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Skeletal Toxicity Resulting from Exposure of Growing Male Rats to Coplanar PCB 126 is Associated with Disruption of Calcium Homeostasis and the GH-IGF-1 Axis and Direct Effects on Bone Formation

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Abstract

Skeletal toxicity has been reported following exposure to polychlorinated biphenyl (PCB) mixtures. However, molecular mechanisms remain poorly understood. We exposed groups of male 4-5 week-old Sprague-Dawley rats to 3,3', 4, 4', 5-pentachlorobiphenyl (PCB 126), a dioxin-like coplanar PCB congener by a single i.p. injection of 5 µmol/kg in soy oil vehicle or vehicle alone. After 4 weeks, rats were euthanized. PCB exposure resulted in hypocalcemia ($P<0.05$) and significant increases in serum PTH without changes in serum phosphorous. Hyperparathyroidism was accompanied by increased expression of mRNAs of vitamin D3 metabolizing cytochrome P450 enzymes CYP27B1 and CYP24 in the kidney ($P<0.05$). PCB exposure also reduced body weight, serum IGF-1 and hepatic expression of mRNAs encoding the male-specific GH-pattern-regulated CYP2C11 and CYP3A2 relative to controls ($P<0.05$). PCB exposure reduced long bone length, diameter and surface area but increased trabecular thickness and volume ($P<0.05$). Serum osteocalcin ($P<0.05$), a marker and a regulator of bone formation, was reduced but PCB exposure had no effect on the bone resorption marker RatLaps. Exposure of human intestinal Caco-2 cells to 10-100 nM PCB 126 in the presence of vitamin D3 resulted in inhibition of mRNAs for the calcium transporters TRPV6 and PMCA1b ($P<0.05$). In addition, PCB 126 suppressed

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CONFLICTS OF INTEREST

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osteoblastogenesis in primary bone marrow mesenchymal stem cell cultures which was blunted by the AhR antagonist CH-223191. These data provide novel evidence that skeletal toxicity after exposure to PCB 126 is a result of disruption of calcium homeostasis and the GH-IGF-1 axis, and involves direct AhR-mediated effects on bone formation.

INTRODUCTION

Osteopenia (low bone density) and osteoporosis are major health problems among the aging population, particularly in post-menopausal women as a result of estrogen loss. Both conditions significantly increase the risk of bone fracture that in turn decreases quality of life and increases the risk of mortality (Cosman et al., 2014; Wright et al., 2014). A decrease in bone growth in early development resulting in reductions in peak bone mass will predispose to osteoporosis in later life (Poole and Compston 2006; Seeman 2002; Weaver et al., 2016). Environmental endocrine disruptors such as polychlorinated biphenyls (PCBs) may affect the modeling and remodeling of bone in this fashion and contribute to osteoporosis risk in exposed populations.

Bone is a multi-functional tissue. In addition to providing structure and protection for vital organs, the skeleton serves as a reservoir for mineral ions, in particular calcium (Ca) and phosphate. 99% of the body's Ca content is contained in the skeleton (Peacock, 2010) and alterations in circulating Ca levels require either the release of the ion from the mineral matrix of bone (during hypocalcemia), or its sequestration into the matrix (during hypercalcemia). This regulatory mechanism requires tight control to maintain a narrow range of circulating Ca (9–10 mg/dL; 2.2–2.6 mM) (Peacock, 2010). Factors both endogenous (e.g. hormones) and exogenous (e.g. toxicants) can modify the balance of bone formation and resorption by affecting Ca homeostasis and via direct effects on bone cell differentiation.

Environmental agonists of the aryl hydrocarbon receptor (AhR) have been suggested to perturb bone turnover through a variety of mechanisms. *In vivo* administration of the highly potent AhR agonist dioxin (2,3,7,8-tetrachlorodibenzodioxin (TCDD)) to rats diminishes bone growth longitudinally and in cross-section while decreasing material strength (Korkalainen et al., 2009). Some of the skeletal effects may be secondary to AhR-mediated endocrine disruption. TCDD was reported to decrease osteoblast activity secondary to increased levels of vitamin D3 (Ryan et al., 2007). AhR-associated generation of oxidative stress or effects on bone marrow immune cells may also impact bone turnover (Jamsa et al., 2001; Yu et al., 2014a). *In vitro*, TCDD has been suggested to suppress bone deposition by inhibiting osteoblastogenesis via AhR-dependent ERK activation and via suppression of Wnt- β -catenin signaling (Iqbal et al., 2013; Yu et al., 2014b; Nishimura et al., 2009; Tanabe 1988). Variable effects of TCDD and AhR ligands have been reported on osteoclastogenesis. Impairment of osteoclast maturation as a result of inhibition of NF- κ B/HIF pathways has been reported (Korkalainen et al., 2009; Pavuk et al., 2014). In contrast, others have shown increases in osteoclast differentiation and activity following AhR agonist exposure (Alvarez-Lloret et al., 2009; Brouwer 1991; Krishnan and Safe 1993).

PCBs are a class of anthropogenic compounds historically produced as electrical insulators. Their highly lipophilic chemistry allows PCBs to accumulate and persist in the environment over decades (Tanabe, 1988; Pavuk et al., 2014), and significant human exposure to these compounds continues despite production being banned in several countries, including the U.S.A. As a class, PCBs consist of 209 compounds identified by the distribution of chlorine atoms around the phenyl rings, where certain arrangements allow for a more co-planar configuration. These co-planar PCBs are termed ‘dioxin-like’ for their structural and chemical similarity to TCDD (Tanabe, 1988). Men that consume high-fat seafood diets have been reported to show an inverse correlation between serum PCB levels and bone density (Glynn et al., 2000). In addition, significantly increased fracture rates have been reported in cohorts of Swedish and Inuit women with high dietary intake of persistent organochlorine pollutants (Alveblom et al., 2003; Downie et al., 2003). Dioxin-like PCBs (e.g. 3,3',4,4',5-pentachlorobiphenyl, aka PCB 126 (Bemis et al., 2005)), have also been reported to suppress bone growth in rats by inhibiting osteoblast differentiation and diminishing collagen content in bone (Lind et al., 2000a; 2000b). Although bone is a known target of PCB toxicity, it is not clear if PCB 126 effects on the skeleton are the result of action on bone cells themselves or secondary to disruption of the Ca-PTH-vitamin D axis. It is further unclear whether toxicity to osteoblastogenesis is a result of AhR activation. The current study was designed to address these questions.

MATERIALS AND METHODS

PCB Synthesis

PCB 126 was synthesized by the Iowa Superfund Synthesis Core using an improved Suzuki-coupling method and its purity verified. A complete description of the synthesis and characterization of the PCB 126 used in this study is provided in the supplemental materials to Gadupudi et al., (2018).

Animal Studies

All experiments were conducted with the approval from the Institutional Animal Care and Use Committee of the University of Iowa. Male Sprague Dawley (SD) rats weighing 75–100 g at an age of 4–5 weeks were fed a defined AIN-93G diet purchased from Envigo (Indianapolis, Indiana) and acclimatized for 1 week. Each animal was individually housed with free access to feed and water and in a controlled environment of 22° C in a 12-h light-dark cycle. Soy oil, used to prepare the PCB 126 and vehicle injections, was purchased from Envigo and was the same oil in the diet. Animals were randomized and received a single ip injection of soy oil (5 ml/kg body weight; n = 12) or PCB 126 at a dose of 5 µmol/kg (1.63 mg/kg body weight; n = 12) body weight after 1 week of acclimatization (Figure 1). This dose of PCB 126 was used previously in several studies with no observable effects on growth during 14 days of exposure, but as a single IP dose was sufficient to induce cytochrome P450 enzyme activity and increase liver weight in Sprague-Dawley rats (Gadupudi et al., 2016; Lai et al., 2010; 2013). The weights of the animals and feed consumed were monitored every alternate day until the end of the study. Four weeks after injection and 12 h prior to euthanasia, feed was withdrawn from a subgroup of the PCB 126-injected (n = 6) and the vehicle-only injected rats (n = 6) for an overnight fast (Figure 1). All

the animals in both “fed” and “fasted” groups were euthanized in the following morning, using carbon-dioxide asphyxiation followed by thoracotomy. Whole blood was obtained from the heart and processed as described below. Left tibia and femur bones were fixed in formalin. Right tibia, femur and vertebral bone, the livers and kidneys were harvested and flash frozen in liquid nitrogen and stored at -80°C until further analysis.

Blood collection and serum analysis

Whole blood samples from the hearts were collected into S-Monovette tubes designed for harvesting serum, after euthanasia. The blood was allowed to clot in the tubes and the serum fractions were separated by centrifugation at $1500 \times g$ for 10 min at 4°C . The serum samples were aliquoted and frozen at -80°C until further analysis. The measurements of serum calcium and phosphorous were measured by Comparative Clinical Pathology Services LLC (Columbia, MO). The intact form of parathyroid hormone (PTH) (Immutopics, Inc., San Clemente, CA, cat# 60–2500), osteocalcin (Immutopics, cat# 60–1505), C-terminal telopeptides (CTXs) of type 1 (Immunodiagnostic Systems, Gaithersburg, MD, cat# AC-06F1) and rat IGF-1 (Abcam plc, Cambridge, U.K., cat# ab213902) were detected in serum by using commercially available enzyme-linked immunosorbent assay kits.

Bone Morphology and Length

The microarchitecture of trabecular bone was assessed by μCT using *ex vivo* fixed tibia as described previously (Bouxsein et al., 2010; Sebastian et al., 2008; Suva et al., 2008). All μCT analyses were consistent with current guidelines for the assessment of bone microstructure in rodents using micro-computed tomography (Bouxsein et al., 2010). Formalin-fixed tibia were imaged using a MicroCT 40 (Scanco Medical AG, Bassersdorf, Switzerland) using a $16\text{-}\mu\text{m}$ isotropic voxel size in all dimensions. Semi-automated contouring was used to select a region of interest (ROI) extending 3.2 mm distal to the proximal tibial metaphysis, but excluding the cortex and subcortical bone, composed of 250 adjacent slices. Three-dimensional reconstructions were created by stacking the regions of interest from each two-dimensional slice and then applying a gray-scale threshold and Gaussian noise filter as described (Suva et al., 2008), using a consistent and pre-determined threshold (300) with all data acquired at 55 kVp, 145 mA, and 275-ms integration time. Fractional bone volume (bone volume/tissue volume; BV/TV) and architectural properties of trabecular bone, trabecular thickness (Tb.Th.), trabecular separation (Tb.Sp.), trabecular number (Tb.N.), and connectivity density (Conn. D.), were calculated using previously published methods (Suva et al., 2008). Similarly, for cortical bone assessment, μCT slices were segmented into bone and marrow regions by applying a visually chosen, fixed threshold for all samples after smoothing the image with a three-dimensional Gaussian low-pass filter ($\sigma = 0.8$, support = 1.0) to remove noise, and a fixed threshold. Cortical geometry was assessed in a 1.5-mm-long region centered at the tibial midshaft. The outer contour of the bone was found automatically using the built-in Scanco contouring tool. Total area was calculated by counting all voxels within the contour, bone area was calculated by counting all voxels that were segmented as bone, and marrow area was calculated as total area - bone area. This calculation was performed on all 40 slices using the average for the final calculation. The outer and inner perimeter of the cortical midshaft was determined by a three-dimensional triangulation of the bone surface (BS) of the 40 slices, and cortical

thickness and other cortical parameters were determined as described (Suva et al., 2008). Parameters assessed included total cross sectional area (Tt.CSA), cortical area (Ct.CSA), cortical thickness (Ct.Th.), medullary area (Me.Ar.), periosteal perimeter (Ps. Pm.), and endocortical perimeter (Ec.Pm). Bone length is represented by the distance from the proximal tibia head to the tibia-fibula junction. Measurements were done in triplicate per tibia using digital calipers (Thermo Scientific, Rockford, IL) on formalin fixed tibia from all groups.

Caco-2 Cell Cultures

Human intestinal Caco-2 cells were obtained from the ATCC (ATCC HTB-37) and were cultured in Minimum Essential Medium Eagle (MEM, 1X) with Earle's salts and L-glutamine, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 0.1mg human transferrin (Roche, Basel, Switzerland) and plated in six-well plates at a density of 10,500 cells/cm². After reaching confluency, each well received 1-alpha-25-dihydroxy-vitamin D (Sigma-Aldrich) at a concentration of 1×10^{-8} M. In addition, cells received either Vh (DMSO) or 5×10^{-6} M CH223191 (Sigma). After 1 hr, PCB 126 was added to appropriate wells at 1×10^{-7} M or 1×10^{-8} M. Stock solutions of CH223191 and PCB 126 were prepared at 2000x concentrations in DMSO to equalize the v/v concentration of DMSO in each well at 0.1%. Media changes and dosing was performed every 2–3 days for one week. Total RNA was extracted into TRI reagent (Molecular Research Center, Cincinnati, OH) and stored in a –80 freezer until further use.

Gene Expression Analysis

Total RNA was isolated from the rat kidney and liver by using TRI reagent as described previously (Chen et al., 2008). Total RNA was reverse-transcribed by using IScript cDNA synthesis (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Subsequent real-time PCR analysis was carried out by using SYBR green and an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA).

For Caco-2 cell extracts and rat renal gene expression, total RNA was reverse transcribed and cDNA amplified using *Power* SYBR green RNA-to-Ct (Thermo-Fisher) one step kit according to manufacturer instructions. Real-time PCR analysis was carried out on a Roche 480 LightCycler system (Roche).

Gene-specific primers were: renal CYP24A1, forward 5'-GACCGCAAACAGCTTGATGTGGAT-3' and reverse 5'-ATA TTCTCAGCTTCCGCCCCGT-3'; renal CYP27B1, forward 5'-AGTGTGGGTTACGGACATTA G-3' and reverse 5'-AGATGGGTGGTGAGAAGTAGA-3'; renal TRPV5, forward 5'-CCTCAGCTTCTGTACCCCTA-3' and reverse 5'-CAGTGGTGACTCCCAAATACTTTTC-3'; renal Klotho, 5'-AACCTACTTTTGGCTCACGCC-3' and reverse 5'-TCTTTGGGTAGTCGCCATCG-3'; hepatic CYP2C11, forward primer 5'-GTCCAACACCTCTCCCAA TC-3'; and reverse 5'-AAGGGCTTCATGCCCAAATAC-3', hepatic CYP3A2, forward 5'- TGGGACCATCTC CATCCA CA-3'; and reverse 5'- TCAAAGGACGAGGACATGGTT A-3', Caco-2 cell

hCYP1B1 forward 5'-CGTACCGGCCACTATCACTG-3' and reverse 5'-TCAGGATACCTGGTGAAGAGGA-3'; hTRPV6 forward 5'-ATTGCAAGGTGCACCAGAGA-3' and reverse 5'-GCAGTCTGACCCTCATAGAGC-3', PMCA1b forward 5'-ACCTTACTTGGAGCTGGAGG-3' and reverse 5'-GGGCTTTTGCTTTGTTGCGA-3'. 18S was used as the housekeeping gene for renal genes: forward 5'-CGGACAGGATTGACAGATTG-3'; reverse 5'-CGG ACA GGA TTG ACA GAT TG-3'. Hprt was utilized as the housekeeping gene for analysis of hepatic mRNAs: forward 5'-TCAAGCAGTACAGCCCCAA-3' and reverse 5'-GCCTGTATCCAACACTTCGAG-3' and TBP was utilized as the house keeping gene in Caco-2 samples, forward 5'-TAAGAGAGCCACGAACCACG-3'; reverse 5'-GGACTGTTCTTCACTCTTGGC-3'.

Mesenchymal Stem Cell Culture

Bone marrow derived mesenchymal stem cells (MSCs) were harvested from femurs of 9-week old wild-type male Sprague-Dawley rats (Charles River Labs, Wilmington, MA) and seeded at a density of 12×10^6 cells/well in 6-well culture plates in minimum essential medium (aMEM) (Gibco (Thermo-Fisher)) with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT, USA) and 100 U/mL each of penicillin and streptomycin (Sigma-Aldrich). After 4 days, half the the medium was changed to remove non-adherent cells. At confluency (7 days), the medium was changed to osteoblast differentiation medium (aMEM as above, plus 50 uM l-ascorbate (Sigma-Aldrich) and 8 mM beta-glycerol phosphate (Sigma-Aldrich)) and Vh, CH223191, and/or PCB 126 were added in the manner and concentrations described above. Media changes and dosings were performed every 2–3 days. After 13 days in differentiation media, mineralizing mature osteoblast colonies were fixed in 10% neutral buffered formalin and calcium deposition was detected by alizarin red staining (Millipore, Burlington, MA) (Gregory et al., 2004).

Western blotting

Femur shaft nuclear protein extracts were isolated by homogenization in fractionation buffer (20 mM HEPES, 10 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM EGTA) containing protease inhibitor cocktail (Thermo-Fisher) following a brief centrifugation to separate the marrow. Homogenates were passed through an 18g needle and spun at $750 \times g$ for 5 minutes. Nuclear pellets were resuspended in 1x TBS with 0.1% SDS and centrifuged again at $500 \times g$ for 1 minute to remove any remaining insoluble tissue. Whole homogenates from the MSC cultures were solubilized in RIPA buffer (Thermo-Fisher). Protein extracts were analyzed for expression of total β -catenin using the Bolt Western Blot system (Thermo-Fisher) and associated reagents, using a goat polyclonal antibody (Cell Signaling #8814, Danvers, MA, USA). Results were normalized to TATA-binding protein (Abcam, Inc., Cambridge, MA #51841).

Statistical Analysis

Data are presented as mean $\pm$ SEM. Statistical significance was determined by one or two-way ANOVA followed by Student-Newman-Keuls post-hoc analysis, unless otherwise noted. All analyses were performed at $\alpha = 0.05$ using SigmaPlot version 11.0 (Systat

Software, San Jose, CA) or Microsoft Excel (2016) and significance ($P<0.05$) is reported as such.

RESULTS

PCB 126 effects on bone morphology and size

Bone morphology was analyzed by μ CT analysis of formalin fixed tibia. No statistical difference was observed between fed and fasted groups and so data from these groups was combined. Structural data are presented in Table 1 and representative 3D reconstructions of the trabecular and cortical compartments are shown in Figure 2A. In the trabecular bone compartment a significant increase in mean bone volume/total volume (BV/TV) was observed in PCB 126-treated rats compared to controls ($P<0.05$) which was associated with an increase in trabecular thickness ($P<0.001$). However, the bone from PCB 126 treated rats was smaller overall with significant decreases in cortical surface area, endosteal and periosteal perimeters, mid-shaft and medullary diameters and medullary area ($P<0.05$) despite a lack of effect on cortical thickness. The tibial bone from PCB 126 treated rats exhibited significantly shorter distance from tibia head to the tibia-fibula junction ($P<0.05$) (Figure 2B).

PCB 126 effects on bone turnover markers

Serum osteocalcin and collagen cross-links (RatLaps, CTX1) were measured by ELISA as markers of bone formation and resorption, respectively. PCB 126 treatment resulted in a greater than 50% suppression of serum osteocalcin relative to vehicle controls ($P<0.001$) but no significant effect was observed on CTX1 levels. (Figure 3A and 3B).

PCB 126 exposure results in endocrine disruption

Mineral homeostasis was also assessed in vehicle and PCB 126 treated rats. Independent of feeding status, PCB 126 treatment resulted in development of hypocalcemia (Figure 4A) ($P<0.01$). However, no significant effects were observed on serum phosphorous concentrations (Figure 4B). Consistent with a homeostatic response to reductions in serum calcium, rats treated with PCB 126 displayed a significant degree of secondary hyperparathyroidism with a 4-fold increase in serum parathyroid hormone (PTH) over vehicle controls (Figure 4C) ($P<0.05$). As has been previously reported (Gadupudi et al., 2018), PCB 126 treatment resulted in substantial weight loss and decreased feed efficiency beginning 14 d following PCB injection. Coincident with these effects on growth and food consumption, we observed significant decreases in serum IGF-1 concentrations in the PCB 126-treated rats at sacrifice relative to the vehicle controls ($P<0.05$) (Figure 4D).

Interestingly, this was accompanied by substantial decreases in expression of mRNAs encoding male-specific cytochrome P450 enzymes CYP2C11 and CYP3A2 in the liver of the PCB 126-treated group ($P<0.05$) (Figures 5A and 5B). In addition, expression of renal mRNAs coding for cytochrome P450 enzymes responsible for vitamin D3 synthesis and degradation, CYP27B1 (25-hydroxycholecalciferol 1-hydroxylase) and CYP24 (1,25-dihydroxycholecalciferol 24-hydroxylase), were significantly elevated in PCB 126 treated rats (Figures 5C and 5D) ($P<0.05$).

PCB 126 exposure inhibits expression of intestinal calcium transporters in vitro but induces mRNAs encoding rat renal calcium transporters in vivo

Exposure of the human intestinal Caco-2 cell line to PCB 126 in the presence of vitamin D3 resulted in a dose-dependent induction of the known AhR target gene cytochrome P450 CYP1B1 and dose-dependent inhibition in expression of mRNAs encoding the vitamin D-dependent calcium transporters TRPV6 and PMCA1b (Figure 6A). In contrast, mRNA expression of renal calcium transporters TRPV5 and Klotho were increased in male rat kidney in animals following dosing with PCB 126 (Figure 6B).

PCB 126 exposure does not affect β -catenin protein expression in bone

Western immunoblot analysis of expression of β -catenin protein expression in femur bone nuclear extracts from male control and PCB 126 treated rats did not demonstrate a significant effect of PCB treatment (Figure 7). Results were verified using total cell extracts from treated BM-MSC cultures (data not shown).

PCB 126 exposure inhibits ex-vivo osteoblastogenesis in primary bone marrow MSC cultures

To investigate whether PCB 126 has a direct effect on bone formation, we performed osteoblast differentiation experiments using in *ex-vivo* bone marrow MSC cultures. We further included the specific AhR antagonist CH223191 in the experiments to ascertain the role of AhR in PCB 126 toxicity. Osteoblast differentiation was inhibited by addition of PCB 126 at doses of both 10 and 100 nM. Addition of the AhR antagonist blunted the inhibition of osteoblastogenesis by PCB 126 (Figure 8). We conclude that PCB 126 exerts a direct effect on osteoblastogenesis in an AhR-dependent manner.

DISCUSSION

The results of the current study confirm previous data in rats suggesting that exposure to coplanar PCB congeners results in skeletal toxicity (Alvarez-Lloret, 2009; Lind et al., 2000a; 2000b). These data are also consistent with epidemiological studies documenting reduced bone density and increased fracture risk in populations with a high intake of persistent organochlorine pollutants (Alveblom et al., 2003; Downie and Fenge, 2003; Glynn et al., 2000). The effects of PCB 126 on bone morphology are consistent with significant reductions in endosteal and periosteal bone growth and the reductions in tibial bone length are also similar to those reported following exposure of rats to the highly potent AhR agonist dioxin (2,3,7,8-tetrachlorodibenzodioxin (TCDD) (Jamsa et al., 2001). The bone turnover data are consistent with *in vitro* studies suggesting dioxin inhibits bone formation and the *ex-vivo* osteoblastogenesis data further support a role for AhR signaling in this process (Korkalainen et al., 2009; Ryan et al., 2007; Yu et al., 2014a). CH-223191 was chosen as the AhR antagonist for this study since it has high affinity (IC50 of 30 nM), does not display agonist activity even at 100 μ M, and is highly specific for AhR with no affinity for the estrogen receptor (Moyer et al., 2016).

In addition, these data are the first to suggest that, at least in part, skeletal toxicity of PCB 126 is the result of endocrine disruption of the calcium-PTH-vitamin D axis and of the

growth hormone (GH)-IGF-1 axis. Although PCBs have been suggested to disrupt calcium signaling in the CNS and cardiovascular system (Lind et al., 2004; Winneke et al., 2002; Rasinger et al., 2014), this is the first report of PCB-induced hypocalcemia. Consistent with reduction in serum calcium levels, we observed significant homeostatic increases in serum PTH and increased expression of renal CYP27B1 which catalyzes the final 1-hydroxylation step in the synthesis of active vitamin D3 (1,25-dihydroxycholecalciferol) which is a known PTH target. Surprisingly, an increase in expression of renal CYP24 mRNA was also observed. CYP24 catalyzes the inactivation of vitamin D3 to 1, 24, 25-trihydroxycholecalciferol and is generally regulated by PTH and vitamin D3 in a reciprocal manner to CYP27B1 (Zierold et al., 2003). It is possible that this represents a secondary response to increased serum vitamin D3. Reduced serum calcium may contribute to the observed reduction in bone formation after PCB 126 treatment since calcium has been shown to stimulate osteoblast proliferation, differentiation and matrix mineralization *in vitro* as a result of stimulation of signaling via the calcium-sensing receptor (CaSR) (Goltzman and Hendy, 2015). Reductions in CaSR signaling and increases in serum vitamin D3 might also explain in part why we observed no increase in bone resorption despite development of secondary hypoparathyroidism. Loss of CaSR has been shown to protect against PTH-induced bone resorption in PTH infused $Pth^{-/-};Casr^{-/-}$ mice (Xue et al., 2012) and long term treatment with 1,25-dihydroxycholecalciferol increases bone mass as a result of suppressing bone resorption (Nakamichi et al., 2017). Suppressed bone formation after PCB 126 exposure and prevention of this by CH-223191 may in addition reflect direct AhR-mediated inhibition of osteoblast differentiation as has been reported *in vitro* using TCDD (Korkalainen et al., 2009; Ryan et al., 2007; Yu et al., 2014a). However, whereas it has been suggested that inhibition of β -catenin signaling mediates dioxin effects on osteoblastogenesis (Yu et al., 2014a), we did not observe changes in β -catenin expression in bone cells in the current study.

We observed dose-dependent increases in expression of CYP1B1 and suppression of mRNAs encoding calcium transporters TRPV6 and PMCA1b in human intestinal Caco-2 cells exposed to PCB 126 *in vitro*. Further studies are required to define AhR binding on the promoters of these genes and whether AhR interacts with Vitamin D response elements. Reductions in bone growth and long bone length may also be related to disruption of the GH-IGF-1 axis. Both systemic GH/IGF-1 and local GH-stimulated IGF-1 synthesis in bone are known to regulate these processes (Yakar and Isaksson, 2015). We observed reductions in both serum IGF-1 – which reflects GH-stimulated hepatic synthesis (Yakar and Isaksson, 2015) – and in expression of male-specific hepatic cytochrome P450 enzymes CYP2C11 and CYP3A2 which are regulated by male-specific patterns of GH secretion (Waxman and O'Connor, 2006). These data would be consistent with either reductions in GH secretion or with impaired GH signaling via the growth hormone receptor (GHR)-JAK-STAT signaling pathway. Despite growth retardation produced by TCDD treatment and stimulation of AhR signaling in rats, previous studies have not observed significant effects on plasma GH concentrations or on secretion of other pituitary hormones (Moore et al., 1989). In contrast, recent studies on TCDD treatment in male mice have reported significant repression of hepatic GHR and IGF-1 mRNA expression (Fader et al., 2018). Further studies are required

to elucidate the molecular mechanisms whereby AhR activation suppresses GH signaling in rat liver and bone.

Interestingly, the current data for skeletal effects of PCB 126 in rats are markedly different from a recent report examining the dose-responsive effects of TCDD on bone in C57BL/6 mice (Fader et al., 2018). Despite similar endocrine effects including increased serum 1,25-dihydroxy vitamin D3 and reduced IGF-1 reported by Fader et al, 2018, in addition to a reduction in bone marrow adiposity was observed in agreement with previously reported *in vitro* suppression of adipogenesis in human pre-adipocytes by PCB 126 (Gadupudi et al., 2015), that study reported a substantial dose-dependent increase in trabecular bone BV/TV and mineral density. This involved reduced trabecular spacing and number, and substantially increased trabecular thickness and increased osteoblast count (Fader et al., 2018). Moreover, no effects of TCDD were observed on bone size or any cortical parameters. The bone morphological effects were ascribed to increased osteoblastogenesis, inhibited osteoclast function and reduced MSC differentiation into adipocytes. Increased trabecular bone occurred despite significant decreases in serum osteocalcin and PiNP bone formation markers and was suggested to be mediated via TCDD-mediated induction of the osteoactivin gene (Fader et al., 2018). Part of the difference in bone phenotypes may be related to the relative potency of PCB 126 vs. TCDD for the AhR. PCB 126 has a TCDD Equivalency Factor (TEF) of 0.1 (Gadupudi et al., 2016) and thus the modest effects of PCB 126 on trabecular bone may reflect its relative lack of affinity for the AhR compared to TCDD. However, there are also substantial species differences between AhR signaling. AhR knockout rats and mice have distinct hepatic and renal phenotypes. Adult AhR $-/-$ rats but not mice develop renal pathology including hydronephrosis, suggesting a more important role for AhR signaling in rat renal function (Boverhof et al., 2006; Harrill et al., 2013). Moreover, AhR-mediated gene expression profiles in rat and mouse hepatocytes differ substantially (Boverhof et al., 2006; Forgacs et al., 2013; Kovalova et al., 2017) and similar differences may occur in AhR signaling in bone cells from rats, mice and humans. Additional *in vitro* dose-response studies with different AhR ligands in primary bone marrow mesenchymal stem cell cultures derived from rats, mice and humans will be required to resolve these issues.

In conclusion, our data demonstrate that, consistent with epidemiological data from populations exposed to persistent organochlorine pollutants, skeletal toxicity occurs in male rats following exposure to the coplanar PCB congener PCB 126. These effects appear to be mediated in part via endocrine disruption and in part by direct effects on bone turnover and bone cell differentiation. Further studies are required to identify the AhR dependent mechanisms underlying skeletal toxicity and AhR-ligand and species differences in these pathways.

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Fig. 1.
Experimental design rat PCB 126 exposure study

Fig. 2.

a. Representative 3D reconstructions of trabecular (left) and cortical (right) compartment of tibiae from male rats treated with oil vehicle or PCB 126. Scale bar = 1.0 mm. **b.** Treatment with PCB 126 decreases tibial bone length in male rats. Control: oil vehicle, PCB: PCB 126, * statistically significant $P < 0.05$ control vs. PCB

Fig. 3.

Treatment of male rats with PCB 126 inhibits bone formation markers but has no effect on bone resorption marker CTX1. a. Serum osteocalcin; b. Serum RatLaps (CTX1). Control: oil vehicle; PCB: PCB 126, *statistically significant $P < 0.05$ control vs. PCB

Fig. 4. Treatment of male rats with PCB 126 results in endocrine disruption. a. Serum calcium; b. Serum phosphorous; c. Serum parathyroid hormone (PTH-1); d. Serum IGF-1. Control: oil vehicle, PCB: PCB 126. *, **Statistically significant $P < 0.05$, $P < 0.005$ soy oil control vs. PCB 126.

Fig. 5.

Treatment of male rats with PCB 126 results in suppression of male specific hepatic CYP mRNAs independent of fasting status. a. *Cyp2c11* b. *Cyp3a2*. Means with differing letters statistically significant $P < 0.05$; $a < b < c$ (two-way ANOVA, Tukey's post-hoc). PCB 126 treatment increases expression of renal cytochrome P450 mRNAs in male rats. c: *Cyp27b1*; d: *Cyp24*. Control: oil vehicle; PCB: PCB 126, * statistically significant $P < 0.05$ control vs. PCB. *Hprt*- and *18s*-normalized expression levels in Control samples have been set to a value of 1.

Fig. 6.

a. PCB 126 exposure to human intestinal Caco-2 cells induces expression of human *CYP1B1* mRNA and suppresses expression of calcium transporter *TRPV6* and *PMCA1b* mRNAs in the presence of vitamin D3 *in vitro*. Vh – vehicle (DMSO, 0.01%) control, *Statistically significant $P < 0.05$ Vh vs. 100 nM PCB 126. *** Statistically significant $P < 0.001$ Vh vs. 10 nM and 100 nM PCB 126. Expression of target genes is reported relative to untreated cultures that did not receive 1×10^{-8} M vitamin D3. b. PCB 126 exposure induces expression of rat renal calcium transporters *TRPV5* and *KLOTHO* mRNAs *in vivo*, * statistically significant $P < 0.05$ control vs. PCB 126

Fig. 7.

Western immunoblot analysis of β -catenin staining in control and PCB 126 treated rat femur shaft nuclear extracts a. Representative blot, b. Quantification of band density normalized to that of TATA binding protein. N = 4 nuclear extracts. n.s.: not significant ($P > 0.05$).

Fig. 8.

Differentiation of bone marrow derived mesenchymal stem cells from 9-week old male Sprague-Dawley rats is inhibited by PCB 126 *in vitro*. PCB effect is blocked by 5 μ M CH223191 AhR inhibitor. a. Representative Alizarin red staining, b. Quantification of calcium nodules. Mean $\pm$ SEM for triplicate cultures unless otherwise noted. * Superscript: $P < 0.05$; $a < b < c$, two-way ANOVA (Tukey's post-hoc)

Table 1.

MicroCT analysis of tibial bone morphology in male rats treated with PCB 126.

Bone Parameter	Oil Control	PCB 126	P Value
<u>Trabecular Bone</u>			
BV/TV	0.125 ± 0.008	0.159 ± 0.012	0.012
Conn.Dens.	59 ± 6.7	70 ± 8.0	NS
Trab. N.	3.4 ± 0.15	3.6 ± 0.18	NS
Trab. Th.	0.058 ± 0.001	0.065 ± 0.001	P<0.001
Trab. Sp.	0.31 ± 0.02	0.29 ± 0.03	NS
<u>Cortical Bone</u>			
Total SA	6.74 ± 0.21	5.15 ± 0.21	P<0.001
Cort. SA	4.18 ± 0.22	3.52 ± 0.13	0.002
Cort. Th.	0.47 ± 0.01	0.45 ± 0.01	NS
Endost. Perim.	5.94 ± 0.16	4.75 ± 0.19	P<0.001
Periost. Perim.	10.5 ± 0.32	9.30 ± 0.40	0.03
Mid Shaft Diam	1.27 ± 0.02	1.10 ± 0.02	P<0.001
Medul. Diam.	0.72 ± 0.01	0.54 ± 0.03	P<0.001
Medul. Area	2.18 ± 0.1	1.30 ± 0.1	P<0.001

Data are mean ± SEM for N = 10 control (fed + fasted) and 11 PCB treated (fed + fasted) rats. BV/TV – bone volume/total volume; ConnDens – connectivity density; Trab.N. – trabecular number; Trab.Th. – trabecular thickness; Trab.Sp. – trabecular separation; Total SA – total surface area; Cort. SA – cortical surface area; Cort. Th. – cortical thickness; Endost. Perim. – endosteal perimeter; Periost. Perim. – periosteal perimeter; Mid Shaft Diam. – mid shaft diameter; Medul. Diam. – medullary diameter; Medul. Area – medullary area.