAGGAGAAT 3', R- 5' GCACCAATAACGGCTCCAGTA 3'; 36B4 (housekeeping): F- 5' AGATGCAGCAGATCCGCAT 3', R- 5' GGATGGCCTTGCGCA 3'. Each sample was analyzed in duplicate.

The amplification reaction was performed with an Eppendorf RealPlex2 Mastercycler® (Hamburg, Germany) using a program that started at 95° for 10 min followed by 40 cycles of two step PCR cycle at 95° for 15 s and 60° for 1 min. Subsequently, a melting curve analysis was performed to verify any false amplification/contamination by primer dimers or non-specific binding. The expression level of each gene in a given sample was corrected with respect to the mean of the control experimental group (corn oil 5ml/Kg;-NAC). The relative gene expression level of CD36 against the housekeeping gene for ribosomal protein 36B4 was calculated using the Pfaffl method (Pfaffl, 2001). The final result for each experimental group was expressed as mean relative fold change of CD36 with respect to housekeeping gene 36B4 (CD36/36B4).

2.11 Statistics

The effect of PCB 126 treatment and NAC on various responses was studied using ANOVA analysis via procedure PROC GLM in the statistical analysis package SAS (version 9.2). Dunnett's test was used to compare PCB 126 treatment with the corn oil control and NAC. This comparison was conducted separately for PCB 126 treatment and NAC level (one-way ANOVA) and also jointly (two-way ANOVA) (Table 2). In two-way ANOVA, the interaction term was removed if it was not significant at level 0.05. The effect of NAC was controlled when applying Dunnett's test to PCB 126 exposure by using lsmeans statement in PROC GLM. The same was done when applying Dunnett's test to NAC level.

3. Results

3.1 Effects on body weight, feed consumption, and organ weights

PCB126 at high dose (5 $\mu\text{mol/kg}$) significantly lowered the weight gain in animals (Figure 1A). NAC supplementation had no overall significant effect. However, the growth rate in the low (1 $\mu\text{mol/kg}$ PCB) dose-treated rats supplemented with NAC was significantly lower than for rats on the control diet. Feed consumption was decreased by both PCB 126 doses, but in the low PCB 126-dose this effect was statistically significant only in the NAC-supplemented group (Figure 1B). NAC alone did not influence feed consumption. Relative liver weights were significantly increased in a dose-dependent manner by PCB 126, but not by NAC (Figure 2A). Relative thymus weights were significantly decreased in a dose-dependent manner by PCB 126 (Figure 2B). Even though the decrease was small at each data point, two-way Anova showed that overall there was a significant decrease in relative thymus weight caused by NAC supplementation (Figure 2B and Table 2).

3.2 Effects on EROD activity (Measurement of CYP1A1 activity)

PCB 126 is a potent inducer of CYP1A1. EROD activity was significantly increased by PCB 126 with the highest induction observed at the lower PCB dose (1 $\mu\text{mol/kg}$) (Figure 3). NAC neither increased nor diminished the CYP1A1 activity.

3.3 Effects on total glutathione and glutathione disulfide (GSSG)

Hepatic total glutathione was significantly diminished in a dose-dependent manner by PCB 126 and in addition, NAC supplementation also significantly diminished total glutathione overall, although this effect was not significant at the level of individual PCB-treatment groups (Figure 4A and Table 2). The effects on GSSG were similar but more pronounced for NAC treatment, with a significant dose-dependent decrease in the PCB 126 groups and additional significant decreases with NAC supplementation (Figure 4B and Table 2). The GSSG-to-GSH ratio, an indicator of oxidative stress, was not changed by PCB 126, but was significantly lower in NAC supplement rats (Figure 4C and Table 2).

3.4 Effects on hepatic glutathione transferase (GST) activity

Hepatic GST activity was significantly increased in a dose-dependent manner by PCB 126 (Figure 4d). The corn oil control rats on the NAC-supplemented diet had a significantly lower GST activity compared to those receiving the control diet without NAC. However, no effect of dietary NAC on GST activity was seen in the PCB-treated rats (Figure 4D).

3.5 Histology

Livers from rats treated with PCB 126 had hepatocellular vacuolation that varied in distribution and severity. All rats treated with PCB 126 had well defined cytoplasmic vacuoles of varying sizes confirmed to be lipid with osmium tetroxide staining (Figures 5 and Supplemental Figure 1). In the low (1 $\mu\text{mol/kg}$) dose group, lipid was predominantly found within centrilobular to midzonal hepatocytes while in the high (5 $\mu\text{mol/kg}$) dose group lipid was present in all zones. Occasionally periportal hepatocytes in the high dose PCB treated livers were enlarged and characterized by cytoplasmic clearing with peripheralization of nuclei (hydropic degeneration). Necrosis, inflammation and copper accumulation were not significant features of any group. NAC supplementation overall significantly reduced the percent hepatic lipid in rats treated with PCB 126 (Table 2), while only the NAC group exposed to 5 $\mu\text{mol/kg}$ PCB 126 was significantly lower than the corresponding PCB group on normal diet (Figure 6A).

3.6 Lipid extraction

Lipids extracted and quantified from frozen liver tissues were consistent with the histological osmium quantification (Figure 6B). PCB 126 increased lipid content significantly at both the 1 $\mu\text{mol/kg}$ and 5 $\mu\text{mol/kg}$ doses in the rats receiving the control diets and at the high dose in the NAC fed animals. However, NAC supplementation significantly reduced hepatic lipid content in the PCB 126-treated rats. As a consequence, hepatic lipids extracted from the low dose PCB group fed NAC were not significantly different from the NAC controls.

3.7 CD36 Expression

PCB 126 treatment overall diminished the expression of CD36 significantly ($P=0.0058$, Table 2) and this effect was also statistically significant when corn oil and PCB 126 5 $\mu\text{mol/kg}$ were compared ($P=0.0015$) (Figure 7). Dietary NAC ameliorated the down regulation of CD36 at the higher dose of PCB 126.

4. Discussion

In addition to N-acetylcysteine's (NAC) well-known role as the antidote to acetaminophen toxicity, there are a wide range of scientific and therapeutic applications for NAC. For example, NAC is widely used to treat chronic obstructive pulmonary disorder, pulmonary fibrosis, and contrast-induced nephropathy (Millea, 2009). Although NAC is only a precursor of the main cellular antioxidant glutathione (GSH), its relative ease of uptake as compared to GSH has supported its wide use as a supplement. With reactive oxygen species (ROS) linked to carcinogenesis, increasing research emphasis has been placed on NAC in chemoprevention, with promising results (Hanczko *et al.*, 2009; Balansky *et al.*, 2010). PCB 77, a less potent dioxin-like PCB was reported to cause cytotoxicity and DNA fragmentation by depletion of GSH and generate reactive oxygen species in vascular endothelial cells (Hennig *et al.*, 1999). The oxidative cellular stress response induced by PCB 77 in these cells was ameliorated by NAC treatment (Slim *et al.*, 2000). Pretreatment with NAC has also been shown to reduce radiation-induced oxidative stress and injury (Mansour *et al.*, 2008). Thus, we set out to determine the efficacy of NAC in reducing the toxicity of a specific polychlorinated biphenyl (PCB) congener of a family of persistent organic pollutants, labeled as "probable carcinogen" by the EPA (IRIS, 2006), while PCB 126 is regarded as "carcinogenic to humans" by IARC (<http://monographs.iarc.fr/ENG/Classification/index.php>).

PCBs were produced for industrial purposes, but have persisted in the environment despite declines in their usage since the 1970s (Safe, 1994). Research into the mechanisms of PCB toxicity and carcinogenesis has proven to be difficult due to the varied effects attributed to the structural and chemical differences of the 209 congeners. Studies have focused on the non-ortho substituted congeners, which can assume a more co-planar conformation similar to dioxin (TCDD). Although these dioxin-like congeners, PCBs 77, 126, and 169, are known for their binding to the aryl hydrocarbon receptor (AhR), the exact mechanisms of toxicity of these PCBs has been under investigation for some time (Janosek *et al.*, 2006; Bock and Kohle, 2009). Various PCBs, including the dioxin-like congeners, have been linked to the generation of ROS (Schleizinger and Stegeman, 2001; Hennig *et al.*, 2002). Dioxin-like PCBs may generate ROS through their efficacious induction of CYP1A1/2, and their interference with the catalytic cycle of these enzymes (De *et al.*, 2010). Because cellular injury from oxidative stress can participate in all stages of carcinogenesis (Klaunig *et al.*, 2011), glutathione homeostasis becomes critical in preventing oxidative stress.

We observed a significant induction of EROD (CYP1A1) activity following PCB 126 exposure (Figure 3), confirming its potent binding to the AhR. In this instance the higher

dose of the toxicant PCB 126 resulted in a lowered induction response of CYP1A1. We have observed this non-linear trend both in vitro and in vivo. In an earlier study using chick embryo hepatocytes, we found that dioxin – like PCBs caused potent induction of total cytochrome P-450 concentrations and EROD activities with increasing concentrations/doses until a maximum was reached. Further concentration/dose increases resulted in marked declines of total cytochrome P-450 concentrations and EROD activities (Rodman *et al.*, 1989). We have seen this effect in the rat as well (Shedlofsky *et al.*, 1991; Lai *et al.*, 2011). Apparently EROD activity (level of induction) does not necessarily follow toxicity, since a point is reached where something required for the synthesis of the holoprotein, for example heme, becomes rate limiting.

Liver hypertrophy observed in rats exposed to PCB 126 (Figure 2A) is consistent with the proliferation of endoplasmic reticulum caused by CYP1A1 induction and steatosis. The growth rate was significantly slowed by reduction in weight gain of the high dose PCB-treated animals (Figure 1A), concomitant with significantly reduced feed consumption (Figure 1B), indicating acute toxicity. Consistent with previous studies with PCB 126 (Lai *et al.*, 2010), total liver glutathione levels were significantly diminished in a dose-dependent manner by PCB 126 (Figure 4A). However, this was also accompanied by diminished levels of the glutathione disulfide (GSSG) in a dose-dependent manner (Figure 4B), possibly due to glutathione disulfide secretion into bile as was reported previously for other compounds that increase oxidative stress (Lauterburg *et al.*, 1984). Contrary to our expectations, rats supplemented with NAC had overall lower levels of total hepatic glutathione, although this was not significant at the individual data point. In our study, NAC-fed animals also had significantly lower levels of GSSG which could indicate that NAC functioned more as a radical scavenger than as a precursor for glutathione synthesis. The lower GSSG-to-GSH ratio in NAC-fed rats suggests that NAC has a protective, antioxidant effect (Figure 4C). However, since PCB 126 did not affect the GSSG/GSH ratio, this may not be related to the PCB 126-treatment. PCB 126 caused a significant increase in GST activity (Figure 4D). Thus the reduction of GSH in PCB-treated animals could be due to an increase in the conjugation of glutathione. GST activity was even higher in PCB-treated NAC animals, but the effect of NAC was not significant. However, NAC controls had significantly lower GST activity than the controls on normal diet. This finding is in contrast with a report showing induction of GST activity after a single or repeated injection of rats with NAC (Arfsten *et al.*, 2007). It can be hypothesized that the route of application, one or few bolus injections vs. continuous, low dose dietary supplementation, may be the reason for this opposing effect.

Based on our results, it is likely that NAC is acting as a protective agent independent of liver glutathione levels. PCBs are known for causing changes to gene expression, some of which have been linked to disruptions in lipid metabolism (Arzuaga *et al.*, 2009). Steatosis is a prominent feature of PCB toxicity (Robertson *et al.*, 1991). In our study as well, PCB 126-induced liver toxicity presented histologically as steatosis (Figures 5, 6A and Supplemental Figure 1) and the most promising of our findings was the reduction in lipid deposition in the livers of rats supplemented with NAC. Quantification of lipids extracted from liver tissue was consistent with these findings (Figure 6B). Previous studies with dioxin-like PCBs have resulted in increases of liver lipids and triglycerides (Azais-Braesco *et al.*, 1990; Matsusue *et al.*, 1997). These results suggest a reduced synthesis of physiologically essential long-chain unsaturated fatty acids (Matsusue *et al.*, 1999). Past studies have shown that activation of the AhR increases expression of CD36, a scavenger receptor involved in fatty acid uptake, and tumor necrosis factor alpha (TNF α), a mediator of inflammation (Lee *et al.*, 2010; Vondracek *et al.*, 2011). Both genes have been implicated in liver steatosis (Greco *et al.*, 2008; Sundaresan *et al.*, 2010). These genes are possible targets of NAC, which has been shown to reduce both liver CD36 and TNF α expression in mouse models of non-alcoholic

steatohepatitis (NASH) (Baumgardner *et al.*, 2008; Ronis *et al.*, 2011). Although no studies have investigated NAC as a possible therapy in simple steatosis, NAC along with metformin have shown promise as treatment for NASH in humans by reducing hepatocellular lipid (de Oliveira *et al.*, 2008). CD36 is a class B scavenger receptor that has an emerging role in controlling hepatic fatty acid uptake. To understand the mechanism of NAC's protection in PCB 126 induced steatosis, we hypothesized that liver from PCB treated rats would have increased expression of CD36 and that the NAC diet may reduce expression of CD36. However, in our study CD36 expression was decreased in livers from PCB treated control diet rats with an amelioration of this effect in NAC supplemented rats at the high PCB dose. Recently, Forgacs *et al.* have reported a comparative microarray analysis of gene expression in both mice and rats treated with TCDD. These studies have also reported similar decrease in expression of fatty acid transporter CD36 in livers of TCDD-treated rats. In contrast, the mice showed increases in expression of CD36 along with increased fatty acid uptake into liver. These studies suggest TCDD and dioxin-like PCBs exhibit a variation in CD36 expression in rats and mice (Forgacs *et al.*, 2012). This expression difference may also be due to the pathophysiology of the steatosis with PCB toxicity resulting from a wasting syndrome as compared to steatosis in NASH as a result of obesity and insulin resistance.

Other factors may be involved in NAC's ability to reduce liver steatosis in PCB 126 treated rats. NAC has been shown to improve mitochondrial metabolic energy production by stimulating the carbon flux through the pyruvate dehydrogenase pathway, which may improve lipid metabolism and reduce steatosis (Zwingmann and Bilodeau, 2006). NAC may also enhance antioxidant protection by increasing the formation of hypotaurine, an intermediate of taurine synthesis (Zwingmann and Bilodeau, 2006). Hypotaurine is a potent radical scavenger and very efficient in protecting DNA and other cellular macromolecules against damage by free radicals (Messina and Dawson, 2000; Acharya and Lau-Cam). Since PCBs are known to increase intracellular oxidative stress, whether by cytochrome P-450 overexpression (Schlezingner *et al.*, 2006), interference with mitochondrial function (Zhu *et al.*, 2009), or other mechanisms, NAC could provide cellular protection via hypotaurine production, a hypothesis that needs to be investigated.

To the best of our knowledge, this is the first *in vivo* study using NAC as a therapeutic agent against dioxin-like PCB toxicity. While steatosis was still a pathologic feature in PCB 126-induced toxicity, we found NAC to be very promising in its ability to reduce steatosis. These results demonstrate that while NAC is a promising therapeutic agent in mitigating PCB 126-induced alterations to lipid metabolism, further studies will be needed to determine its exact mechanisms and efficacy in protection against PCB toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Weight and feed consumption of vehicle- (Corn Oil) and PCB 126- (1 μmol/kg and 5 μmol/kg) treated animals

(A) Weight percentage is measured as the weight gained relative to initial weight. High (5 μmol/kg) dose PCB 126 significantly slowed growth relative to vehicle-treated control indicating acute toxicity. Animals fed NAC treated with low (1 μmol/kg) PCB 126 had significantly slowed growth. (B) Feed consumption is defined as total feed consumed in grams following injection. PCB 126 diminished feed consumption in a dose-dependent manner. Error bars represent SEM. * $p < 0.05$ as compared to Corn Oil vehicle control. † $p < 0.05$ as compared to AIN-93G control diet.

Figure 2. Relative liver and thymus weights of vehicle- (Corn Oil) and PCB 126- (1 μ mol/kg and 5 μ mol/kg) treated animals

(A) Relative liver weight is defined as final liver weight as a percentage of total body weight. PCB 126 significantly increased liver weight in a dose-dependent manner. (B) Relative thymus weight is defined as final thymus weight as a percentage of total body weight. PCB 126 caused significant dose-dependent thymic involution. Error bars represent SEM. * $p < 0.05$ as compared to Corn Oil vehicle control.

Figure 3. Liver ethoxyresorufin-O-deethylase (EROD) activity of vehicle- (Corn Oil) and PCB 126- (1 $\mu\text{mol/kg}$ and 5 $\mu\text{mol/kg}$) treated animals
 PCB 126 significant induced EROD activity. Error bars represent SEM. * $p < 0.0001$ as compared to Corn Oil vehicle control. ** $p < 0.0005$ as compared to Corn Oil vehicle control.

Figure 4. Liver total Glutathione (GSH), oxidized glutathione (GSSG), ratio of liver oxidized glutathione (GSSG) to liver total glutathione (GSH), and glutathione transferase (GST) activity of vehicle- (Corn Oil) and PCB 126- (1 $\mu\text{mol/kg}$ and 5 $\mu\text{mol/kg}$) treated animals

(A) PCB 126 decreased total GSH in a dose-dependent manner, significantly at the high (5 $\mu\text{mol/kg}$) dose. (B) PCB 126 decreased GSSG in a dose-dependent manner, significantly at the high (5 $\mu\text{mol/kg}$) dose. GSSG was significantly decreased in animals fed NAC-supplemented diets. (C) Liver GSSG to GSH ratio was diminished significantly in animals fed NAC-supplemented diets and treated with PCB 126. (D) GST activity was significantly induced in a dose-dependent manner by PCB 126. Error bars represent SEM. * $p < 0.05$ as compared to Corn Oil vehicle control. † $p < 0.05$ as compared to AIN-93G diet.

Figure 5. Osmium tetroxide staining for lipid (stains lipid black) of liver from control and PCB 126- (1 $\mu\text{mol/kg}$ and 5 $\mu\text{mol/kg}$) treated animals

There is little hepatocellular lipid in control treated rats fed the control diet (A) and the NAC-supplemented diet (B). Livers from rats treated with 1 $\mu\text{mol/kg}$ PCB fed the control (C) and NAC-supplemented diet (D) had increased hepatocellular lipid in centrilobular to midzonal hepatocytes. Livers from rats treated with 5 $\mu\text{mol/kg}$ PCB fed the control (E) and NAC-supplemented diet (F) had lipid in all zones. The percent parenchymal lipid staining was quantified and showed that PCB 126 treatment increased liver lipid in a dose-dependent manner.

Figure 6. Quantification of liver lipid staining and lipids extracted from liver tissue of control and PCB 126- (1 $\mu\text{mol/kg}$ and 5 $\mu\text{mol/kg}$) treated animals

(A) Rats treated with 5 $\mu\text{mol/kg}$ PCB and fed the NAC-supplemented diet had significantly lower percent liver lipid than rats fed the control diet. (B) Rats treated with 5 $\mu\text{mol/kg}$ PCB and fed the NAC-supplemented diet had significantly lower percent liver lipid than rats fed the control diet. Error bars represent SEM. * $p < 0.05$ as compared to Corn Oil vehicle control. † $p < 0.05$ as compared to AIN-93G diet. 200X

Figure 7. CD36 Expression in rat liver

Hepatic expression levels of CD36 were significantly decreased in the high dose PCB 126 treated animals ($P=0.0058$). NAC supplemented high PCB 126 dose animals were significantly different from their corresponding corn oil control ($P=0.0015$).

Table 1

Composition of AIN-93G and modified NAC supplemented diets

Constituent	AIN93-G g/kg	AIN-93G w/1% NAC g/kg
Casein, low Cu & Fe	200.	200.
L-Cystine	3.0	3.0
Corn Starch	397.	387.
Maltodextrin	132.	132.
Sucrose	100.	100.
Soybean Oil	70.0	70.0
Cellulose	50.0	50.0
Mineral Mix, AIN-93G-MX	35.0	35.0
Vitamin Mix, AIN-93-VX	10.0	10.0
Choline Bitartrate	2.5	2.5
THBQ, antioxidant	0.014	0.014
N-acetylcysteine	0.0	10.0

Table 2

Two-way ANOVA analysis of the effects of NAC and PCB 126

	NAC	PCB 126
Growth Rate (%)	-	<.0001
Feed Consumption	-	<.0001
Relative Liver Weight (%)	-	<.0001
Relative Thymus Weight (%)	0.0168	<.0001
EROD Activity	-	<.0001
Total GSH	0.0499	<.0001
GSSG	<.0001	<.0001
GSSG/GSH Ratio	<.0001	-
GST Activity	-	<.0001
Liver Lipid %	0.0134	<.0001
CD36 Expression	-	0.0046

Only p-values <0.05 are reported.