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PCB126 Inhibits Adipogenesis of Human Preadipocytes

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Abstract

Emerging evidence indicates that persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs), are involved in the development of diabetes. Dysfunctional adipocytes play a significant role in initiating insulin resistance. Preadipocytes make up a large portion of adipose tissue and are necessary for the generation of functional mature adipocytes through adipogenesis. PCB126 is a dioxin-like PCB and a potent aryl hydrocarbon receptor (AhR) agonist. We hypothesized that PCB126 may be involved in the development of diabetes through disruption of adipogenesis. Using a newly developed human preadipocyte cell line called NPAD (Normal PreADipocytes), we found that exposure of preadipocytes to PCB126 resulted in significant reduction in their subsequent ability to fully differentiate into adipocytes, more so than when the cells were exposed to PCB126 during differentiation. Reduction in differentiation by PCB126 was associated with downregulation of transcript levels of a key adipocyte transcription factor, PPAR γ , and late adipocyte differentiation genes. An AhR antagonist, CH223191, blocked this effect. These studies indicate that preadipocytes are particularly sensitive to the effects of PCB126 and suggest that AhR activation inhibits PPAR γ transcription and subsequent adipogenesis. Our results validate the NPAD cell line as a useful model for studying the effects of POPs on adipogenesis.

Keywords

Adipocyte; Preadipocyte; PCB126; Adipogenesis; AhR; PPAR γ ; Diabetes; Metabolic Syndrome

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SUPPLEMENTARY DATA DESCRIPTION

The supplementary data section contains extended data showing a more complete dose response to PCB126 pre-exposure (Supplementary figure 1), the MTT assay that confirms no significant cytotoxicity (Supplementary figure 2), the protein levels of adiponectin on PCB126 treatment (Supplementary figure 3), the qRT-PCR data from the non-differentiated cultures (Supplementary Figure 4), and the micrographs and FACS plots from the antagonist studies (Supplementary Figure 5). In addition, Supplementary Table I provides raw Ct values for the qRT-PCR assays.

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INTRODUCTION

There is now compelling evidence that exposure to persistent organic pollutants (POPs) is associated with an increased risk of developing metabolic syndrome and its associated pathologies, including diabetes and hypertension (Hotamisligil, 2006; Ruzzin et al., 2010; Thayer et al., 2012). One group of POPs, the Polychlorinated Biphenyls (PCBs), was originally manufactured for industrial applications because of their insulating and flame retardant properties. PCBs are biphenyls with 1 to 9 chlorines; PCB mixtures can contain up to 209 individual congeners, which differ in the number and pattern of chlorines on the biphenyl rings. While intentional commercial production of PCBs was discontinued in the late 1970s, PCBs are considered significant POPs that continue to accumulate in the environment because of their lipophilicity and persistence (Alonso-Magdalena et al., 2011; Everett et al., 2011; Lee et al., 2011; Narbonne and Robertson, 2014; Roos et al., 2013; Silverstone et al., 2012; Tang-Peronard et al., 2011; Ward et al., 2010). Toxic and biological effects of PCB congeners can vary widely depending on chlorination patterns. Exposure to certain PCB congeners is associated with the development of metabolic syndrome (Everett et al., 2011; Silverstone et al., 2012; Thayer et al., 2012). The coplanar PCBs PCB77 and PCB126, have been associated with the development of glucose intolerance in mice (Baker et al., 2013). However, the mechanisms by which these PCBs potentially cause metabolic syndrome are unknown.

Adipocytes provide a link between obesity and the insulin resistance that occurs in type II diabetes (Mlinar and Marc, 2011). Adipocytes are critical players in energy storage and metabolism. It is becoming clear that adipocyte dysfunction, rather than adipocyte number, is causally associated with the development of metabolic syndrome (Guilherme et al., 2008; Harwood, 2012). Adipocytes in diabetic patients exhibit aberrant production of adipokines, including reduction in secretion of adiponectin, a hormone that modulates a number of metabolic processes (Dunmore and Brown, 2013). Both obesity and lipodystrophies have been reported to cause insulin resistance, suggesting the critical need for functional adipocyte mass.

The mechanisms by which dysfunctional adipocyte tissue plays a role in the development of insulin resistance and diabetes are now beginning to be understood. Adipokines interact mainly with peripheral tissues (liver, brain, muscle, and pancreas) and regulate carbohydrate and lipid metabolism, energy expenditure, and feeding behaviors. Insensitivity or inability to produce leptin leads to increased fat mass whereas decreased adiponectin causes insulin resistance and increases free fatty acid circulation. Adipocytes producing normal levels of both leptin and adiponectin are thus essential for effective insulin signaling, and their dysfunction leads to disruption of insulin signaling (Mlinar and Marc, 2011). Adipocytes and fat tissue in general accumulate lipophilic toxicants such as PCBs and thus are likely to be affected by them (Regnier and Sargis, 2014).

Adipose tissue is comprised of progenitor preadipocytes and differentiated adipocytes along with other cells which include multipotent mesenchymal stem cells (MSCs) also referred as adipose tissue stem cells (ASCs) (Cawthorn et al., 2012). Although both ASCs and

preadipocytes can be differentiated into white adipocytes, the latter are more committed down the lineage to form adipose (Cinti, 2012; Hausman et al., 2001). Preadipocytes also make up a significant portion of fat tissue (15 to 50%) (Tchkonia et al., 2010). Under normal conditions, adipocyte tissue development begins during gestation and proceeds until adolescence by increased proliferation of preadipocytes and their subsequent differentiation into adipocytes (Knittle et al., 1979). After adolescence, the changes in fat mass are mostly attributed to changes in lipid accumulation with very little change in total cell number (Spalding et al., 2008). During adulthood, adipocyte death is balanced by proliferation and differentiation of preadipocytes to adipocytes (Tchkonia et al., 2010). Adipose tissue alters its mass by increase or decrease in adipocyte size and/or numbers in response to various stimuli. Adipocyte size increases by synthesis and accumulation of lipids. Too much lipid accumulation can lead to hypertrophy and dysfunction. Adipocyte number is increased by the proliferation of preadipocytes that later differentiate into adipocytes.

On physiological and nutritional demand, the preadipocytes are modulated by various hormones and growth factors to initiate a transcriptional cascade that programs adipogenesis (Gregoire et al., 1998). The most important event in this cascade is the transcription and activation of the nuclear receptor peroxisome proliferator-activated receptor- γ (PPAR γ), also called the master regulator of adipogenesis (Rosen et al., 1999). PPAR γ activates the transcription of genes that are involved in the development of mature functional adipocytes. Alterations in adipogenesis would be expected to lead to adipocyte dysfunction and thus increase the likelihood of developing insulin resistance and, subsequently, type II diabetes (Mlinar and Marc, 2011).

While primary preadipocytes can be isolated from adipose tissue and differentiated into mature adipocytes, they can be expanded for only a very short time *in vitro*, a characteristic that makes it difficult to assess the effects of environmental factors on adipocyte differentiation and function. Most studies have been limited to the use of immortal mouse preadipocytes called 3T3-L1 that differentiate into adipocytes (Green and Kehinde, 1975). Employing this model, certain POPs including PCB congeners were shown to alter adipocyte differentiation and fatty acid and cytokine release when applied to cells during the differentiation process (Arsenescu et al., 2008; Taxvig et al., 2012). Mouse cells provide one means by which the effects of PCBs on adipocytes can be tested. However, there are significant species-to-species variations in their sensitivity and in how PCBs affect physiology and fatty acid metabolism across rodents and humans (Forgacs et al., 2012). Studies using primary human MSCs to assess the effects of POPs on adipogenesis have been described (Li et al., 2008) but they are uncommon, most likely because primary MSCs are difficult to isolate and have a limited lifespan in culture. There have been reports on the immortalization of human MSCs or preadipocytes that retain the ability to differentiate into adipocytes (Darimont et al., 2003; Rodriguez et al., 2004; Terai et al., 2005; Zhang et al., 2006). Immortal MSCs can be differentiated into osteocyte, chondrocyte, or adipocyte lineages and are difficult to maintain in a preadipocyte state (Rodriguez et al., 2004; Terai et al., 2005; Zhang et al., 2006). An immortal human preadipocyte line, referred to as Chub-S7, was reported but, for unknown reasons, has not been widely used (Darimont et al., 2003).

We have recently developed an immortal human preadipocyte cell line from primary subcutaneous preadipocytes that can be readily differentiated into mature adipocytes that accumulate lipid droplets and express all the expected normal markers of adipocyte differentiation (Vu et al., 2013). This provides a valuable tool for the assessment of how POPs such as PCBs affect adipocyte differentiation and function in human cells. In the current study, we were interested in determining how adipogenesis was affected by PCB126, a PCB that has been implicated in the development of diabetes and metabolic disorders. We hypothesized that pre-exposure of human preadipocytes to PCB126 would subsequently reduce their ability to differentiate into mature, properly functioning adipocytes. Our results indicate that exposure of preadipocytes to PCB126 is effective at inhibiting subsequent adipocyte differentiation. We also found that activation of AhR by PCB126 was associated with reduction in PPAR γ transcript levels. These results suggest that preadipocytes may be an important target for POPs and point to a potential mechanism in which disruption of adipogenesis could lead to the development of metabolic syndrome.

MATERIALS AND METHODS

Preadipocyte culture and differentiation

All experiments were performed with extended lifespan Normal PreADipocytes (NPADs) developed from primary human preadipocytes that were derived from the subcutaneous fat tissue of a non-diabetic donor as recently described (Vu et al., 2013). The NPADs were cultured as a monolayer in Preadipocyte Basal Medium 2 (PBM-2) (Lonza, MD) supplemented with 10% fetal bovine serum (FBS), L-glutamine, gentamycin, and amphotericin according to the manufacturer's instructions. This media is referred to as preadipocyte growth media 2 (PGM-2). For differentiation, the NPADs were seeded into 35 mm tissue culture plates at 30,000 cells/plate and allowed to grow in PGM-2 until confluent (usually 5–6 days). The cells were then induced to differentiate into adipocytes with differentiation medium consisting of PGM-2 plus dexamethasone, 3-isobutyl-1-methyl-xanthine, indomethacin, and extra insulin prepared according to the manufacturer's instructions (Lonza, MD). The cells were left in the differentiation medium for 11 days until full development of lipid droplets occurred. Comparative control groups of confluent NPADs (preadipocytes) were cultured in normal PGM-2 without any differentiation factors for the duration of the normal time required for differentiation.

Reagents and treatment

PCB126 was obtained from the Synthesis Core of the Iowa Superfund Research Program (courtesy of Dr. Hans Joachim-Lehmmler). The AhR antagonist, CH223191, and all other chemicals were purchased from Sigma unless otherwise specified. PCB126 or CH223191 were dissolved in dimethylsulfoxide (DMSO) to a final concentration less than 0.01% (v/v) unless otherwise stated. Equivalent volumes of DMSO were used in treatments and negative controls. NPADs were treated with PCB126 mixed into PGM-2 in the pre-differentiation phase until confluence (5–6 days) after which the PCB was removed and the cells were differentiated into mature adipocytes without PCB126 for 11 days. We refer to this exposure regimen as “pre-exposure”. To assess the effects of PCB126 during differentiation, cells received PCB126 only during differentiation for 11 days. In the pre-exposure regimen,

preadipocytes (NPADs) are treated with PCB126 while they are still dividing, before addition of differentiation inducing agents. This is in contrast to exposure during the course of differentiation that occurs for 10–11 days after confluence and induction of differentiation.

Assessment of NPAD proliferation and growth

In order to assess the effects of PCB126 on cell proliferation, NPADs were seeded into 6 well plates at a density of 20,000 cells per well. On the following day (day 1), the cells were treated with the stated concentrations of PCB126 dissolved in DMSO (0.01% v/v final). The treatments were removed on day 6 and the media was changed with regular PGM-2 media or differentiation media. Cell growth was assessed by counting the number of cells at various time points (1, 3, 6 and 17 days) using a Z1 Coulter Counter (Beckman Coulter, CA). Cells treated with DMSO (0.01%) were used as a comparative negative control.

Assessment of NPAD viability using MTT assay

In order to determine the effects of PCB126 on cell viability, NPADs were seeded into 24 well plates at a density of 10,000 cells per well. On the following day, the cells were treated with the noted concentrations of PCB126 dissolved in DMSO (0.01% v/v). Cell viability was assessed at various time points (1, 3 and 6 days) using MTT assay. Briefly, the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) mixed into the media for 4 hrs (Mosmann, 1983). After 4hrs, the formazan was extracted into acidified isopropanol and incubated on the shaker for 15 min. Absorbance was measured at 570 nm using micro plate reader (Biotek, VT).

Triglyceride staining and microscopy

Differentiated cultures from various treatment groups were visualized by microscopy for lipid droplet formation. The cells were stained with Adipored™ (Lonza, MD) for 15 min. as described by the manufacturer and then washed with phosphate-buffered saline (PBS; 0.005 M NaPO₄, 0.15 M NaCl). Adipored™ is a fluorescent dye that binds to triglycerides. The photomicrographs of the stained cells were taken using a fluorescent microscope (Nikon) at 200X magnification. The images post-acquisition were processed using the Image J software package.

Quantification of differentiation using flow cytometry

The treated and differentiated cultures were quantified on a single cell basis by a flow cytometric approach as previously described (Barnes et al., 2003; Bernstein et al., 1989; Lee et al., 2004) with slight modifications. Cells were trypsinized by Trypsin-EDTA (0.25%) for 10 min and the trypsin was inhibited by adding an equal volume of 2% FBS in PBS. The cells were pelleted by centrifugation at 450 g (RCF) or 10 min., washed with PBS and re-suspended in 10% neutral buffered formalin (NBF). The fixed cells were washed with PBS and incubated with Adipored™ in PBS (0.025% v/v) for 15 min. The excess unbound dye after the staining was thoroughly washed with PBS and the cells were finally suspended in PBS for flow cytometry using a BD Accuri™ C6 machine (BD biosciences, CA). A total of 25,000 cells (events) were counted. Cells were gated based on cellular triglyceride

fluorescence stained by Adipored™ (excitation at 488 nm and emission at 530 nm). The mature/differentiated cell populations were identified based on the Adipored™ fluorescence (lipid content) and side scatter (complexity) caused by the lipid accumulation inside the cell. The flow cytometry analysis and gating were performed with BD Accuri C6 Software. Samples were analyzed in triplicate.

Quantification of differentiation markers using quantitative RT-PCR

RNA was collected from various treatment groups with and without induction of differentiation for analysis of RNA transcript levels. Briefly, cells were washed with PBS and the total RNA was extracted into 1 ml of Trizol reagent (Invitrogen, NY) and treated with DNase (Qiagen, CA) as described by the manufacturers. The mRNA was purified with RNeasy Mini Columns (Qiagen, CA) and was reverse transcribed to cDNA using a Retroscript kit (Ambion, CA) as described in the manufacturer's protocol. The relative quantification of gene transcripts was performed by quantitative reverse transcriptase polymerase chain reaction (q-RT-PCR) in triplicate as described (Gourronc et al., 2010; Taura et al., 2009; Vu et al., 2013). The primers used for the quantification of selected genes are listed in Table 1. The transcript levels of the analyzed genes in each sample were normalized to the transcript levels of a house keeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), which we found to be at consistent levels across treatments. The Ct values of both the house keeping gene (GAPDH) and the genes of interest obtained for all the samples are included in Supplementary Table 1. For graphing, the transcript levels were calculated relative to the normalized transcript levels of in undifferentiated NPADs treated with DMSO.

Quantification of Adiponectin using ELISA

Secreted adiponectin was measured by using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's protocol. Briefly, preadipocytes were pre-exposed to PCB126 or vehicle as described previously, allowed to become confluent, and differentiated for nine days. Consequently, the media was changed and four days later the whole media aliquots were collected and frozen at -80° C. ELISA was performed in replicate using the media supernatants. The unknown concentrations of adiponectin (ng/ml) were calculated using standards supplied by the manufacturer.

Statistics

The effects of PCB 126 treatment on NPADs at various concentrations and treatment groups across this study were compared with respect to vehicle control using one-way ANOVA. The possible interaction of both CH223191 and PCB126 during their co-incubation in the antagonist study was tested using two-way ANOVA. In two-way ANOVA, the interaction term was not reported if not significant ($P < 0.05$). All the error bars represent standard deviations (SD) from the mean in triplicate assays. All the statistical analyses were performed using GraphPad Prism (GraphPad Software Inc, CA)

RESULTS

Effects of PCBs on adipocyte differentiation

Previously reported studies (using mouse or human adipocytes) have mostly assessed the effects of various test chemicals, including PCBs, on adipocyte differentiation by treating cells with compounds during the differentiation process. To test our hypothesis that certain compounds might be active during the preadipocyte stage (before differentiation), causing changes that are revealed upon subsequent differentiation, we used a “pre-exposure” regimen of treatment and compared exposure during differentiation (see Materials and Methods). For both treatments, we used a higher dose of 10 μ M to ensure maximum changes in differentiation if it occurred. After 11 days in differentiation media, both the cultures were stained with Adipored™ for the visualization of lipid droplets by microscopy (Figure 1). Interestingly, pre-exposure of preadipocytes to 10 μ M PCB126 resulted in reduced differentiation compared to exposure during differentiation (Figure 1). The pre-exposure of NPADs to PCB126 caused a clear dose-dependent effect on differentiation (Supplementary Figure 1). Because of the non-homogenous distribution of differentiation in culture plates, it was difficult to precisely quantify levels of differentiation by microscopy alone. In order to better quantify differentiation, we analyzed the cells by flow cytometry. The differentiated cultures were trypsinized, stained with Adipored™, and the number of mature adipocytes in the suspension were identified by fluorescence and side scatter (SSC). As was found with microscopy, pre-exposure of the preadipocytes to PCB126 resulted in a significant, dose-dependent reduction in their differentiation compared to vehicle control (DMSO), and the decrease in differentiation was greater than that observed in cultures that had been treated with PCB126 during differentiation (Figures 2A and 2C). Even the low concentration of 0.5 μ M caused a significant reduction in differentiation compared to DMSO-treated controls (Figure 2C). These results suggest that the preadipocytes are profoundly sensitive to PCB126 compared to cells that are exposed during differentiation. This finding emphasizes that preadipocytes might be an important target for POPs. Because of these results, subsequent experiments were carried out using the pre-exposure regimen only.

Reduced differentiation on PCB126 exposure is independent of effects on proliferation

To rule out the possibility that the effects we observed were due to the selective cytotoxicity or growth inhibitory effects of PCB126 on preadipocyte proliferation, we performed cell counts at various time points during our pre-exposure treatment period while the cells were still proliferating. We found no significant differences in preadipocyte growth during pre-exposure to PCB126 at the concentrations used (Figure 3). These results indicate that the reduced differentiation caused by PCB126 is due to intrinsic changes in cell function(s) rather than selective inhibition or killing of specific cell subpopulations. In addition to no changes in cell counts from PCB exposure, we also did not observe significant changes in cell viability, as measured by the MTT assay (Supplementary Figure 2). It has been previously reported that murine preadipocytes undergo mitotic clonal expansion, even after they have reached confluence, after induction of differentiation during adipocyte development (Tang and Lane, 2012). Similar effects in human preadipocytes have not been yet reported. To determine whether pre-exposure to PCB126 affected any further clonal expansion during differentiation, the cells were trypsinized and counted after they had fully

differentiated (11 days after differentiation induction). While we observed an increase in the number of the cells after differentiation compared to their numbers at confluence after 6 days, no significant difference was observed in the PCB126 treated cultures compared to untreated controls (Figure 3). These results indicate that the immortal human preadipocytes behave similarly to what has been observed for mouse preadipocytes in that they approximately double after induction of differentiation, but that PCB126 treatment does not affect this process.

PCB126 pre-exposure reduces expression of adipocyte marker genes and increases expression of CYP1A1

To further examine how PCB126 affects adipocyte differentiation, we examined the transcript levels of specific genes important for adipocyte differentiation and function. Adiponectin (ADIPOQ) is an important hormone specifically secreted by functionally differentiated adipocytes. Lower levels of adiponectin are strongly associated with the development of insulin resistance caused by adipose tissue dysfunction (Ye and Scherer, 2013). Quantitative RT-PCR analysis of differentiated adipocytes pre-exposed to PCB126 demonstrated a dose-dependent reduction in transcript levels of adiponectin (Figure 4A, Supplementary Table I). We also found that the protein levels of adiponectin secreted into the medium were also reduced in PCB126 treated cultures (Supplementary Figure 3). In addition to adiponectin, another adipocyte specific gene FABP4 (fatty acid binding protein 4) also showed a similar dose dependent decrease in transcript levels after exposure to PCB126 (Figure 4B, Supplementary Table I). The most important event during the adipocyte differentiation is the transcriptional activation of PPAR γ , and it has been demonstrated that activation of PPAR γ is necessary and sufficient for induction of adipogenesis (Rosen et al., 1999). We found that PPAR γ transcript levels were downregulated by pre-exposure to PCB126 (Figure 4C, Supplementary Table I), indicating a possible mechanism by which PCB126 modulates differentiation.

To confirm the known AhR agonist activity of PCB126, we measured the transcript levels of CYP1A1, a classic downstream target of AhR which is activated by dioxin-like compounds (Mimura and Fujii-Kuriyama, 2003). CYP1A1 levels increased dose dependently in the PCB126 pre-exposed cells, both in differentiated cultures and undifferentiated cultures (Figure 4D and Supplementary Figure 4), indicating activation of the AhR by PCB126. Notably, the CYP1A1 induction persisted over an 11-day period after the PCB126 pre-exposure.

PCB126 induced AhR activity reduces differentiation of preadipocytes

In addition to AhR mediated toxicity, PCB126 and other dioxin-like compounds have also been implicated in causing several AhR independent effects in various tissues (Patel et al., 2009). To determine whether the observed inhibition of differentiation by PCB126 was through the AhR, we utilized an AhR antagonist CH223191 (Choi et al., 2012). Co-incubation of pre-adipocytes during the PCB126 pre-exposure period with CH223191 prevented upregulation of CYP1A1 transcript levels, determined at the end of the differentiation period (Figure 5C, Supplementary Table I). This indicated that AhR activation was effectively inhibited by the antagonist. CH223191 also partly hindered the

ability of PCB126 to inhibit adipocyte differentiation as assessed by flow cytometry (Figure 5A) and microscopy (Supplementary Figure 5). The reduction in transcript levels of both the early differentiation marker PPAR γ (Figure 5B) and late differentiation marker ADIPOQ by PCB126 was also mitigated on co-treatment with CH223191 (Figures 5B and 5D, Supplementary Table I). Interestingly, CH223191 treatment alone increased adipocyte differentiation, suggesting that it has effects on its own or, possibly, that a low level of endogenous AhR activation exists at baseline that partially inhibits or regulates differentiation. The exposure to CH223191 alone may be preventing this activation. Overall, our results indicate that the effect of PCB126 on adipocyte differentiation is at least partly mediated through the AhR.

DISCUSSION

The current studies demonstrate that pre-exposure of human preadipocytes to PCB126 significantly affects the ability of these cells to differentiate into mature adipocytes. The PCB126 treated cells showed decreased transcript levels of PPAR γ , a key regulator of adipogenesis/adipocyte differentiation, concomitant with reduction in transcript levels of adipogenic marker genes such as ADIPOQ and FABP4. These studies, using a newly developed human preadipocyte cell line, identify preadipocytes as important, sensitive targets for environmental toxicants such as PCBs and point to a potential mechanism by which PCB exposure can lead to the development of metabolic disorders such as diabetes.

Epidemiological studies provide a link between PCB exposure and the development of diabetes (Silverstone et al., 2012). Elevated PCB levels in serum were found to be associated with a higher incidence of diabetes in individuals of the Anniston, AL community that were subjected to high level PCB exposures due to their historic release into the environment from a nearby Monsanto chemical factory (Silverstone et al., 2012). Other cohorts exposed to contaminated oil comprised of dioxins and dioxin-like PCBs were reported to have an increased risk of type II diabetes (Everett et al., 2011; Henriksen et al., 1997). Body burden of dioxin-like PCB congeners (PCB 77, 81, 126) along with other PCBs were also found to be associated with increased risk of metabolic disorders in an exposed Japanese population (Uemura et al., 2009). A significant positive correlation between serum concentrations of PCB126 and the risk of diabetes was demonstrated in a U.S. study (National Health and Nutrition Examination Survey) (Everett et al., 2011; Everett et al., 2007). In addition to epidemiological evidence, mouse studies support a role for coplanar PCBs such as PCB126 and PCB77 in the impairment of glucose homeostasis along with elevation of inflammatory cytokine TNF α , specifically in adipose tissue (Baker et al., 2013).

Compromising the ability of preadipocytes to differentiate into functional adipocytes, either during development or later in life, would be expected to disrupt insulin signaling and metabolism, thus potentially leading to the development of diabetes. Adipose tissue development can be affected by various factors including genetics, diet, lifestyle and environment and POPs such as PCB126 (Knittle et al., 1979; Spalding et al., 2008; Tchkonina et al., 2010). Changes in the ability of preadipocytes to differentiate or proliferate in children and adolescents put them at risk for altered adipose tissue development caused by POP exposure. However, chronic exposure to POPs in adults may also lead to dysfunctional

adipose tissue because of the need for replacement of mature adipocytes caused by cell death.

POPs, such as PCBs, can accumulate in lipophilic tissues and thus the adipose tissue is considered to be the principal contributor to the total body burden of these accumulated compounds (La Merrill et al., 2013). Uptake of PCBs into adipocytes is dependent on their lipophilicity and the concentration of PCBs that accumulate is proportional to the triglyceride (TG) content of the adipocytes (Bourez et al., 2013). *In vitro* studies with mouse adipocytes have demonstrated that lipophilic congeners such as PCB126 can reach concentrations as high as 15 mol PCB/mol TG in mature adipocytes (Bourez et al., 2012). There is evidence that accumulated toxicants can be released from triglyceride storage pools during weight loss and thus may be released into the adipose tissue cellular microenvironment. This would further expose preadipocytes and potentially disrupt proper differentiation into adipocytes. In addition, the release of PCBs from adipose tissue into the blood may further affect other tissues and organs in the body (Kim et al., 2011).

How preadipocyte exposure to PCBs disrupts subsequent adipocyte differentiation is not entirely clear. Circulating levels of PCBs have been related to changes in visceral and subcutaneous fat mass (Roos et al., 2013). Previous studies with both dioxin-like and other PCBs have reported inhibition of adipocyte differentiation in mouse 3T3-L1 adipocytes *in vitro* (Arsenescu et al., 2008; Hsu et al., 2010; Shimba et al., 2001; Taxvig et al., 2012). Dioxin treatment of mouse embryonic fibroblasts inhibits lipid metabolism and adipogenesis and, using cells from AhR knockout mice, it has been shown that much of this effect is mediated by AhR (Alexander et al., 1998). While not previously characterized for its ability to inhibit adipogenesis, PCB126's main mechanism of action is thought to be through activation of AhR (Hestermann et al., 2000; Ovando et al., 2010; Safe et al., 1998; Safe, 1994). Our experiments using the AhR antagonist, CH223191, would support a model in which AhR activation by PCB126 causes a reduction in adipocyte differentiation. On ligand binding, AhR changes its conformation in the cytosol to expose a nuclear localization signal (NLS) and thus translocates into the nucleus of a cell where it forms a heterodimer with Aryl hydrocarbon Receptor Nuclear Translocator (ARNT) to recognize xenobiotic recognition elements (XREs) in the promoter regions of responsive genes and activate their expression (Denison and Nagy, 2003; Okey, 2007). The transcriptional response mediated by AhR is regulated by various other coactivators and repressors in a cell-type specific manner (Beischlag et al., 2008; Puga et al., 2009). PCB126 and other dioxin-like molecules have been shown to induce expression of a number of genes including CYP1A1 (Mimura and Fujii-Kuriyama, 2003). We found that PCB126 exposure caused downregulation of PPAR γ , which would be expected to limit activation of adipocyte specific genes during the differentiation process. The downregulation of PPAR γ suggests that AhR inhibits PPAR γ transcription or activity, either directly or indirectly, or through activity of an upstream inhibitor of PPAR γ (Tontonoz and Spiegelman, 2008). Interestingly, the AhR antagonist alone caused some increase in the differentiation of preadipocytes, suggesting either that endogenous levels of AhR activity play a repressive role in adipocyte differentiation or that the antagonist has other targets. Another possibility is that PCB126 also affects adipocyte

differentiation through AhR independent mechanisms. Further studies will be needed to clarify these issues.

Regardless of the mechanism of inhibition of adipocyte differentiation by PCB126, it would appear that the effect is relatively persistent in that pre-exposure of preadipocytes to PCB126, followed by removal of the medium, still results in subsequent down-regulation of adipocyte specific transcripts. It is interesting to note that in our studies CYP1A1 transcripts were found to be up-regulated long after PCB126 removal, thus suggesting persistent AhR activation. The effects of PCB126 on adipocyte differentiation could be mediated by relatively transient epigenetic alterations such as histone modifications or by more permanent epigenetic alterations such as DNA methylation. Such mechanisms would not be unexpected for dioxin-like compounds. Dioxin exposure is associated with DNA hyper-methylation and certain effects of dioxin have been linked to epigenetic changes that are trans-generational (Lind et al., 2013; Manikkam et al., 2012). Interestingly, studies using the pluripotent mouse cell line, C3H10T1/2, have also indicated that dioxin exposure of these cells before differentiation inhibits their subsequent ability to differentiate into adipocytes, suggesting that dioxin induces sustained epigenetic alterations (Cimafranca et al., 2004). In addition, it has been demonstrated that exposure of mouse 3T3-L1 preadipocytes to the flame retardant BDE-47, another POP, resulted in changes in methylation, specifically at the PPAR γ promoter (Kamstra et al., 2014). Considering the potential long-term consequences of early life exposure to PCBs on future health, it will be of significant interest to determine whether PCB126 exposure of human preadipocytes causes persistent epigenetic alterations. Because of their significantly extended lifespan compared to primary preadipocytes, the immortal NPAD cells may allow such an assessment using human cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- PCB126 decreases adipogenesis through its effects on preadipocytes.
- PCB126 does not decrease preadipocyte viability.
- PCB126 reduces transcript levels of PPAR γ , the master regulator of adipogenesis.
- The effects of PCB126 on adipogenesis are likely mediated by AhR.
- Immortal human preadipocytes are useful for assessing how POPs affect adipogenesis.

Figure 1. Preadipocytes show reduced ability to differentiate after pre-exposure to PCB126
NPADs were exposed to PCB126 (10 μ M) or DMSO before induction of differentiation (pre-exposure) (A) or during differentiation (B). After the differentiation period, the cells in both treatment regimens were stained for triglycerides with AdipoRed™ (red fluorescence). Fluorescent images are shown in the bottom panels and comparative bright field images are shown in the top panels.

Figure 2. Exposure to PCB126 causes a dose-dependent reduction in the number of differentiated adipocytes

Preadipocytes were treated with PCB126 at the concentrations of 0, 0.5 and 10 μM before differentiation (pre-exposure) (A) or during differentiation (B) and the differentiated cultures were analyzed by flow cytometry as described in the Materials and Methods. Differentiated cells were gated based on fluorescence and side scatter (represented as red dots). (C) The number of differentiated adipocytes is represented as the percentage of differentiated cells in a total of 25,000 events. Readings were performed on triplicate cultures. Preadipocytes that were pre-exposed to PCB126 before differentiation (black bars) exhibited significantly less differentiation than preadipocytes exposed during differentiation

(grey bars). Pre-exposure to PCB126 resulted in a statistically significant reduction in differentiated cells compared to vehicle control (* represents $P < 0.05$; ANOVA) and compared to exposure to the same concentrations during differentiation (# represents $P < 0.05$; ANOVA).

Figure 3. Effects of PCB126 on differentiation are independent of the cell proliferation
Cells were counted on days 1, 3, and 6 during the pre-exposure regimen to PCB126 concentrations of 0.5, 2 and 10 μ M. The cultures were confluent after 6 days and were subsequently induced to differentiate for 11 days and counted at 17 days post-seeding. DMSO was used as a vehicle control. All counts were performed on triplicate cultures. No significant variation in cell number (ANOVA, one-way; $P < 0.05$) due to PCB126 exposure either before or after differentiation was observed.

Figure 4. PCB126 exposure decreases transcript levels of adipogenic markers and increases transcript levels of CYP1A1

Preadipocytes were pre-exposed to PCB126, allowed to differentiate, and analyzed for expression of adipogenic markers and CYP1A1 expression using quantitative reverse transcriptase PCR (qRT-PCR). The transcript levels of both late adipogenic markers ADIPOQ (A) and FABP4 (B) and the early adipogenic marker PPAR γ (C) were decreased in a dose-dependent manner by exposure to PCB126 (ADIPOQ: adiponectin; FABP4: fatty acid binding protein; and PPAR γ : proliferator activated receptor gamma). (D) PCB126 induced a sustained and dose-dependent increase in transcript levels of CYP1A1. Transcript levels are expressed relative to the housekeeping gene GAPDH and normalized to undifferentiated preadipocytes treated with DMSO and cultured for the same period of time as the differentiated cultures (Supplementary Fig 2).

Figure 5. The AhR antagonist CH223191 partially blocks the inhibitory effects of PCB126 on adipocyte differentiation

Preadipocytes were treated with 2 μ M PCB126 (grey bars) or DMSO (black bars) and concurrently co-incubated with the AhR antagonist CH223191 (10 μ M) or DMSO and subsequently allowed to differentiate. (A) The percentage of differentiated cells was quantified using flow cytometry. There was no significant interaction between CH223191 and PCB126 during co-incubation and hence not reported (ANOVA, two-way; $P < 0.05$). The relative transcript levels of adipogenic markers PPAR γ (B) and ADIPOQ (D) in each condition were measured using qRT-PCR. (C) Transcript levels of CYP1A1 were measured to validate the efficiency of the CH223191 antagonist. Transcript levels are represented as relative to GAPDH and normalized to cultures treated with DMSO only.

Table I

Primers for the genes that were analyzed by qRT-PCR.

Gene name	Forward primer (5'–3')	Reverse primer (5'–3')
Peroxisome proliferator activated receptor gamma (PPAR γ)	GCC CAG GTT TGC TGA ATG TG	TGA GGA CTC AGG GTG GTT CAG
Adiponectin (ADIPOQ)	GCT CAG CAT TCA GTG TGG GA	GTA CAG CCC AGG AAT GTT GC
fatty acid binding protein (FABP4/ap2)	AAC TGG TGG TGG AAT GCG TC	TGC GAA CTT CAG TCC AGG TC
Cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1)	CCT TGG AAC CTT CCC TGA TCC	CGT GGC CGA CAT GGA GAT TG
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AAG GTC ATC CAT GAC AAC TTT G	GTA GAG GCA GGG ATC ATC TTC T