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Role of oil vehicle on hepatic cell proliferation in PCB-treated rats

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Abstract

Here we report the role of dietary glycine and the type of oil used as a vehicle on hepatotoxicity in 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153)-treated or control rats. In the first study, glycine or valine (as control) was fed in an unrefined diet at 5% for the entire study (5 days) to inhibit Kupffer cell activity. PCB-153 (100 or 300 $\mu\text{mol/kg}$) dissolved in medium chain triglyceride (MCT) oil, was injected i.p. two days before euthanasia; the peroxisome proliferator Wy-14,643 was included as a positive control. MCT oil decreased cell proliferation by about 50%. PCB-153 slightly increased hepatic cell proliferation, but dietary glycine did not reduce cell proliferation. Because of the inhibition of cell proliferation in rats receiving MCT oil compared to rats receiving no injection, we hypothesized that MCT oil may have been inhibiting the hepatocyte proliferation in PCB-153-treated rats. We therefore performed another experiment using three types of oil as a vehicle for PCB-153: MCT oil, corn oil and olive oil. Rats were injected with PCB-153 (300 $\mu\text{mol/kg}$) or one of the vehicles, again two days before euthanasia. MCT oil again decreased the hepatocyte proliferation by about 50%. In rats receiving PCB-153, hepatocyte proliferation was slightly higher than their respective vehicle controls for corn oil and olive oil but not for MCT oil. These studies show that the oil vehicle is important in cell proliferation after PCB exposure, with MCT oil appearing to be protective.

Keywords

2,2',4,4',5,5'-hexachlorobiphenyl; hepatocyte proliferation; MCT oil; glycine; Kupffer cells

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Introduction

Polychlorinated biphenyls are ubiquitous lipophilic environmental contaminants. In addition to environmental contamination from intentionally-synthesized PCBs, PCBs are also unintended by-products of dye/paint manufacture and the disposal of these materials, including through municipal solid-waste incineration.¹⁻³ 2,2', 4,4', 5,5'-hexachlorobiphenyl (PCB-153) is one of the most abundant congeners in the environment, in the food web, and in both animals and humans.⁴⁻¹¹ In humans, PCB-153 was found at levels of 121 ng/g fat of mammary gland,¹² 21.6 ng/g hair,⁶ 9.5 ng/g muscle, 7.5 ng/g liver, 3.1 ng/g brain, and 2.6 ng/g kidney.⁴ Exposure of humans to this pollutant is unavoidable via air and food. Fat-rich foods are an important source of contamination.¹³ A recent study of persistent organic pollutant (POP) levels in foods purchased from supermarkets in Dallas, TX in 2009 found that PCB-153 is especially abundant in fish, with salmon being the most contaminated.¹⁴ Two accidental exposures (Yusho and Yucheng) involved human consumption of cooking oil contaminated with PCB mixtures,^{15,16} and one (Belgian PCB/dioxin crises) involved human consumption of animal products from animals fed with PCB- and dioxin-contaminated feed.¹⁷

The significant PCB-153 levels present in the environment, and the ability of PCB-153 to increase cytochrome P450 2B1/2 activity¹⁸ raise a series of concerns. Increased cytochrome P-450 activity was reported to increase the formation of reactive oxygen species.¹⁸⁻²⁴ Although an inducer of cytochrome P-450, PCB-153 is not a substrate of this enzyme. However, the induction of cytochrome P-450 and continued high levels of the enzyme in the absence of substrate produces the maximal amount of reactive oxygen species, compared with the case when the substrate is present.²⁵ Reactive oxygen species are known regulators of cell proliferation and thus influencing the risk of developing tumors.²⁶⁻²⁸ PCB-153 has been shown to promote hepatic preneoplastic lesions.²⁹ Previous experiments carried out in our laboratory have shown that PCB-153 increases hepatocyte proliferation two days after one single i.p. administration³⁰ and that dietary glycine diminished hepatocyte proliferation in preneoplastic foci.³¹

Based on these results, we hypothesized that promotion of the preneoplastic lesions by PCB-153 involves hepatocyte proliferation prompted by the secretory activity of the Kupffer cell and that these processes take place shortly after PCB-153 administration. Glycine is a known inhibitor of the secretory activity of Kupffer cells.^{32,33} Glycine preserves the phagocytic activity (colloidal carbon uptake) of Kupffer cells,³⁴ but inhibits the secretory activity of these cells.³⁵ This model was successfully used in a number of studies examining liver toxicity induced by alcohol,^{36,37} endotoxin,³⁸ peroxisome proliferators,^{39,40} and CCl₄.⁴¹ The main mechanism of Kupffer cell influence upon hepatotoxicity seems to be through TNF- α and appears to involve cAMP, PKC and NF- κ B upstream of TNF- α . Other important mediators are NO, reactive oxygen species and PGE₂.^{38,42-44}

Another important consideration in PCB studies is the route by which PCBs are administered. PCBs can be fed in the diet, or dissolved in a vehicle, usually oil, and then injected, usually p.o. or i.p.⁴⁵ The type of oil used can influence endpoints induced by xenobiotics, including cell proliferation. For example, corn oil administered p.o. was found to increase cell proliferation and NF- κ B activation in the liver, whereas olive oil and medium chain triglyceride (MCT) oil did not.⁴⁶

In this study, we investigated the interactions between dietary glycine, PCB-153 injection, and the oil vehicle used on the cell proliferation rate and CYP2B1/2 activity in rats. Glycine or valine (as control) was fed in an unrefined diet at 5% for the entire study (5 days). PCB-153 was injected i.p. two days before euthanasia. Several oil vehicles were used to

dissolve PCB-153: MCT oil, olive oil, and corn oil. We observed that cell proliferation was reduced about 50% when MCT oil was used as the vehicle compared to other oils.

Materials and Methods

Materials

PCB-153 (2,2',4,4',5,5'-hexachlorobiphenyl) was synthesized and characterized as described previously.⁴⁷ The purity of this PCB was greater than 98%, as determined by gas chromatography. Vitamin E-stripped corn oil (Acros Organics, Morris Plains, NJ), medium chain triglyceride (MCT) oil (Mead Johnson, Evansville, IN), olive oil (Sigma St. Louis, MO), Wy-14,643 ([4-chloro-6-(2, 3- xylindino)-2- pyrimidinylthio]-acetate) (Chemsyn Science Laboratories, Lenexa, KS), Alzet osmotic pumps (model 2ML1) (Alza Scientific Products, Palo Alto, CA), glycine and valine (Sigma, St. Louis, MO), the anti-5-bromo-2'-deoxyuridine antibody (Becton-Dickinson, San Jose, CA), Antigen Retrieval Citra solution (BioGenex, San Ramon, CA), and poly (deoxyinosinicdeoxycytidylic acid) (Amersham Pharmacia Biotech, Piscataway, NJ) were obtained from the sources indicated. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental design and animal treatment

Male Sprague-Dawley rats weighing 175–200 g (Harlan Sprague Dawley, Indianapolis, IN), were housed three per cage in a temperature- and light-controlled room and fed an unrefined rodent diet *ad libitum* in feeding cups (Global 18% protein Rodent Diet 2018, Harlan Teklad, Madison, WI). The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Upon arrival, the animals were allowed to adjust for one week before starting the experiment.

In the first study (Study 1), glycine or valine (as control) was fed in the diet at 5% for the entire study (5 days). Glycine was fed at the 5% level because of previous studies showing that this level inhibited the induction of cell proliferation and hepatic tumors by peroxisome proliferators such as Wy-14,643.^{39, 40} PCB-153 was injected i.p. three days after starting the diets and 48 hours before euthanasia. Two doses were used: 100 $\mu\text{mol/kg}$ body weight and 300 $\mu\text{mol/kg}$ body weight, using medium chain triglyceride (MCT) oil as the vehicle (0.2 ml oil/100g rat). As a positive control for hepatocyte proliferation, Wy-14,643 (0.05% in diet) was fed for 48 hours. The rats were implanted with Alzet osmotic pumps containing BrdU (20mg BrdU/ml PBS, 10 $\mu\text{L/h}$) 48 h before euthanasia.

In the second study (Study 2), the effect of vehicle on the induction of cell proliferation by PCBs was examined. Rats were injected with PCB-153 (300 $\mu\text{mol/kg}$ body weight) or vehicle and euthanized 48 hours later. MCT, corn oil or olive oil was used as the vehicle (1ml oil/100g rat). The rats were injected s.c. with 5-bromodeoxyuridine (BrdU, 100 mg/kg; 20mg BrdU/ml PBS) 2 h before euthanasia.

In both studies, rats were euthanized by overexposure to carbon dioxide. The livers were immediately removed and weighed; a portion of each was frozen in liquid nitrogen and stored at -80°C until used. For each rat, a piece of liver was randomly taken from 4 different lobes and fixed in 10% buffered formalin.

BrdU immunohistochemical staining

The formalin-fixed liver tissues were paraffin-embedded, sectioned, laid on glass slides, stained with an anti-BrdU antibody to identify nuclei that had incorporated BrdU (using diaminobenzidine (DAB) as the staining agent), and counter-stained with hematoxylin. At least 3000 hepatocellular nuclei randomly per slide (rat) with 1000 nuclei from each of three

different lobes were counted. The labeling index was expressed as the percentage of the number of labeled hepatocyte nuclei out of the total number of hepatocyte nuclei counted.

Alkoxyresorufin O-dealkylation assay

The microsomal fraction was isolated from the whole-liver homogenate as described by Schramm et al.⁴⁷ Microsomal 7-benzyloxyresorufin (BROD) was used as specific substrate for the CYP2B1/2 isozyme in the alkoxyresorufin O-dealkylase method.⁴⁸ The absorbance of resorufin was detected with a fluorescence spectrophotometer at an excitation wavelength of 556 nm and an emission wavelength of 589 nm.

Fatty acyl-CoA oxidase assay

Fatty acyl-CoA oxidase assay was performed using lauroyl-CoA as the substrate.⁴⁹ The readings were carried out with a Gilford Fluoro IV fluorometer at the excitation wavelength of 318 nm and the emission wavelength at 405 nm.

Statistical analysis

All statistical analyses were realized using SYSTAT V.8 (SPSS, Chicago, IL) software. Results were first analyzed by two-way analysis of variance (ANOVA) for study 1 and one-way ANOVA for study 2. Individual differences between the treatment means were determined with the LSD post-hoc test. The results were reported as means $\pm$ standard error of the mean (SEM). Results were defined as statistically significant at $P \leq 0.05$.

Results

The aim of these experiments was to determine if dietary glycine and the type of oil used as vehicle modulated PCB-153 hepatotoxicity as quantified by cytochrome P450 2B1/2 activity and hepatocyte proliferation rate. In the first experiment, two groups of rats received PCB-153 (100 $\mu\text{mol/kg}$ or 300 $\mu\text{mol/kg}$) dissolved in MCT oil, another group received only MCT oil, one group received no injection, and the final group received Wy-14,643 in the diet as a positive control for hepatocyte proliferation. We used MCT oil in this study because it had been reported that orally administered corn oil, but not MCT oil, increased hepatocyte proliferation in rats, via increased NF- κ B DNA binding activity in Kupffer cells and TNF- α release.⁴⁶ In each of the groups, half of the animals received glycine and the other half received valine in the diet for the entire study (5 days). PCB-153 was injected 3 days after starting the diets and the animals were euthanized 48h after the PCB injection. The body weights in rats treated with PCB-153 (300 $\mu\text{mol/kg}$) were lower, but generally were not different among the other groups (Table 1). The relative liver weights were significantly increased only by Wy-14,643 administration (Table 1). The liver slides stained with hematoxylin and eosin presented no abnormalities.

Microsomal BROD activity was measured as an indication of cytochrome P-450 2B1/2 levels, while fatty acyl CoA oxidase activity was measured to estimate peroxisome proliferation as well as peroxisomal β -oxidation. BROD activity was significantly increased by both doses of PCB-153, in both the valine- and glycine-fed rats (Figure 1). Glycine slightly decreased BROD activity ($P = 0.075$ in two-way ANOVA with no significant interaction). Fatty acyl-CoA oxidase activity was increased by Wy-14,643 significantly but was not affected by PCB or glycine treatment (Figure 2).

Hepatocyte proliferation was quantified by infusing BrdU over a 3-day period and then performing labeling indexes in hepatocytes (Figure 3). Wy-14,643 significantly increased cell proliferation compared to all other groups; glycine significantly decreased cell proliferation only in rats receiving Wy-14,643. Glycine increased cell proliferation in the

low-dose PCB group. MCT oil decreased the cell proliferation by about 50% compared with the groups that did not receive any vehicle. Neither dose of PCB significantly increased hepatic cell proliferation in the valine groups, although the rate was increased about two-fold in the high-dose PCB group. Cell proliferation was increased by the low dose of PCB-153 in glycine-fed rats.

In contrast to our previous experiments with PCB-153,^{30, 50} we did not observe an increase in cell proliferation in rats receiving PCB-153, in either the valine or glycine groups. Because of the inhibition of cell proliferation in rats receiving MCT oil compared to rats receiving no injection, we hypothesized that MCT oil may have been inhibiting the hepatocyte proliferation in PCB-153-treated rats. We therefore performed another experiment using three types of oil as a vehicle for PCB-153: MCT oil, corn oil and olive oil. Rats were injected with PCB-153 (300 µmol/kg) and one of the vehicles or the vehicle alone; other rats received no injection. The liver weights were not significantly different among groups, although the MCT-PCB-treated rats had a lower body weight compared to all of the other groups (Table 2). PCB-153 significantly increased BROD activity (Figure 4). In PCB-treated rats, BROD activity was higher in rats that received corn oil as the vehicle compared to rats that received MCT as the vehicle, with olive oil not having a significant effect.

Hepatocyte proliferation was quantified by administering BrdU s.c. two hours before euthanasia and then performing labeling indexes in hepatocytes. MCT oil again decreased the hepatocyte proliferation by about 50% compared to the group that did not receive any injections (Figure 5). Cell proliferation was higher in PCB-treated rats receiving corn oil ($P < 0.05$) or olive oil ($P = 0.08$) than in PCB-treated rats receiving MCT oil. Hepatocyte proliferation was slightly higher in rats receiving PCB-153 than their respective controls for corn oil and olive oil but not for MCT oil.

Discussion

In this study, we examined whether the type of oil vehicle or Kupffer cell inhibition by dietary glycine modulates PCB-153 hepatotoxicity. The primary end point was hepatocyte proliferation. While normally quiescent, hepatocytes proliferate after liver injury to regenerate the tissue. In addition, xenobiotics such as peroxisome proliferators, phenobarbital, and PCBs induce additive hyperplasia, which increases the risk of hepatocellular carcinoma.⁵¹ The studies presented here show for the first time that medium chain triglyceride (MCT) is able to decrease *in vivo* hepatocyte cell proliferation. The studies also show that dietary glycine does not influence hepatic cell proliferation in rats administered PCB-153, but diminishes CYP2B1/2 induction by PCB-153.

To quantify the rate of hepatocyte proliferation, we determined the labeling index after rats were administered BrdU. Wy-14,643 significantly increased cell proliferation compared to the controls. Glycine significantly decreased cell proliferation only in Wy-14,643-treated rats. The increase in cell proliferation by Wy-14,643 is well established⁴⁰ and our positive control rats behaved as expected. The inhibition of Wy-14,643-induced cell proliferation by dietary glycine has previously been reported,⁴⁰ and was used as a control in our experiments. PCB-153 treatment led to an increase in cell proliferation when corn oil or olive oil was used as the vehicle, but not when MCT oil was used. This is in agreement with the study of Lu et al.,³⁰ which found that cell proliferation in rats was increased 2 days after 300 µmol/kg PCB-153 treatment, in which corn oil was used as the vehicle.

In both studies, when we used MCT oil, cell proliferation was decreased by about 50% compared with the groups that did not receive any injections. But when we used corn oil or

olive oil, hepatocyte proliferation in PCB-treated rats was increased 2–3 fold compared to MCT oil. When injected p.o., corn oil but not olive oil or MCT oil was found to increase cell proliferation in hepatocytes in rats.⁴⁶ MCT oil has been found to inhibit ethanol-induced liver injury and endotoxin-induced TNF- α production by Kupffer cells in rats,^{52, 53} and to inhibit the induction of CYP2E1 by ethanol.⁵⁴ Several human and animal studies have observed modulation of the immune response by MCT oil.⁵⁵ The vehicle-dependent effects of PCB 153 in our study are most likely due to differences in its redistribution from the peritoneum to the liver after i.p. administration, which alters the toxicokinetics at critical sites.^{56–58} The observation that i.p. administration results in much higher PCB tissue levels compared to oral administration⁵⁹ supports our interpretation that i.p. administration drastically alters the toxicokinetics of PCBs due to sustained and vehicle-dependent PCB release.

Cytochrome P-450 2B1/2 activity was increased by PCBs as expected, and fatty acyl-CoA oxidase activity was increased by Wy-14,643 as expected. Glycine inhibited the PCB-induced increase of cytochrome P-450 2B1/2 activity, which was in contrast to the earlier study of Bunaciu et al.,³¹ which was a long-term study in which rats were administered glycine for over two months. However, glycine did not affect the Wy-14,643-induced increase of fatty acyl-CoA oxidase activity, in agreement with the finding of Rose et al.⁴⁰

In summary, these studies show that the oil vehicle is important in modulating cell proliferation after PCB exposure. The use of MCT oil as a vehicle inhibits cell proliferation compared to corn oil or olive oil. Kupffer cell inhibition by dietary glycine did not consistently inhibit cell proliferation in PCB-treated rats, although it did inhibit the induction of cytochrome P-450 2B1/2 activity.

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List of abbreviations

BrdU	bromodeoxyuridine
BROD	Benzyloxyresorufin O-dealkylase
EROD	Ethoxyresorufin O-dealkylase
LPS	lipopolysaccharide
PCBs	polychlorinated biphenyls
PCB-153	2,2',4,4',5,5'-hexachlorobiphenyl
MCT	medium chain triglyceride

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Figure 1. Effect of PCB-153 and glycine on cytochrome P-450 2B1/2 activity
 Cytochrome P450 2B1/2 was determined at 48h after administration, by 7-benzyloxyresorufin O-dealkylase (BROD) activity. Results are expressed as mean $\pm$ SEM. The values were analyzed by ANOVA followed by LSD, and the level of significance was $p < 0.05$. Significantly different values are designated by different letters.

Figure 2. Effect of PCB-153 and glycine on fatty acyl-CoA oxidase (FAO) activity
 Results are expressed as mean $\pm$ SEM. The values were analyzed by ANOVA followed by LSD, and the level of significance was $p < 0.05$. Significantly different values are designated by different letters.

Figure 3. Effect of PCB-153 and glycine on hepatocyte proliferation (Study 1)

Results are expressed as mean $\pm$ SEM. The values were analyzed by ANOVA followed by LSD, and the level of significance was $p < 0.05$. Significantly different values are designated by different letters.

Figure 4. Effect of PCB-153 and vehicle on cytochrome P-450 2B1/2 activity (Study 2)
Results are expressed as mean $\pm$ SEM. Significantly different values are designated by different letters.

Figure 5. Effect of PCB-153 and vehicle on hepatocyte proliferation (Study 2)
Results are expressed as mean $\pm$ SEM. Significantly different values are designated by different letters.

Table 1

Body and Liver Weights in Study 1

Dietary Treatment	PCB Treatment	Number of rats per group	Liver weight (g)	Body weight (g)	Relative liver weight (%)
Valine	---	6	12.7±0.7 ^a	279.8±7.1 ^{bc}	4.5±0.2 ^a
	MCT only	6	13.1±0.5 ^a	277.2±3.6 ^{bc}	4.7±0.1 ^a
	PCB 100	6	12.8±0.4 ^a	269.2±6.7 ^{ab}	4.7±0.1 ^a
	PCB 300	6	12.5±0.2 ^a	264.5±1.9 ^a	4.7±0.1 ^a
	Wy	4	15.7±0.2 ^b	279.5±3.3 ^{bc}	5.6±0.0 ^b
Glycine	---	6	13.5±0.3 ^a	286.7±3.8 ^c	4.7±0.1 ^a
	MCT only	6	13.5±0.3 ^a	283.0±2.3 ^c	4.8±0.1 ^a
	PCB 100	6	13.6±0.4 ^a	281.8±6.8 ^c	4.8±0.1 ^a
	PCB 300	6	12.9±0.3 ^a	263.3±2.0 ^a	4.9±0.1 ^a
	Wy	5	15.9±0.6 ^b	280.4±5.0 ^c	5.7±0.1 ^b

Values are means ± SEM. Values with different superscripts are significantly different.

Table 2

Body and Liver Weights in Study 2

PCB-153	Vehicle	Number of rats per group	Liver weight (g)	Body weight (g)	Relative liver weight (%)
Absent	---	6	11.8±0.4	256.3±2.6 ^b	4.6±0.1
	MCT	6	11.4±0.5	251.5±3.7 ^b	4.5±0.1
	Corn oil	6	12.3±0.5	255.2±4.0 ^b	4.8±0.1
	Olive oil	6	12.4±0.3	258.8±4.0 ^b	4.8±0.1
Present	MCT	6	11.7±0.4	240.2±3.0 ^a	4.9±0.2
	Corn oil	6	13.2±0.5	260.7±3.6 ^b	5.1±0.2
	Olive oil	6	12.7±0.5	258.7±4.0 ^b	4.9±0.1

Values are means ± SEM. There were no significant differences in liver weights or relative liver weights. For body weights, values with different superscripts are significantly different.