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Identification of Lipidomic Markers of Chronic 3,3',4,4',5-Pentachlorobiphenyl (PCB 126) Exposure in the Male Rat Liver

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Abstract

Exposure to PCB 126, an environmentally relevant aryl hydrocarbon receptor agonist, is an environmental factor causing hepatic steatosis in rodent models; however, the lipidome of PCB 126-exposed rats has not been investigated in-depth. The objective of the present study was therefore to characterize dose-dependent changes in the lipid profile in the liver of male Sprague-Dawley rats exposed to PCB 126. Rats were exposed for three month to intraperitoneal injections of 0.01, 0.05 and 0.2 $\mu\text{mol/kg}$ bw PCB 126 in corn oil. Control animals were exposed in parallel and received corn oil alone. Lipids were extracted from whole liver homogenate and levels of polar lipids and fatty acids incorporated into triglycerides (FA_{TAGs}) were determined with tandem mass spectrometry using electrospray ionization. PCB 126 exposure increased the hepatic content of polar lipids and FA_{TAGs}. Protein adjusted levels of several polar lipid classes, in particular phosphatidylserine levels, decreased, whereas FA_{TAGs} levels typically increased with increasing PCB 126 dose. Sensitive, dose-dependent endpoints of PCB 126 exposure included an increase in levels of adrenic acid incorporated into triglycerides and changes in levels of certain ether-linked phospholipid and 1-alkyl/1-alkenyl diacylglycerol species, as determined using partial least square discriminant analysis (PLS-DA) and ANOVA. These changes in the composition of polar lipids and fatty acid in the liver of PCB 126 exposed rats identified several novel markers of PCB 126-mediated fatty liver disease that need to be validated in further studies.

Keywords

Adrenic acid; diacyl glyceryl ethers; ether lipids; lipidomics; phosphatidylserine; polyunsaturated fatty acids

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1. Introduction

Polychlorinated biphenyls (PCBs) are a class of environmental contaminants that have been banned world-wide under the Stockholm Convention on Persistent Organic Pollutants. PCBs were manufactured and sold as complex mixtures containing over 150 individual PCB congeners and used in many commercial applications, such as carbonless copy paper, oil-based paints, oils in motors and hydraulic systems, caulking and plastics (Agency for Toxic Substances and Disease Registry, 2000). PCBs are still used in enclosed applications, including transformers, capacitors and fluorescent light ballasts. PCBs are inadvertently manufactured as byproducts of various industrial processes, such as the production of certain paint pigments (Anezaki and Nakano, 2013; Hu and Hornbuckle, 2010). PCBs have been released into the environment and are present in many environmental samples, including soil, sediments, water and air. Humans are exposed to PCBs primarily *via* the diet (Schechter et al., 2010; Shin et al., 2015; Voorspoels et al., 2008) and by inhalation of indoor and outdoor air (Harrad et al., 2006; Lehmann et al., 2015). Exposure to PCBs has been implicated in a range of health effects, including immune, reproductive, neurological and endocrine effects (Agency for Toxic Substances and Disease Registry, 2000). A considerable body of evidence demonstrates that PCBs and their metabolites are carcinogens (Robertson and Ludewig, 2011; Silberhorn et al., 1990), and the International Agency for Research on Cancer (IARC) recently classified PCB as known human carcinogens (Lauby-Secretan et al., 2013). An analysis of NHANES data demonstrates that exposure to PCBs is associated with nonalcoholic fatty liver disease (NAFLD) (Cave et al., 2010), a complex disease associated with complex changes in the liver lipid profiles (Alonso and Matos, 2014; Gorden et al., 2015; Loomba and Sanyal, 2013).

Animal studies demonstrate that PCBs, either as individual congeners or mixtures, are possible etiologic factors in fatty liver disease (Kopeck et al., 2011; Mailloux et al., 2014; NTP, 2006; Shi et al., 2012; Wahlang et al., 2013). Exposure to both technical PCB mixtures and individual PCB congeners, either alone or in combination with a high fat diet, affects the fatty acid, polar lipid and triacylglyceride (TAG) composition of the liver (Azaïs-Braesco et al., 1990; Borlakoglu et al., 1990; Borlakoglu et al., 1991). PCB 126, a highly toxic PCB congener, is of particular concern because both histopathological evaluations and gravimetric lipid determinations demonstrate that exposure to PCB 126 results in hepatic lipid accumulation in rodent studies (Boucher et al., 2015; Lai et al., 2010a; Lai et al., 2011; Lai et al., 2012; Wu et al., 2016). The steatosis in the liver of PCB 126-exposed rats is accompanied by significant changes in the balance of polyunsaturated fatty acids (PUFAs) and major lipid classes in the liver of PCB 126 exposed rats (Matsusue et al., 1997, 1999). Similarly, PCB 77 (3,3',4,4'-tetrachlorobiphenyl), which is structurally related to PCB 126, increases total levels of phospholipids and triglycerides in the liver and levels of cholesterol and phospholipids in liver microsomes from PCB 77 exposed male rats (Azaïs-Braesco et al., 1990).

Both PCB 77 and PCB 126 are dioxin-like PCB congener and potent agonists of the aryl hydrocarbon receptor (AhR) (Bandiera et al., 1982). Exposure to PCB 126 causes wasting syndrome and alters redox and metal homeostasis in rodent models (Klaren et al., 2015; Lai et al., 2010a; Lai et al., 2011; Lai et al., 2012) by mechanisms involving the AhR (van den

Berg et al., 2006). PCB 126 exposure also decreases hepatic glucose levels, followed by a decrease in peroxisomal fatty acid oxidation in male rats (Gadupudi et al., 2016). These changes in glucose homeostasis precede alterations in hepatic lipid and fat accumulation following PCB 126 exposure. Moreover, PCB 126 exposure alters the activity of enzymes involved in the hepatic synthesis of PUFAs (Matsusue et al., 1999) and causes a dose-dependent decrease in the ratios of several PUFA epoxides to diol metabolites formed *via* the cytochrome P450 (P450) monooxygenase-soluble epoxide hydrolase (sEH) pathway in the liver (Wu et al., 2016). The later finding suggests a more inflammatory phenotype in the liver of PCB 126 exposed rats, which is consistent with the hepatic inflammation observed in female rats exposed to PCB 126 as part of a two year, chronic bioassay (NTP, 2006).

Despite the well documented hepatotoxicity of PCB 126, changes in the hepatic profiles of polar lipids and FA_{TAGs} following PCB 126 exposure remain poorly characterized. The present study uses a lipidomics approach to characterize changes in both polar lipid profiles and profiles of FA_{TAGs} in the liver of male rats exposed chronically to PCB 126. Our results demonstrate profound, dose dependent effects of PCB 126 exposure on the hepatic lipid composition and suggest that changes in the levels of phosphatidylserine, adrenic acid and certain ether-linked polar lipid and 1-alkyl/1-alkenyl diacylglycerol (ADAG) species are sensitive markers of chronic PCB 126 exposure in the male rat liver.

2. Materials and Methods

2.1 Animals and experimental design

The animal experiment was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Iowa, Iowa City. Details regarding the animal study, including general toxicity outcomes, dose-dependent effects of liver and serum oxylipin levels, and activities of enzymes in the cytochrome P450 monooxygenase-soluble epoxide hydrolase pathway, have been reported previously (Wu et al., 2016). Briefly, thirty-four male Sprague-Dawley rats (4 weeks old) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed in a controlled environment maintained at 22 °C with a 12-hour light and dark cycles (Wu et al., 2016). Rats were fed a basal diet (Harlan 7913 with 18% protein, 6% fat and 5% fiber) and water *ad libitum*. Animals were acclimatized to the animal facility for one week, randomized into 4 groups (n = 8-10 per group, 147 ± 8 g) and exposed to six biweekly intraperitoneal injections of corn oil (vehicle; 5 mL/kg b.w.) or PCB 126 in corn oil (5 mL/kg b.w.) at the doses of PCB 126: low (0.01 µmol/kg bw), medium (0.05 µmol/kg bw) and high (0.2 µmol/kg bw) (Table 1). These doses were selected based on published studies (Alvarez-Lloret et al., 2009; Lai et al., 2010b) and expected to perturb lipid and oxylipin profiles in our animal model without causing overt toxicity. Two-week after the last dose, the rats were euthanized by CO₂ asphyxiation followed by thoracotomy as confirmation of death. Tissues were excised *en bloc* and samples stored at -80 °C until further analysis.

2.2 Lipid extraction from liver homogenate

Lipid was extracted from whole liver homogenate following a procedure from the Kansas Lipidomics Research Center with some modification (Kansas Lipidomics Research Center,

2016). Briefly, the liver samples (0.51 ± 0.08 g) were homogenized in 10 mL water. One aliquot of 1.6 mL homogenized liver was used for lipid extraction. Another aliquot of 1.0 mL homogenized liver was used for protein analysis. For the aliquot of liver sample for lipid extraction, two mL chloroform containing 0.01% butylated hydroxytoluene (BHT) and 4 mL methanol were added to a 1.6 mL aliquot of homogenized liver sample. Samples were inverted for 5 min, followed by addition of 2 mL chloroform with BHT and 2 mL water. Samples were inverted for an additional 5 min and centrifuged at 3000 g for 5 min. The lower layer were removed (the samples were kept on ice as much as possible). The top layer was extracted two more times with 2 mL chloroform with BHT. The combined organic layers were washed with 0.5 mL 1M KCl and 0.5 mL water. The samples were evaporated under a gentle stream of nitrogen gas, vacuum dried, and stored at -80°C . Samples were shipped on dry ice to the Kansas Lipidomics Research Center for analysis. Protein concentrations of liver homogenate were measured with the Lowry assay (Lowry et al., 1951), with bovine serum albumin as standard.

2.3 Lipidomic analyses

Lipidomic analyses were performed by the Kansas Lipidomics Research Center (<http://www.kstate.edu/lipid/>). The details of the analyses were described previously (Welti et al., 2002; Welti et al., 2003). Briefly, Applied Biosystems API 4000 and Applied Biosystems Q-TRAP (Foster City, CA, USA) tandem mass spectrometers connected to a collision cell and working in electrospray ionization mode were used for the polar lipid and FA_{TAG} analysis, respectively. Solutions of the lipid extracts were introduced by continuous infusion. Depending on the lipid class, either precursor scan or neutral loss scans were used, and the characteristic scans were performed sequentially during a single analysis. Polar lipids and FA_{TAG} species, including ether-linked polar lipid and ADAG species, containing saturated (SFAs), monounsaturated (MUFAs) and PUFAs were quantified after background subtraction, smoothing, integration, correction for isotope overlap and comparison of sample peaks to peaks in the internal standard. Individual polar lipid and FA_{TAG} species were normalized against the internal standards listed in Table S1 as described previously (Welti et al., 2002; Welti et al., 2003). The normalization was performed in such a manner that one unit of the signal corresponds to 1 nmol of the respective internal standard. Levels of all polar lipid species were subsequently expressed as normalized signal per mg protein. Because the response of individual species within a particular class of polar lipids is typically similar to the response of the respective internal standard, polar lipid data expressed as normalized signal/mg protein were considered to be equal to nmol/mg protein. The response of individual FA_{TAG} species varies compared to the internal standard and only allows a comparison of the relative amount of lipid species across samples. All FA_{TAG}s levels are reported as normalized signal/mg protein.

Lipids species were identified based on the m/z of the intact ion and the mass of a fragment formed during the analysis, such as the head group fragment for polar lipids or an acyl fragment for neutral lipids. The nomenclature used for polar lipids indicates the polar lipid species (see Table S2), the total number of acyl/alk(en)yl carbon, and the total number of double bonds. For example, PS(38:6) is a phosphatidylserine species with a total of 38 acyl carbons and 6 double bonds. The FA_{TAG} nomenclature indicates the mass $[M + \text{NH}_4]^+$ of the

acyl fragment (Table S3), the number of carbons: number of double bonds of the acyl/alk(en)yl fragment, and the number of total acyl/alk(en)yl carbons: total number of double bonds of a TAG species. For example, NL299 18:1 54:4 represents an acyl fragment of an octadecenoic acid (*i.e.*, 18:1), most likely oleic acid, incorporated into a TAG containing a total of 54 acyl carbons and a total of 4 acyl double bonds (*i.e.*, 54:4). This approach does not allow an identification of the other two acyl chains in the TAG nor the position of the acyl chain on the glycerol backbone. However, the fatty acid composition of polar lipids and TAGs in rats is well established and allows a tentative identification of specific lipid species. A summary of the polar lipid and TAG classes analyzed and the respective abbreviations are shown in Tables S2 and S3.

2.4 Statistical analysis

Univariate ANOVA with Tukey post-hoc test on lipids by group was performed in R (www.rproject.org, version 3.1.2). The differences were considered significant for $p < 0.0005$. Multivariate analysis and accompanying ANOVA with Fisher LSD post-hoc test was performed in MetaboAnalyst on data adjusted by mg protein (<http://www.metaboanalyst.ca>) (Xia and Wishart, 2011a, b). Differences were considered significant for $p < 4 \times 10^{-11}$.

3. Results

Dose-dependent changes in the profiles of polar lipids and FA_{TAGs} were investigated in the liver of male rats following chronic PCB 126 exposure. The sum of polar lipids in the liver increased in a dose-dependent manner from vehicle-exposed animals (390 μmol per liver) to the high dose exposure group (590 μmol per liver; 1.5-fold increase) (Fig. 1A). The sum of FA_{TAGs} in the liver increased 2-fold in livers from the low dose exposure group (Fig. 1B). An even more pronounced increase in total FA_{TAGs} in the liver was observed in the medium and high dose exposure groups, with a 6- and 8-fold increase in total FA_{TAGs} compared to vehicle-exposed animals, respectively. These observations are consistent with our earlier, gravimetric determination of the extractable lipid content in the liver from the same animals investigated in this study (Wu et al., 2016). Despite the overall increase in the polar lipid content of the liver, protein adjusted levels of polar lipids remained relatively constant across all four exposure groups, which is due to the previously described increase in the protein content of the liver from the same PCB 126 exposed rats investigated herein (Wu et al., 2016) (Fig. 2A). The protein adjusted levels of FA_{TAGs} increased with increasing PCB 126 dose (Fig. 2B).

3.1. PCB 126-mediated changes in classes of polar lipids

The lipidomics approach allowed the quantification of a total of 362 individual polar lipid species belonging to 13 different lipid classes, including the major phospholipid classes as well as their ether-linked and lyso derivatives (Table S2). On a molar basis, the major polar lipids in the vehicle-exposed rat liver were PC (66%), followed by PE (16%) and SM & DSM (7%) (Fig. S1). The remaining lipid classes, including PI, ePC, PS, LPC, ePE, PG, LPG, PA and ePS, PE-Cer, accounted for less than 4 % of total polar lipids in the liver from control animals. No drastic change in the relative composition of the polar lipid classes investigated was observed in the liver following PCB 126 exposure. However, PCB 126

exposure caused a significant, dose-dependent decrease in protein adjusted total levels of 6 out of the 13 polar lipid classes investigated in the liver (Fig. 3). Specifically, total PS, ePS and ePE levels decreased in a dose-dependent manner compared to vehicle-exposed animals; however, this change did not reach statistical significance for total ePS and ePE levels in the low dose exposure group. PCB 126 exposure also resulted in a significant decrease of total ePC, LPE and PG levels in the high dose exposure group. Total PC, PA, LPC, PE-Cer and SM & DSM levels were not affected by PCB 126 exposure at any PCB 126 dose investigated.

3.2 Effect of PCB 126 on levels of individual polar lipids in rat livers

The profiles of individual polar lipids in the liver from control animals and their dose-dependent changes in response to PCB 126 exposure are shown in Figs. S3-S14. The major PC, PE, PS and PI lipids in the liver from control animals were 38:4 species that, based on their molecular weight and the well documented positional distribution of fatty acids in these polar lipid classes in the rat liver (Wood and Harlow, 1969a; Wood and Harlow, 1969b), likely contained AA and stearic acid. Moreover, the major ether-linked PC, PE and PS species observed in our study are precursors of AA and included ePC(38:4), ePE(38:4) and ePS(38:4). In addition, phospholipids lipids incorporating linoleic acid appeared to be major lipid species present in the liver of control animals, including PC(36:4) and PC(34:2); PE(36:4) and PE(34:2); PA(36:4) and PA(34:2); and PG(34:2). Major lyso derivatives of PC and PE also contained AA or linoleic acid, including LPC(20:4), LPC(18:2) and LPE(20:4). In contrast, the major SMs and DSMs detected in the livers from vehicle exposed animals contained mostly saturated fatty acids and included SM(24:0) and SM(24:1), followed by SM(16:0), SM(18:0) and DSM(22:0). PE-Cer lipids were detected at comparatively low levels in a few liver samples.

PCB 126 exposure had no clear, dose-dependent effect of the levels of individual lipids within the lipid classes investigated, with levels increasing for some lipids species, but decreasing for other lipid species with increasing PCB 126 dose (Figs. S3-S14). For example, protein adjusted PC(38:4) levels increased with increasing PCB 126 dose, whereas levels of several other 38:4 phospholipids decreased with increasing dose. Partial least square-discriminant analysis (PLS-DA) was therefore used to identify individual polar lipids showing the most pronounced dose-dependent change in the rat liver (Fig. 4A4). Components 1 and 2 clearly distinguished the different dose groups and together explained 35% of the data variation. The list of individual lipids with high Variable Importance in Projection (VIP) scores identified by Component 1 overlapped well with the lipids identified based on their low p-values in the ANOVA (Figs. 4B and 4C), with 16 lipids identified using both approaches (Table S4). The twenty most important lipid species identified based on their VIP score belong to the following classes: PC (6 lipid species), PS (4 lipid species), ePC (3 lipid species), PI (3 lipid species), PE (2 lipid species) and ePE (2 lipid species).

Specific lipid species identified with the PLS-DA include PC(34:2), PC(36:2), ePC(36:4), PE(34:2), PE(36:4), PS(36:2), PS(36:4) and PS(38:6), major phospholipids in the liver (Fig. 4B). The levels of the majority of individual lipid species with a high VIP score decrease with increasing dose of PCB 126; however, some lipid species, including PC(40:5),

PC(36:2), PS(44:3) and PI(36:2), increased with increasing PCB 126 dose (Fig. 5). Although the molecular structure of these lipid species cannot be unambiguously determined, it is likely that they contain one chain of a saturated fatty acid at the C-1 position and one chain of a PUFA at the C-2 position (McMurray and Magee, 1972). Thus, the presence of two or four double bonds in a majority of lipids identified using multivariate analysis (*i.e.*, 12 out of the top 20) suggests that they contain arachidonic or linoleic acid. Additionally, eight (out of the top 20) lipids with a high VIP score appear to contain 16:0 combined with either 20:4 or 22:6. The presence of ether-linked lipids, such as ePCs and ePEs, among lipids with a high VIP score is a particularly interesting observation. These lipids include ePE(36:4), ePE(38:6) and ePC(36:4), with ePE(36:4) being the major lipid responsible for the grouping of samples by PCB 126 exposure group (Figs. 4 and 5).

3.3 Effect of PCB 126 of profiles of fatty acids incorporated into TAGs

A total of 607 FA_{TAG} species containing 15 individual fatty acids, including three SFAs (*i.e.*, 14:0, 16:0 and 18:0), three MUFAs (*i.e.*, 14:1, 16:1 and 18:1) and 9 PUFAs (*i.e.*, 18:2, 18:3, 20:2, 20:3, 20:4, 20:5, 22:4, 22:5, and 22:6), were quantified in the liver, thus allowing an assessment of the composition of fatty acids incorporated in hepatic TAGs (Table S2). Based on the total hepatic FA_{TAG} signal, the majority of FA_{TAG} in the liver from vehicle-exposed rats were 18:2 (33%) and 18:1 (26%), followed by 16:0 (23%) (Fig. S2). The remaining FA_{TAGs} accounted for 5% or less of the total fatty acids incorporated into TAGs. ADAGs were minor tissue components compared to TAGs and accounted for approximately 3% of the total hepatic signal. No drastic change in the relative composition of FA_{TAGs} was observed in the liver following PCB 126 exposure.

The protein adjusted levels of FA_{TAGs} in the liver typically increased with increasing dose of PCB 126 (Fig. 6). Specifically, levels of 16:1, 16:0, 18:3, 18:2, 18:1, 18:0, 20:4, 20:2, 22:5 and 22:4 fatty acids increased significantly in the liver from animals in the medium and high dose exposure groups compared to controls, whereas no significant differences were observed for any FA_{TAG} between control and low dose exposure groups. Adrenic acid (22:4) showed the most pronounced increase with increasing PCB 126 dose and, on a molar basis, was 20-fold higher in the high dose compared to the control group. Changes in hepatic FA_{TAG} levels varied only slightly between the medium and high dose exposure groups and no consistent trend was observed. Stearic acid was the only exception and increased significantly between the medium and high dose exposure groups.

PLS-DA was used to assess the effect of increasing doses of PCB126 on levels of individual FA_{TAG} species in the male rat liver. Components 1 and 2 distinguished the different dose groups and explained 69% of the data variation (Fig. 7A). The list of individual FA_{TAG} species with high VIP scores identified by component 1 (Fig. 7B) included 16:1, 18:0, 18:1, 18:2, 18:3 and 20:4 fatty acids. There was a good overlap with FA_{TAG} species identified based on their low p-value in the ANOVA (Fig. 7C), with 13 FA_{TAG} species identified by both approaches (Table S5). Two of the 18:1 (NL299 18:1 54:4 and NL299 18:1 54:3 in Fig. 8) and one of the 18:2 containing TAG species (NL297 18:2 54:4 in Fig. 8) identified are relatively major constituents of hepatic TAGs, with the respective FA_{TAGs} accounting for approximately 2 to 4% of the total FA_{TAGs} in the liver of control animals. Interestingly, PLS-

DA identified six and ANOVA identified three ADAGs incorporating 18:0, 18:1 and 18:2 among the top 20 species identified (Fig. 7B; Table S5). These ADAG species are only minor TAG constituents in the rat liver. Overall, all FA_{TAGs} shown in Fig. 8 followed the same trend observed for the total TAGs and increased with increasing PCB 126 dose.

4. Discussion

PCB 126 is a potent AhR agonist and a hepatotoxicant that causes oxidative stress, wasting syndrome and disturbs metal homeostasis. One hallmark of PCB 126 exposure is liver steatosis in rodent models (Klaren et al., 2015; Lai et al., 2010a; Lai et al., 2011; Lai et al., 2012). For example, histological examinations revealed that acute intraperitoneal exposure to PCB 126 caused a gradual increase in hepatic lipid levels, with lipid levels being significantly higher compared to controls of days 6 and 12 after exposure (Gadupudi et al., 2016; Klaren et al., 2015). These changes were accompanied by diffuse vacuolar changes that are apparent as early as 18 h after PCB 126 exposure. Other AhR agonists, for example TCDD (Angrish et al., 2013; Angrish et al., 2011; Angrish et al., 2012; Forgacs et al., 2012; Nault et al., 2017) and polycyclic aromatic hydrocarbons (Jin et al., 2014; Kawano et al., 2010), also cause fatty liver in rodent models. PCB 126-mediated hepatic steatosis is accompanied by changes in the profiles of polyunsaturated fatty acids (PUFAs) and polar lipids (Matsusue et al., 1997, 1999; Wu et al., 2016). As we have shown in tissues from the same animals used in this study, PCB 126 exposure also had a pronounced effect on liver and serum levels of PUFA-derived oxylipins (Wu et al., 2016). This observation is important because changes in the balance between pro- and anti-inflammatory oxylipins may play an important, but poorly characterized role in NAFLD in humans (Wells et al., 2016). Studies with AhR null mice demonstrate that fatty liver and altered oxylipin homeostasis caused by TCDD exposure are AhR-mediated effects (Bui et al., 2012; Fernandez-Salguero et al., 1996). Although studies in AhR null rats are needed to confirm this hypothesis, it is likely that the hepatic lipid accumulation and changes in oxylipin levels observed in PCB 126 exposed rats are similarly mediated by AhR.

Because changes in the global lipid profiles are potential markers of PCB 126-mediated hepatotoxicity, the present study characterized dose-dependent changes in profiles of polar lipids and FA_{TAG} in the liver from rats chronically exposed to PCB 126. The major polar lipid classes in the liver from control rats were PC, followed by PE, SM and DSM, which is in agreement with earlier studies reporting the polar lipid composition of the rat liver (Belina et al., 1975; Meftah and Skett, 1989; Patton et al., 1982). The major PC, PE, PS and PI lipids in the liver from control animals were 38:4 species, likely containing AA and stearic acid. Similarly, several other studies demonstrate that 38:4 species are major phospholipids in the rat liver (Burdge et al., 1994; Wood and Harlow, 1969b). Moreover, the fatty acid composition of TAGs in our study was similar to earlier studies (Fukuchi et al., 2004; Tanaka et al., 2013; Wood and Harlow, 1969b), with linoleic acid, oleic acid and palmitic acid being major FA_{TAGs} in the whole rat liver. These three fatty acids are also major FA_{TAGs} in the mouse liver (Nault et al., 2017) and are abundant constituents of rodent chow (Angrish et al., 2012). While the polar lipid and FA_{TAG} composition observed in these earlier studies is consistent our findings, the rank order of individual lipid species differed between studies. These minor differences are not surprising because of differences in the

analytical approach (*i.e.*, whether lipids and TAGs were analyzed together or separately) as well as experimental parameters, such as the rat strain, age, sex and diet.

Chronic PCB 126 exposure resulted in a dose-dependent decrease in the total levels of several polar phospholipids, in particular total PS, in the whole rat liver. The dose-dependent decrease in the levels of PS(38:4), the major PS species in the liver, likely made the major contribution to the overall decrease in the hepatic levels of this lipid class. Similarly, a decrease in PS levels has recently reported in TCDD exposed male and female mice (Nault et al., 2017). PLS-DA and ANOVA identified several individual PS species, including PS(38:6), PS(36:2) and PS(44:3), as sensitive markers of PCB 126 exposure in the rat liver. These three PS species showed no consistent trend with increasing PCB 126 dose, with PS(38:6) and PS(36:2) showing a dose dependent decrease and PS(44:3) displaying a dose dependent increase in liver levels. Although PS is a relatively minor polar lipid constituent in the mammalian liver, PS lipids comprise a considerable percentage of plasma and endoplasmic reticulum membranes. Because of the increase in the volume of the endoplasmic reticulum in PCB-exposed animals (Connell et al., 1999), the decrease in PS levels following PCB 126 exposure is unexpected. Changes in the expression and activity of enzymes involved in the synthesis of PS, such as PS synthases or PS decarboxylases, or post synthesis remodeling of the fatty acid composition by phospholipases may contribute to the decrease in PS levels following PCB 126 exposure. While PCB 126 does not alter the activities of acyltransferases involved in the post synthesis remodeling of polar lipid (Matsusue et al., 1999), there is some evidence that TCDD increases the expression of phospholipase A2, another key enzyme involved in post synthesis lipid remodeling (Bui et al., 2012; Forgacs et al., 2012). The effect of acute and chronic PCB 126 exposure on the expression and activities of PS synthases or PS decarboxylases have not been investigated to date; however, a recent study in mice demonstrates that TCDD exposure affects the expression of enzymes in PS synthetic pathways, possibly by mechanisms involving AhR (Nault et al., 2017). Moreover, it is unknown if the PCB 126-mediated decrease in hepatic PCB levels affects the intra- and extracellular functions of PS within the liver, for example their role in apoptosis and the modulation of the catalytic activity of intracellular signaling proteins, such as Akt (Vance and Tasseva, 2013).

In addition to effects on hepatic PS levels, PCB 126 exposure decreased total levels of ether-linked lipids, including ePS, ePE and ePC, in a dose-dependent manner. Ether-linked lipids identified in this study by PLS-DA and ANOVA included ePE(36:4), ePE(38:6) and ePC(36:4), suggesting that these lipids are potential markers for PCB 126-mediated fatty liver in rodents. It is noteworthy that a recent study identified ether-linked phospholipids, in addition to other plasma metabolites, as potential clinical markers differentiating NASH and steatosis (Gorden et al., 2015). Ether-linked phospholipids contain either 1-*O*-alkyl and/or 1-*O*-(alk-1'-enyl)-*sn*-glycerol moieties. ePC and ePE are major ether-linked lipids that typically occur at relatively low levels (<5%) in the mammalian liver (Braverman and Moser, 2012), but constitute 15-20% of cell membranes and are important component of subcellular membranes (Braverman and Moser, 2012; Nagan and Zoeller, 2001). The storage of PUFAs is one important cellular function of these lipids (Nagan and Zoeller, 2001). In addition, ether-linked lipids are structural components of cell membranes, can protect membrane lipids from oxidative damage and have been implicated in a range of diseases,

including metabolic disease (Dean and Lodhi, 2017). The observed decrease in hepatic reservoirs of ether-linked lipids with increasing PCB 126 dose is consistent with observations that PCB 126 exposure causes oxidative stress (Lai et al., 2010a; Lai et al., 2012) and activates metabolic pathways forming PFUA epoxide and diol lipid mediators in the liver (Wu et al., 2016).

Analysis of FA_{TAGs} revealed a significant, dose-dependent increase in levels of 16:1, 16:0, 18:3, 18:2, 18:1, 18:0, 20:4, 20:2, 22:5 and 22:4 fatty acids. Adrenic acid (22:4), a PFUA synthesized by elongation of AA (Harkewicz et al., 2007), showed the most pronounced increase with increasing PCB 126 dose, with a 20-fold increase in adrenic acid levels in the high dose exposure compared to the control group. The increase in levels of adrenic acid incorporated in hepatic TAGs may be due to an increase in the elongation of AA to adrenic acid and/or a decrease in adrenic acid catabolism by peroxisome-based β -oxidation (Hagve and Christophersen, 1986; Tran and Christophersen, 2001). Exposure to PCBs, including PCB 126, results in a decrease in peroxisomal fatty acid oxidation in male rats (Gadupudi et al., 2016; Robertson et al., 2007), which at least in part explains the observed decrease in adrenic acid levels incorporated into TAGs. It is noteworthy that a recent study implicated increasing levels of adrenic acid in NAFLD progression in both mice and humans, possibly by exacerbating hepatic inflammation (Nababan et al., 2017).

The effect of sub-acute PCB 126 exposure on the fatty acid composition in the rat liver has been studied previously; however, a comparison of our results with earlier studies (Matsusue et al., 1997, 1999) is challenging because of differences in the experimental approach: while our study determined profiles in polar lipids and fatty acids incorporated into TAGs in the whole rat liver, the studies by Matsusue *et al.* determined the fatty acid composition in the whole liver as well as the microsomal fraction. In the earlier study, exposure to a single i.p. dose of PCB 126 resulted in a significant increase in 18:2, 18:1 and 20:3, but a significant decrease in 16:0, 20:4 and 22:6 fatty acids five days after PCB administration in the whole rat liver. Particularly intriguing is the decrease in the levels of AA in the whole liver as well as the microsomal fraction. In contrast, we observed an increase in the levels of AA incorporated into TAGs and polar lipids, especially at the highest PCB 126 dose. It is interesting to note that TCDD increased total hepatic fatty acid content in mice compared to controls, but elicited no consistent effects on levels of individual fatty acids (Angrish et al., 2013). Time, dose, species and other experimental factors acting together likely modulate the complex biological processes leading to an increase in hepatic lipid accumulation in mammals exposed to PCB 126 and other AhR agonists. Indeed, Gadupudi *et al.* showed that the time-dependent increase in hepatic lipid accumulation was more severe on days 6 and 12 after PCB 126 exposure compared to earlier time points (Gadupudi et al., 2016).

In addition to changes in fatty acids incorporated into TAG, several ADAG species were identified as potential markers of PCB 126 exposure in the rat liver. The levels of all ADAG species in this study increased with increasing PCB 126 dose, which is opposite to the effect of PCB 126 exposure on hepatic levels of ePS, ePE and ePC species discussed above. Both ADAGs and ether-linked polar lipids are formed *via* 1-*O*-alkyl-2-acyl-*sn*-glycerol intermediates by the same intraperoxisomal synthetic pathway (Magnusson and Haraldsson, 2011). The 1-*O*-alkyl-2-acyl-*sn*-glycerol intermediates are subsequently metabolized to

neutral ADAGs or ether-linked polar lipids. The final fatty acid composition of ADAGs is the result of subsequent remodeling processes. In addition, the catabolism of neutral ADAGs vs. ether-linked polar lipids may contribute to the different effect of PCB 126 exposure on the hepatic levels of these both ether lipid classes. Although the physiological consequences of the increase in certain ADAG species are unclear, it is worth noting that higher ADAG levels have been reported in certain tumors, including human hepatocellular carcinoma, compared in normal tissue (Lin et al., 1978; Snyder and Wood, 1968). Taken together, these observations raise the question if changes in ADAG levels are also associated with and, possibly, markers of PCB 126-induced liver cancers in rodents (NTP, 2006).

5. Conclusions

PCB 126 exposure alters the hepatic lipid composition in the liver of rats. The lipidomic analysis of both polar lipids and FA_{TAGs} in the liver following chronic PCB 126 exposure identified not only overall changes in the levels of polar lipids and TAGs, but also allowed the identification of several sensitive markers of PCB 126 exposure, including changes in levels of total PS and several individual PS species; adrenic acid incorporated into TAGs; and several ether-linked polar lipids and ADAGs. These changes may be useful markers of the effect of PCB 126 exposure on the rat liver; however, additional studies are needed to determine if the changes observed in the liver are also present in the blood lipid profiles and, ultimately, if these changes are also present in humans exposed to PCB 126 and other dioxin-like compounds. Moreover, changes in ether-linked polar and neutral lipids and adrenic acid may play an unrecognized role in the complex etiology of fatty liver disease, and changes in the expression and activity of genes involved in their synthesis and catabolism warrant further attention, possibly as therapeutic targets for the treatment of fatty liver diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

PCB 126 exposure resulted in a dose dependent increase in the total amount of (A) polar lipids and (B) FA_{TAGs} in the rat liver. Data for polar lipids and FA_{TAGs} are expressed as the total, normalized signal per liver, see Materials and methods. The composition of polar lipids and FA_{TAGs} in the liver is shown in Figures S2 and S3. * Different from vehicle exposed rats, # different from the low dose exposure group. ANOVA with Tukey post-hoc test, $p < 0.0005$.

Figure 2.

PCB 126 exposure did not affect protein adjusted levels of (A) polar lipids, but increased protein adjusted levels of (B) FATAGs in a dose-dependent manner in the rat liver. Data for polar lipids and FATAGs are expressed as the total, normalized signal per mg protein, see Materials and methods. * Different from vehicle exposed rats, # different from the low dose exposure group. ANOVA with Tukey post-hoc test, $p < 0.0005$.

Figure 3.

Protein adjusted levels of six out of 13 classes of polar lipids, including ePC, ePE, LPE, PG, PS and ePS, decreased significantly with increasing dose of PCB 126 in the whole rat liver. Data are the sum of all polar lipid species within a lipid class (Table 1). * Different from vehicle exposed rats, # different from the medium dose exposure group, ^ different from the low dose exposure group. ANOVA with Tukey post-hoc test, $p < 0.0005$.

Figure 4.

Partial Least Squares-Discriminant Analysis (PLS-DA) and one-way ANOVA allowed the identification of polar lipid species showing pronounced, dose-dependent changes following PCB 126 exposure. (A) PLS-DA of the polar lipid data reveals clear groupings of rat liver samples according to PCB 126 dose, with Components 1 and 2 accounting for 35.4% of the data variability. There is a good overlap of (B) lipid species identified as the top 20 variables based on the Variable Importance in Projection (VIP) score of Component 1 and (C) polar lipid species with p values $< 4 \times 10^{-11}$ in the oneway ANOVA. See Table S4 for a detailed list of lipid species identified in panel C using one-way ANOVA.

Figure 5.

Individual polar lipid species identified by PLS-DA show clear, dose-dependent changes following PCB 126 exposure. Sixteen representative polar lipid species with high VIP scores are shown (see Fig. 4B). Most of these lipids were also identified using ANOVA (names of polar lipids that were not identified using ANOVA are shown in *italics*; also see Table S4). The nomenclature indicates the polar lipid species (see Table S2), the total acyl/alk(en)yl carbon, and the total number of double bonds. Box plots show normalized concentrations.

Figure 6.

The increasing dose of PCB 126 resulted in an increase in the levels of several important classes of FA_{TAGs} in rat liver. Data are expressed as the sum of normalized signals per mg protein, see Materials and methods. * Different from vehicle exposed rats, # different from the low dose exposure group, ^ different from medium dose. ANOVA with Tukey post-hoc test, $p < 0.0005$.

Figure 7.

Partial Least Squares-Discriminant Analysis (PLS-DA) and one-way ANOVA allowed the identification of FA_{TAG}s showing pronounced, dose-dependent changes following PCB 126 exposure. (A) PLS-DA of the FA_{TAG} data reveals partial groupings of rat liver samples according to PCB 126 dose, with Components 1 and 2 accounting for 69% of the data variability. There is a good overlap of (B) FA_{TAG}s identified as the top 20 variables based on the Variable Importance in Projection (VIP) score of Component 1 and (C) FA_{TAG}s with p values $< 4 \times 10^{-11}$ in the one-way ANOVA. See Table S5 for a detailed list of the FA_{TAG} species identified in panel C using one-way ANOVA.

Figure 8.

Individual FA_{TAG}s identified by PLS-DA showed clear, dose-dependent changes following PCB 126 exposure. Sixteen representative FA_{TAG}s with high VIP scores are shown (see Fig. 7B). Most of these FA_{TAG}s were also identified using ANOVA (FA_{TAG}s that were not identified using ANOVA are indicated in *italics*; also see Table S5). The FA_{TAG} nomenclature indicates the mass of the FA_{TAG} [M + NH₄]⁺, the number of carbons: double bonds of the FA_{TAG} and the total acyl/alk(en)yl carbons: total number of double bonds of the TAG species. Box plots show normalized concentrations.

Table 1

Summary of individual and cumulative PCB 126 dose, expressed on a molar or weight basis, for different exposure groups.^a

Group	Number of rats	PCB 126 dose/injection		Cumulative PCB126 dose	
		µmol/kg b.w.	µg/kg b.w.	µmol/kg b.w.	µg/kg b.w.
Vehicle (corn oil)	10	0	0	0	0
Low dose	8	0.01	3.3	0.06	19.8
Medium dose	8	0.05	16.3	0.3	97.8
High dose	8	0.2	65	1.2	390

^a Animals were acclimatized to the animal facility for one week, randomized into the four exposure groups, and exposed with six biweekly i.p. injections of corn oil (vehicle; 5 mL/kg b.w.) or PCB 126 in corn oil (5 mL/kg b.w.) for 3 months as described previously (Wu et al., 2016).