Toxicology 302 (2012) 25–33

Contents lists available at SciVerse ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

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

Ian K. Lai[a,b,1], Kiran Dhakal[a,b,2], Gopi S. Gadupudi[a,b], Miao Li[a,b,3], Gabriele Ludewig[a,b,4], Larry W. Robertson[a,b,*], Alicia K. Olivier[c,5]

[a] Interdisciplinary Graduate Program in Human Toxicology, University of Iowa, United States
[b] Department of Occupational and Environmental Health, The University of Iowa, College of Public Health, 100 Oakdale Campus #121 IREH, Iowa City, IA 52242-5000, United States
[c] Department of Pathology, University of Iowa, 500 Hawkins Drive, 1163ML, Iowa City, IA 52242, United States

ARTICLE INFO

*Article history:*
Received 14 May 2012
Received in revised form 9 July 2012
Accepted 15 July 2012
Available online 21 July 2012

*Keywords:*
CD36
Fatty liver
PCB 126
Dietary supplementation
Growth
CYP1A1

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 μmol/kg body weight PCB 126 by i.p. 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 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.



## 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

* Corresponding author at: Department of Occupational and Environmental Health, The University of Iowa, College of Public Health, 100 Oakdale Campus #219 IREH, Iowa City, IA 52242-5000, United States. Tel.: +1 319 335 4346; fax: +1 319 335 4225.
*E-mail addresses:* ian-lai@uiowa.edu (I.K. Lai), kiran-dhakal@uiowa.edu (K. Dhakal), miao-li@uiowa.edu (M. Li), gabriele-ludewig@uiowa.edu (G. Ludewig), larry-robertson@uiowa.edu (L.W. Robertson), alicia-olivier@uiowa.edu (A.K. Olivier).
[1] Tel.: +1 408 507 1308; fax: +1 319 335 4290.
[2] Tel.: +1 573 529 2187; fax: +1 319 335 4290.
[3] Tel.: +1 520 205 1368; fax: +1 319 335 4290.
[4] Tel.: +1 319 335 4650; fax: +1 319 335 4290.
[5] Tel.: +1 319 384 4668; fax: +1 319 335 8453.

0300-483X/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tox.2012.07.007

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 (Schlezinger 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 for publication), 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 et al. (2009) and 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 $^{13}$C 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 g 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 ml/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 10,000 × *g* for 20 min. The resulting supernatants were then centrifuged at 100,000 × *g* 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).

**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 |

### 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 100,000 × *g* supernatants were determined by the methods of Griffith (1980) and Anderson (1985). Absorbance change at 412 nm over 5 min 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 μm and antigen unmasking was performed in citrate buffer (pH 6.0) for 3 × 4 min in the microwave (1000 W). 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 h in running tap water and then processed normally and embedded in paraffin. Sections were cut at 4 μm and baked in a 60 °C oven overnight. Slides were cooled, deparaffinized and counterstained with nuclear fast red for 5 min. Slides were then dehydrated and coverslipped. Osmium stained slides were examined with a high resolution microscope (BX51, Olympus), digital images collected at 100× magnification (DP72, Olympus)