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Toxicity evaluation of exposure to an atmospheric mixture of polychlorinated biphenyls by nose-only and whole-body inhalation regimens

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Abstract

The health risk of inhalation exposure to polychlorinated biphenyls (PCB) cannot be assessed due to the lack of rigorous inhalation studies providing a low dose effect level. One large uncertainty rests on the exposure regimen. Whole-body exposure systems allow oral PCB intake that confounds the exposure. Thus, we sought to compare the whole-body and nose-only exposure methods by conducting contemporaneous PCB inhalation exposures. Vapor-phase PCBs were generated from the Chicago Air Mixture supplemented with PCB 11 (CAM+). Female Sprague-Dawley rats were exposed concurrently to a PCB concentration of $533 \pm 93 \mu\text{g}/\text{m}^3$, 4 h/day, 6 days/week, for 4 weeks. Congener-specific analysis using gas chromatography–mass spectrometry (GC/MS) showed higher total PCB concentration in the lungs of nose-only exposed animals than the whole-body exposed, resulting in a higher dose level for nose-only group. Congener profiles were consistent among exposure groups and tissue types and were dominated by PCB 28/31 and higher-chlorinated congeners reflecting rapid metabolism of other lower-chlorinated PCBs. No significant change was seen regarding metabolic enzyme expression, glutathione, or histopathology. However, diminished weight gain and reduced plasma total thyroxine levels were found in both groups compared with controls, with stronger response in the nose-only group. Lipid peroxidation was also elevated in the liver of nose-only exposed animals. We conclude that nose-only exposure was the preferred regimen for 4-wk PCB inhalation studies and thyroid hormone dysregulation was observed at an estimated dose of $1320 \mu\text{g}/\text{kg}$ b.w., providing information on a preliminary lowest-observed-adverse-effect-level (LOAEL).

Keywords

Polychlorinated biphenyl; Inhalation; Nose-only exposure; Whole-body exposure; Thyroid hormone; Risk assessment

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Supplementary Material

Chemical sources, tables listing instrumental calibration, quality assurance measures, ΣPCB concentrations in tissue samples of individual animals, individual congener concentrations in tissue samples (per gram of tissue weight and per gram of lipid weight) and hematology measurements; and figures showing elution chromatogram, XAD homolog profiles, tissue congener profiles, histopathology of liver tissue, free thyroid hormone levels, GSH/GSSG levels and gene expression results.

Introduction

Inhalation exposure to polychlorinated biphenyls (PCBs) in non-occupational settings has long been neglected, until recent recognition of its role in human exposure (Lehmann et al. 2014). Vapor-phase PCBs are emitted from a wide range of outdoor and indoor sources, including industrial facilities, waste sites, contaminated soil and water bodies (Martinez et al. 2010), building materials (Herrick et al. 2004) and household products (Rodenburg et al. 2010). While dietary intake of PCBs has declined dramatically over recent decades (Schechter et al. 2010), atmospheric PCB concentrations remain mostly unchanged (Sun et al. 2006). Moreover, exceptionally high PCB levels were found in caulk and other building materials, contributing to indoor air concentrations of orders-of-magnitude greater than in ambient air (Ampleman et al. 2014; Herrick et al. 2004). Inhalation can be a major exposure route in some indoor settings and for certain vulnerable population such as school children (Ampleman et al. 2014; Harrad et al. 2006; Lehmann et al. 2014; Thomas et al. 2012). It was estimated that the child PCB exposure level in New York City public schools exceeded the reference oral dose for Aroclor 1254, and over 70% of the dose was ascribed to inhalation (Thomas et al. 2012). In a recent cohort study investigating adolescent children and their mothers, inhalation exposure was calculated to account for as much as one-third of the total dietary plus inhalation exposure, in particular for many lower-chlorinated PCBs (Ampleman et al. 2014).

Well-characterized PCB inhalation exposure studies are urgently needed to establish dose-response relationships and to support health risk assessment (Lehmann et al. 2014). In particular, low-chlorinated congeners that were more volatile and thus dominate the airborne PCBs (Norstrom et al. 2009) were barely assessed for their exposure-induced health effects. The only study that has reported an adverse health outcome (Casey et al. 1999) found histopathological changes in the thyroid and thymus, increases in serum thyroid hormone and decrease in open field activeness of adolescent male Sprague-Dawley rats after inhalation exposure to Aroclor 1242 vapor. However, uncertainties in the Casey et al. study regarding exposure assessment and deprivation of food and water make it difficult to establish a reliable human health risk assessment (Lehmann et al. 2014). For example, animals exposed via the whole-body exposure regimen used in this study might have unquantified oral exposure as a result of grooming after PCB deposition on their fur (Thorne 2000). A nose-only exposure system would avoid the oral exposure by delivering the PCB-laden air stream directly to the nose of a restrained animal, yet the confinement might stress the animals during prolonged duration of exposure (Thorne 2000). It is therefore necessary to establish an exposure regimen that allows accurate assessment of the exposure dose as well as the associated toxicological responses.

Many PCBs that are released from non-Aroclor sources are still being produced during manufacturing of pigments and other industrial products, resulting in unexpected high environmental levels and widespread contamination (Anezaki and Nakano 2014; Anezaki and Nakano 2015; Hu and Hornbuckle 2009; Hu et al. 2010a). 3,3'-Dichlorobiphenyl (PCB 11), a signature congener for the non-legacy PCBs, is one of the most abundant and frequently detected congener throughout the Chicago airshed (Hu et al. 2010a). We supplemented the previously developed (Zhao et al. 2010) Chicago Air Mixture (CAM) with

PCB 11, seeking to create a mixture (named CAM+) for improved representation of Chicago air. For evaluation of the potential health effects, we targeted a higher dose level as our previous exposure to the CAM vapor (446 µg/(kg b.w.) generated minimal toxicity in female rats (Hu et al. 2012). We also tested for more endpoints as potentially sensitive biomarkers. For instance, thyroid hormone levels can be affected by extremely low concentrations of endocrine-disrupting chemicals (Vandenberg et al. 2012). These quantitative measures will be of great importance to inform inhalation risk assessment for PCBs, since most of adverse health effects have been reported from high dose studies using oral delivery and one congener at a time.

In this study we conducted contemporaneous PCB inhalation exposures in nose-only and whole-body exposure systems. The primary purpose was to compare the body burden and toxicity caused by the two exposure methods and to answer the following specific questions: 1) Do whole-body exposed animals have increased body burden, potentially attributed to additional oral exposure from grooming? 2) Do rats tolerate repeated 4 hour confinement during nose-only exposure? 3) Do the exposures cause biological effects compared to control animals and do the effects differ by the exposure regimens? Further, we anticipated that the information from this study would help establish the exposure dose range and toxicity endpoints for a future investigation with multiple dose levels and large numbers of animals.

Experimental Section

Chemicals

Congeners are designated by their IUPAC identities, numbered PCB 1 (2-chlorobiphenyl) through PCB 209 (decachlorobiphenyl). Sources of chemicals are listed in supplementary material.

Exposure to the CAM+ vapor

The CAM+ was composed of Aroclor 1242 and Aroclor 1254 (electrical grade, Monsanto lots KB-05-415 and KB-05-612) (Zhao et al. 2010) supplemented with PCB 11 (64.0%: 34.5%: 1.5%, w/w). The 4 L/min PCB-laden vapor was generated as previously described (Hu et al. 2012), and was diluted with 16 L/min clean air inside a mixing chamber and delivered at an equal flow rate of 9.0 L/min to a custom built whole-body exposure chamber (O'Shaughnessy et al. 2003) and to a radial nose-only exposure tower (SCIREQ InExpose System, Montreal, Canada) (Figure 1). Rats were housed individually inside the whole-body exposure chamber. Each rat in the nose-only group received direct positive flow from the tower to open-restraint tubes. The tubes were unsealed at the rear to allow for thermoregulation via the tail as well as for the drainage of rat excreta. Exhaust was captured in a secondary chamber and then driven through a PCB trap consisting of activated charcoal and HEPA filter at a flow rate of 12 L/min. The exposure atmosphere was sampled from the mixing chamber at a flow rate of 2 L/min with a XAD-filled metal cartridge collected every third day for analysis of PCB exposure concentrations. The whole system was placed in a 6 m³ glass and stainless steel walk-in chamber (not shown in Figure 1) for exhaust control and operated at negative pressure to ensure no leakage outward.

Animal treatment

All protocols were approved by the Institutional Animal Care and Use Committee. Animals were housed in our on-site vivarium with food and water provided *ad libitum* prior to exposure. After 2 wk acclimation period, 11-wk old female Sprague-Dawley rats (Harlan, Inc., Indianapolis, IN) were assigned to the nose-only group ($n = 8$; 236 ± 4 g), whole-body group ($n = 7$; 228 ± 3 g) or control group ($n = 6$; 218 ± 2 g). Heavier animals were systemically assigned to the nose-only group because their snouts provided an optimal fit in the nose-only system. The animals in the nose-only and whole-body groups were exposed to the CAM+ atmosphere 4 h/day, 6 days/week, for 4 wk. Between exposures, two animals from each exposed group were housed in metabolic cages in a fume hood to monitor fluid input/output and to sample excreta. The rest of the nose-only group were housed in our vivarium, while the rest of the whole-body group were housed in identical cages held within fume hoods in order to guard against PCB contamination of the vivarium. Upon termination of the exposure, animals were immediately euthanized with isoflurane followed by cervical dislocation. Whole blood was collected via cardiac puncture to prepare serum and plasma samples for analysis.

Analytical methodology for congener-specific PCB quantification

Five types of tissue samples were collected and analyzed to reflect PCB body burden after exposure: lung+trachea, serum, liver, brain and adipose tissue (taken from retroperitoneal fat depot). PCBs were extracted from XAD and tissue samples that were homogenized into diatomaceous earth (0.5 g each of lung+trachea, liver, brain, 1 mL serum and 0.2 g adipose tissue) using pressurized liquid extraction (ASE 200, Dionex, Sunnyvale, CA) as described previously (Kania-Korwel et al. 2007). Every sample was spiked with surrogate standards (20 ng deuterium-labeled PCB 30 and PCB 65). Prior to GC-MS analysis, extracts were further cleaned using concentrated sulfuric acid (Kania-Korwel et al. 2007), concentrated to a sample volume of 100 μ L, and then spiked with 20 ng ^{13}C -PCB 9 and ^{13}C -PCB 194 as internal standards. Each PCB mass was corrected for surrogate recovery.

A congener-specific analytical method was developed using a congener calibration set (AccuStandard, New Haven, CT) containing all 209 congeners. The quantification was carried out employing an Agilent 7890A GC system coupled with an Agilent 5975C Inert Mass Selective Detector operated in electron ionization mode. A capillary Supelco SLB-5MS GC column (Sigma-Aldrich, St Louis, MO, $30 \text{ m} \times 250 \mu\text{m} \times 0.25 \mu\text{m}$) was used under the following temperature program: hold at 80 $^{\circ}\text{C}$ for 1 min, 2 $^{\circ}\text{C}/\text{min}$ to 160 $^{\circ}\text{C}$, 1 $^{\circ}\text{C}/\text{min}$ to 170 $^{\circ}\text{C}$ and hold for 15 min, 1 $^{\circ}\text{C}/\text{min}$ to 180 $^{\circ}\text{C}$ and hold for 15 min, 1 $^{\circ}\text{C}/\text{min}$ to 245 $^{\circ}\text{C}$, then 10 $^{\circ}\text{C}/\text{min}$ to 300 $^{\circ}\text{C}$ and hold for 10 min. The injector temperature was 280 $^{\circ}\text{C}$ and the MS temperatures were 280 $^{\circ}\text{C}$, 230 $^{\circ}\text{C}$ and 150 $^{\circ}\text{C}$ for transfer line, source and quadrupole, respectively. The flow rate of the carrier gas helium was 1.1792 mL/min. After the mass spectra were acquired, the quantitation database was established with selected ion monitoring (SIM) at m/z of the two most abundant ions for each individual congener (Table S1, S: Supplementary material). The GC method was locked for the compound retention times to maintain SIM acquisition.

Chromatographic separation and quantification of 209 PCB congeners were achieved on GC-MS in SIM mode. The method separated the 209 congeners into 162 peaks (Figure S1). The first 81 eluted peaks were quantified by internal standard ^{13}C -PCB 9, while the second 81 eluted peaks were quantified by ^{13}C -PCB 194. Linearity of response were achieved for all peaks as determined from their respective calibration curves (linearity $r^2 > 0.999$, Table S2). Measures of quality control and assurance are described in Supplementary material. The quality of the analytical method was assessed by the method blank samples and the recovery of the ongoing precision and recovery standard (Tables S3–S5) analyzed in parallel to all tissue and serum samples.

Toxicity assays

The right caudal lung lobe and the right lateral lobe of liver were perfused with saline for lipid peroxidation and glutathione measurements. Malondialdehyde (MDA) as an end-product of lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS) assay in plasma, liver and lung homogenate (Cayman Chemical Company, Ann Arbor, MI). Erythrocyte lysate, homogenized liver and lung samples were immediately deproteinated and assessed for GSH/GSSG levels according to the GSH-5,5'-dithio-bis-(2-nitro-benzoic acid) (DTNB) recycling assay (Griffith 1980) (Cayman Chemical Company, Ann Arbor, MI). Total protein was assayed by the bicinchoninic acid method (Sigma-Aldrich, St. Louis, MO) for normalization.

Blood samples were collected for hematological testing by an automatic hematology analyzer (Sysmex XT-2000i, Kobe, Japan). Plasma concentrations of total T_4 , free T_4 , total T_3 and free T_3 were measured by the Diagnostic Center for Population and Animal Health at Michigan State University using radioimmunoassays. The left lung with trachea and thyroid, the left lateral lobe of liver, lymph nodes, one kidney lobe, thymus, uterus and spleen from the same animals were collected, formalin-fixed and embedded in paraffin. Sections were stained with hematoxylin and eosin and evaluated by a certified veterinary pathologist.

RNA was extracted with the RNeasy mini kit (Qiagen, Germantown, MD) from 20–30 mg of unperfused liver and lung tissues that were preserved in RNA_{later} Stabilizing Reagent (Qiagen). A selection of metabolic enzymes (CYP1A1, 1A2, 1B1, 2A1, 2B1, 3A23/3A1, UGT1A1, GSTA2, SULT1A1, 2A1 and 1E1) was assayed for mRNA with custom designed RT² Profiler PCR array (Qiagen), while the expression of 84 key genes related to oxidative stress was measured with cataloged RT² Profiler rat oxidative PCR array (Qiagen). SYBR green fluorescence was quantified with Bio-Rad CFX96 real-time PCR detection system. Gene expression was considered to be significantly different in exposed liver if greater than a twofold difference was found in mRNA levels and with a threshold p value of 0.05.

Data Analysis

Statistical analyses were performed using SAS (version 9.2; SAS, Inc., Cary, NC). Summary data are expressed as the arithmetic mean and standard error unless stated otherwise. Two-way analysis of variance (ANOVA) with time as a repeated measure was used to determine differences in body weight gain between groups of animals. Two sample t -tests for equal and unequal variances were used to compare other measurements between exposure and

control groups. A p value < 0.05 was considered significant. Data from gene expression measurements were analyzed using Qiagen online software RT² Profiler PCR Array Data Analysis version 3.5.

Results

Characterization of CAM+ Atmosphere

The CAM was developed (Zhao et al. 2010) in an attempt to mimic the average PCB profile recorded from Chicago (Sun et al. 2006). Compared to the CAM vapor, the CAM+ vapor in this study also contained mono- to octachlorobiphenyls, with tetra- and lower-chlorinated PCBs being the major homologue groups (Figure 2). The differences lay in the presence of PCB 11 in the CAM+ vapor, a non-Aroclor congener that was absent in any previous Aroclor-derived vapor mixture (Hu et al. 2012; Hu et al. 2010b) in spite of the abundance in atmospheric environment (Hu et al. 2010a). In addition, reduced levels of pentachlorobiphenyls and increased levels of some di- and trichlorobiphenyls compared to the CAM were seen in the CAM+ vapor, possibly owing to different equilibrium of gas-phase PCBs with laboratory glassware surfaces. The CAM+ vapor shared many leading congeners with the Chicago airshed (Hu et al. 2010a). However, like the CAM vapor, the CAM+ vapor was also enriched in lower-chlorinated PCBs. The underrepresentation of higher-chlorinated PCBs that were found in Chicago air could result from differential volatilization of PCB congeners from heterogeneous surfaces in the city as compared to volatilization from pure PCB liquid in laboratory (Hu et al. 2012). The congener profile was consistent among exposure days with only a slight increase in representation of lower-chlorinated PCBs and slight decrease in that of higher-chlorinated PCBs with time (Figure S2). Animals were exposed to an average concentration of $533 \pm 93 \mu\text{g}/\text{m}^3$ total PCBs.

PCB Distribution in Nose-only versus Whole-body Exposed Tissues

Compared to the CAM+ vapor profile (Figure 2), the profiles in the tissue samples contained fewer congeners with higher chlorination (Figure 3), consistent with our previous results (Hu et al. 2012; Hu et al. 2010b; Hu et al. 2013) showing fast elimination of many congeners within a few hours after inhalation exposure. Most of the remaining congeners (PCBs 8, 15, 18, 28/31, 49, 66, 74 and 99) in tissue samples had the *para* position occupied, reflecting disfavored metabolism by cytochrome P450 enzymes (Brown Jr 1994). PCB 28/31 was the most abundant congener in all types of tissues and in both groups, accounting for 17 to 39% of ΣPCBs (Figure S3). In contrast, PCB 11, the supplemented congener in CAM+, was found at low to minimal levels in tissues other than the lung, indicating minimal propensity of bioaccumulation (Figure 3).

Total PCB concentrations (ΣPCBs) in tissues of all exposed rats were 24-fold (serum) to 450-fold (adipose tissue) higher than background levels in control animals (Table 1). Compared to the whole-body exposure, rats exposed in the nose-only system accumulated higher mean ΣPCBs in almost all tissues except for the serum, yet statistical difference was found only for the lung. The largest differences in mean ΣPCBs was also found in the lung, with the nose-only group showing 1.5-fold of ΣPCBs compared to the whole-body group ($p=0.0024$). Although not statistically significant, moderate differences in the ΣPCBs

between the two groups were seen in the brain and the adipose tissue (Table 1). Interestingly, variation of Σ PCBs correlated among different tissue types: within the nose-only group, the animals that had relatively higher PCB concentrations in the lung also had consistently higher levels in the brain and the adipose tissue as compared to the rest of the group (Table S6). The profiles of the detected congeners among the two exposure groups as well as in different tissue types shared principal congeners. However, comparing individual PCB congeners, many of them had significantly higher levels in the lungs of nose-only exposed animals than those exposed in the whole-body system (Figure 3). These congeners included mainly di- to tetrachlorobiphenyls such as PCBs 11, 15, 18, 25/26, 28/31, 32, 47/48/62/65/75, 49, 43/52, 66, 70, 74 and 85. A few of them also showed significant differences in the brain between the two exposure methods, while other tissues presented only minor differences. Concentrations of individual congeners in tissue samples are reported in online Supplementary material (Tables S7–S16).

Toxicological Response

The nose-only exposed rats displayed a significant lower weight gain than the whole-body group, while the weight gain of both exposure groups were diminished compared to controls (Figure 4, repeated measures ANOVA, $p = 0.0049$). Statistical analysis showed that at any given time of measurement, the mean weight gain of the nose-only group was significantly lower than the whole-body group ($p < 0.0001$) (Figure 4). No interaction was found between the two variables of treatment and time. No significant differences were found in either absolute or body-mass-normalized weights of liver, lung, brain, uterus, spleen or heart among different treatment groups (Table S17).

Histopathological evaluation revealed a subtle zonal difference in the hepatocytes of PCB exposed animals in that the centrilobular hepatocytes had more granular and pale cytoplasm as compared to the periportal hepatocytes, yet the clinical significance of this change was minimal (Figure S4). Other organs including lung, trachea, thyroid, lymph nodes, kidney, brain, thymus, uterus and spleen did not exhibit any differences between control and exposed animals. Minor changes of hematology parameters including mean corpuscular volume, mean corpuscular hemoglobin concentration and reticulocyte counts (Table S18) were within normal physiological range (Bernardi et al. 1996; Mathers et al. 2012).

Although the average T_4 of the nose-only group was lower than the whole-body group, the difference was not statistically significant (Figure 5). Plasma total thyroxine (T_4) levels were significantly reduced in exposed animals compared to controls ($p = 0.0006$), and compared to sham-exposed female Sprague-Dawley rats at the same age in our previous study ($p = 0.0179$) (Hu et al. 2012). No change of free T_4 , total triiodothyronine (T_3) or free T_3 was seen either (Figure 5 and Figure S5).

Lipid peroxidation was assessed by measuring levels of malondialdehyde (MDA). Increased formation of MDA in liver was found in the nose-only group ($p = 0.0316$) compared to controls (Figure 6). No significant change was found in lung tissue or in plasma after exposure. No significant changes in glutathione (GSH) and glutathione disulfide (GSSG), another indicator of oxidative stress, were found in lung, liver tissues or erythrocyte lysate (Figure S6). Oxidative-stress-induced gene regulation was investigated with real-time PCR

in liver tissue. Out of the 84 genes that were tested, six genes were found down-regulated after nose-only exposure and five were up-regulated. Five genes were changed after whole-body exposure (Figure S7). The only gene that was found to be statistically different in both exposure groups is FA complementation group C gene (FANCC), showing a consistent twofold down-regulation in nose-only and whole-body exposed animals (Figure S7). This gene is associated with redox cycling. No consistently significant change was found in mRNA levels of CYP1A1, 1A2, 1B1, 2A1, 2B1, 3A23/3A1, UGT1A1, GSTA2, SULT1A1, 2A1 and 1E1 in either lung or liver samples (Figure S8).

Discussion

We sought to compare whole-body and nose-only inhalation exposure methods to gain insight on the use of exposure regimen in PCB inhalation studies. Contemporaneous whole-body and nose-only exposures were carried out in a specially designed system to concurrently feed both exposure chambers with the same atmosphere and at equal flow rate (Figure 1). Thus, the two groups of animals inhaled air with equal concentrations of airborne PCBs. However, congener-specific analysis of post-exposure body burden revealed that the nose-only group had higher Σ PCBs in tissues than the whole-body group. The differences were statistically significant in the lung (Table 1), and were most evident for the more volatile lower-chlorinated congeners (e.g. PCB 28/31, Figure 3), suggesting a connection to inhalation. Moreover, similar organ weights (Table S17) indicated that the differences of PCB tissue concentrations between the two exposure groups were not due to growth dilution. It has been shown that minute volume and breathing frequency of confined F344 rats in nose-only tubes were the highest during the first 15 min and gradually declined to a steady state after 4–6 h of exposure (Mauderly 1986). Therefore, it is probable that the confinement led to somewhat higher ventilation (i.e. minute volume and breathing frequency) of the nose-only exposed animals and thus resulted in a higher dose compared to the unrestrained animals in the whole-body chamber. Among the congeners that showed differences in the lung tissue of the two groups, many (e.g. PCB 11) were also significantly different in the brain but not in other tissues. It is possible that these congeners may translocate to the brain directly through the olfactory bulb (Apfelbach et al. 1998; Oberdörster et al. 2004) circumventing the blood-brain barrier and leading to higher uptake in the brain.

We surmised that the nose-only group was exposed to a higher dose level than the whole-body group, resulting in a higher body burden. These female Sprague-Dawley rats were exposed to an average of 533 $\mu\text{g}/\text{m}^3$ total PCBs for a total of 96 hours. At 11-wks old they have a minute ventilation volume of 143 mL/min (Lai 1992). Our previous characterization showed a pulmonary uptake efficiency of 99.8% for PCB 11 (Hu et al. 2014), yet considering the presence of higher-chlorinated congeners in the exposure atmosphere here we assumed an efficiency of 70% as used by US EPA (personal communication). The whole-body exposed animals are thus estimated to inhale a dose of 1320 $\mu\text{g}/\text{kg}$ b.w. total PCBs. Using the value of minute ventilation (216 mL/min) measured by Mauderly (1986) for female F344 rats confined in nose-only inhalation tubes, we estimate that the nose-only group were exposed to a dose of 1980 $\mu\text{g}/\text{kg}$ b.w..

Although dose-response relationship has been established for oral exposure to higher chlorinated Aroclors, no study has ever reported any effects relative to different doses of inhalation exposure. Diminished weight gain of animals has been found in our 2-wk nose-only inhalation study, in which male rats were exposed to Aroclor 1242 vapor at an equivalent dose of 4630 $\mu\text{g/kg b.w.}$ (Hu et al. 2010b). In a 4-wk nose-only inhalation study, female rats were exposed to a dose of 446 $\mu\text{g/kg b.w.}$ with no effects found in weight gain or thyroid hormone (Hu et al. 2012). In that study, total T_4 concentrations of both sham-exposed (56 ± 2 nmol/L) and PCB-exposed groups (61 ± 5 nmol/L) (Hu et al. 2012) were of similar values as the controls in the current study (59 ± 2 nmol/L), all being significantly higher than the PCB-exposed animals in the current study (46 ± 3 nmol/L). This suggests that the reduction in T_4 level may be associated with a PCB dose level between 446 and 1320 $\mu\text{g/kg b.w.}$ (Table S18). This observed effect level appeared to be lower than what has been found with most oral exposure studies (ATSDR 2000; Hallgren et al. 2001), which could be ascribed to differences in bioavailability (Shu et al. 1988) between exposure routes and in studied PCB composition.

Our results from this exploratory study showed that plasma thyroid hormone level may serve as a sensitive marker for inhalation studies. The reduction of total T_4 in response to PCB exposure has been widely reported in studies with both animal models and human populations (see review articles (Grimm et al. 2015; Hagmar 2003)). Several mechanisms have been established for the depression of thyroid hormones including: 1) disruption of thyroid gland function and regulation, often accompanied by altered thyroid gland morphology; 2) interference with thyroid hormone metabolism including T_4 glucuronidation and sulfation; and 3) interference with plasma transporter transthyretin (TTR) (Brouwer et al. 1998). In our study, neither histopathological changes of thyroid gland nor reduction of serum T_3 levels were present in PCB-exposed rats, suggesting that thyroid hormone synthesis was unlikely disrupted. Upregulation of T_4 glucuronidation has been well described as a dominant mechanism for T_4 depression caused by dioxin-like congeners (Brouwer et al. 1998). This usually occurs through interaction between aryl hydrocarbon receptor (AhR) and CYP1A inducers (Shelby and Klaassen 2006; Vansell and Klaassen 2002). Since CYP1A and UGT1A6 were often co-induced by AhR ligands (Van Birgelen et al. 1995), the lack of change in hepatic CYP1A mRNA level (Figure S7) suggested minimal likelihood of this mechanism. UGT1A1 is another major isoform that can glucuronidate T_4 (Vansell and Klaassen 2002), yet the unchanged mRNA level (Figure S7) is also inconsistent with a UGT-mediated mechanism. It is most likely that the reduction of T_4 was caused by displacement of T_4 from its binding sites on the transporter TTR, as it has been found that the hydroxylated metabolites and PCB sulfates can bind to TTR with affinities much higher than T_4 (Grimm et al. 2015).

Our results contradicted the elevated T_3 and T_4 concentrations reported by Casey et al. (1999), which were accompanied by increased intracellular vacuolization of follicular epithelial cells of thyroid gland (Casey et al. 1999) after inhalation exposure to a dose much lower than ours (Table 2). However, it is difficult to compare their results to our findings for the following reasons: 1) The animals were adolescent males that were 5 weeks younger and probably at different life stage than our females. 2) Exposure assessment was limited with only a subset of congeners identified in the analytical method. No evidence was shown to

support minimal ingestion exposure due to grooming. 3) Uniformity of chemical distribution inside the unorthodox whole-body chamber was uncertain, and information on breathing zone concentration was missing. 4) The animals were exposed 23 h per day with no access to food or water. Whether this prolonged food and water deprivation would cause any stress or alter PCB disposition was not addressed.

On the other hand, we are aware of the following limitations in our current study: 1) A low number of animals were studied. 2) Although all animals were born on the same day, the baseline weight of the nose-only group was 17.7 g heavier than the control group at the start of exposure due to the consideration of animal snout fit in the nose-only system. The slight differences in the baseline weight were considered not sufficient to cause differential growth rate (Harlan Sprague-Dawley 104-Week Data). 3) Other than control animals that were not exposed, sham-exposed animals were not included. Nevertheless, the thyroid hormone levels of these controls were not different from those of 4-wk sham-exposed rats in our previous inhalation study (Hu et al. 2012) as discussed above. Since stress was a potential concern for nose-only exposure, animals were carefully monitored yet no signs of stress were observed. Surprisingly, the rats willingly walked into the nose-only tubes for the next repeated exposure, further suggesting that they tolerated the confinement well. In contrast to closed-end nose-only tubes (Hu et al. 2010b), the open tubes of our SCIREQ exposure system allowed efficient heat transfer via the tails of the rats and guarded against hyperthermia. The tube also supported the rat on a platform for enhanced comfort and the excreta drained without contaminating the fur (Figure 1). Although no evidence was found indicating additional oral exposure that would cause increased body burden in whole-body exposed rats, the concern still exists that the PCBs may contaminate the fur of whole-body exposed animals thus necessitates housing animals outside the vivarium.

Some oxidative-stress related effects were seen in the nose-only exposure group. Increased lipid peroxidation indicated by *in situ* levels of MDA has been reported commonly with PCB exposure (Dogra et al. 1988; Fadhel et al. 2002). One possible mechanism has been proposed that the induction of CYP increases the release of active oxygen species based on the finding that both PCB CYP1A and 2B inducers enhanced hepatic lipid peroxidation (Fadhel et al. 2002). However, since the time frame of MDA elevation did not correlate entirely with CYP induction, it was speculated that other oxidative enzyme or sources of reactive oxygen species might contribute to the elevation as well. Our results showed that hepatic MDA was increased without change in CYP mRNA levels, suggesting that MDA formation may be a more sensitive endpoint compared to CYP induction. The observation that MDA was not statistically significantly increased in the whole-body group compared to controls and that the levels were lower than in the nose-only group (Figure 6) may again reflect the different dose levels. On the other hand, FANCC gene that was down-regulated in both groups has a close link with redox activation of many xenobiotics as it can interact with CYP2E1 (Futaki et al. 2002) and glutathione S-transferase (Cumming et al. 2001). It is also involved in the attenuation of electron transport in the microsomal membrane (Kruyt et al. 1998). Further investigation of this gene and related pathways will shed light on the mechanism as well as adequate molecular biomarkers.

In conclusion, our study showed that the nose-only exposure in our system was well-tolerated with no signs of stress to the rats and minimal effort was needed in regard to animal care and training. It is therefore preferred to use nose-only regimen for long-term, repeated inhalation studies. It is important to take into consideration that animals exposed in the nose-only system may have elevated minute ventilation compared to the whole-body exposure, resulting in a higher dose and body burden. Biological responses were observed associated with the dose levels, thereby providing useful data for derivation of a preliminary reference concentration (RfC) for PCB inhalation exposure. Further investigation with a larger number of animals, longer exposure period and more dose levels are planned to fully establish a dose-response relationship.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CAM	Chicago Air Mixture
CAM+	PCB 11 supplemented Chicago Air Mixture (CAM+)
GSH	glutathione
GSSG	glutathione disulfide
MDA	malondialdehyde
ΣPCBs	total PCB concentrations
SIM	selected ion monitoring
T₃	triiodothyronine
T₄	thyroxine
TBARS	thiobarbituric acid reactive substances

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Highlights

- ✦ We conducted contemporaneous PCB inhalation exposures using different regimens.
- ✦ The nose-only exposed animals had higher PCB tissue levels reflecting higher dose.
- ✦ Diminished weight gain and reduced plasma total T₄ levels were found postexposure.
- ✦ Nose-only regimen is preferred over whole-body for future PCB inhalation studies.

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Figure 2.

Average congener mass percentage in generated CAM+ vapor, with inset plot showing congeners from PCB 75 to PCB 209. Values are expressed as mean mass percent $\pm$ standard error.

Figure 3.

Average mass of all PCB congeners in lung, serum, liver, brain and adipose tissue after 4 week inhalation exposure to CAM+ vapor via nose-only and whole-body regimen, with inset plot showing congeners from PCB 120 to PCB 209. Values are expressed as mean mass (ng/g wet tissue weight) $\pm$ standard error ($n = 7$ /group for serum, $n = 4$ for other tissues). Major congeners ($>5\%$ of total PCBs in either group) are labeled for every type of tissue, and asterisks indicate significant differences between the nose-only and whole-body group: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Treatment	Least square means of weight gain (g)	<i>p</i> -value for group comparison	
control	11.8	control vs nose-only	<0.0001
nose-only	-1.8	control vs whole-body	<0.0001
whole-body	4.4	nose-only vs whole-body	<0.0001

Figure 4.

Average weight gain compared to pre-exposure (day 0) of control and exposed rats over 4 week inhalation exposure to CAM+ vapor. Asterisk indicates that treatment had significant effect on weight gain analyzed by ANOVA repeated measures. The lower table shows differences between two treatment group at any given measurement of rat weight.

Figure 5.

Serum total thyroxine (T4) and total triiodothyronine (T3) levels in control and exposed rats after 4 week inhalation exposure to CAM+ vapor. Values are expressed as mean mass (ng/g wet tissue weight) $\pm$ standard error ($n = 6$ for control, $n = 8$ for exposed groups). Asterisks indicate a significant difference to the control group: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.0001$.

Figure 6.

Liver lipid peroxidation (malondialdehyde formation) in control and exposed rats after 4 week inhalation exposure to CAM+ vapor. Values are expressed as mean mass (ng/g wet tissue weight) $\pm$ standard error ($n = 3$ for control, $n = 4$ for exposed groups). Asterisks indicate a significant difference to the control group: * = $p < 0.05$, ** = $p < 0.01$.

Table 1

Differences of total PCB levels (Σ PCBs, ng/g tissue weight) in lung, serum, liver, brain and adipose tissue from the nose-only exposed, whole-body exposed and control animals. Values are expressed as mean $\pm$ standard error.

Tissue type	Σ PCBs (ng/g tissue weight)				Group comparison of Σ PCBs			
	nose-only	whole-body	all exposed	control	nose-only vs whole-body		all exposed vs control	
					p-value	fold difference	p-value	fold difference
Lung	200 $\pm$ 12	130 $\pm$ 7.5	160 $\pm$ 15	6.5 $\pm$ 1.0	0.0024	1.5	<0.0001	25
Serum	16 $\pm$ 1.4	15 $\pm$ 1.3	16 $\pm$ 0.9	0.68 $\pm$ 0.16	0.55	1.1	<0.0001	24
Liver	150 $\pm$ 12	120 $\pm$ 10	140 $\pm$ 10	2.7 $\pm$ 1.6	0.12	1.2	<0.0001	52
Brain	340 $\pm$ 32	270 $\pm$ 10	300 $\pm$ 21	12 $\pm$ 1.4	0.073	1.3	<0.0001	25
Adipose tissue	2800 $\pm$ 330	1900 $\pm$ 30	2300 $\pm$ 220	5.1 $\pm$ 1.4	0.058	1.5	<0.0001	450

Table 2

Comparison of dose levels and health effects from different inhalation studies.

Exposure Regimen	Animals	PCB		Dose		Observed effects	References
		Vapor source	Conc. ($\mu\text{g}/\text{m}^3$)	μg	$\mu\text{g}/(\text{kg b.w.})$		
whole-body	rats	Aroclor 1254	1500 ^a	13000 ^a	59000 ^a	Histopathologic lesions in liver	(Treon et al. 1956)
whole-body	adolescent male rats	Aroclor 1242	0.9	2.3 ^b	19.2 ^b	Histopathological changes in the thyroid and thymus, increases in serum T ₃ and T ₄ , decrease in exploratory behavior, diminished weight gain ^c .	(Casey et al. 1999)
nose-only	male rats	Aroclor 1242	8200	985	4630	Diminished weight gain	(Hu et al. 2010)
nose-only	female rats	CAM	520	100	446	No effects in weight gain or thyroid hormone, only minor change in blood GSH/GSSG.	(Hu et al. 2012)
nose-only ^d	male rats	PCB 11	106	2	8	No overt toxicity	(Hu et al. 2013)
nose-only ^d	female rats	PCB 3	2060	35	150–180	No effects in serum chemistry parameters including T ₄ , or 8-oxo-dG levels in urine.	(Dhakal et al. 2014)
whole-body	female rats	CAM+	533	307	1320	Diminished weight gain, decrease in T ₄	Current study
nose-only				464	1980	Diminished weight gain, decrease in T ₄ , increase in hepatic lipid peroxidation.	

^a Estimated by measuring hydrochloric acid formation after thermal decomposition of Aroclor vapor.

^b Estimated using a minute ventilation volume that is lower than reported by others in literature.

^c No statistical analyze was presented.

^d Acute exposure that lasted 2 h.