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Hydroxyl metabolite of PCB 180 induces DNA damage signaling and enhances the DNA damaging effect of benzo[a]pyrene

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ABSTRACT

Non-dioxin-like (NDL) polychlorinated biphenyls (PCBs) and their hydroxyl metabolites (OH-PCBs) are ubiquitous environmental contaminants in human tissues and blood. The toxicological impact of these metabolites is poorly understood. In this study rats were exposed to ultrapure PCB180 (10–1000 mg/kg bw) for 28 days and induction of genotoxic stress in liver was investigated. DNA damage signaling proteins (pChk1Ser317 and γH2AXSer319) were increased dose dependently in female rats. This increase was paralleled by increasing levels of the metabolite 3′-OH-PCB180. pChk1 was the most sensitive marker. In *in vitro* studies HepG2 cells were exposed to 1 μM of PCB180 and 3′-OH-PCB180 or the positive control benzo[a]pyrene (BaP, 5 μM). 3′-OH-PCB180, but not PCB180, induced CYP1A1 mRNA and γH2AX. CYP1A1 mRNA induction was seen at 1 h, and γH2AX at 3 h. The anti-oxidant N-Acetyl-L-Cysteine (NAC) completely prevented, and 17β-estradiol amplified the γH2AX induction by 3′-OH-PCB180. As 3′-OH-PCB180 induced CYP1A1, a major BaP-metabolizing and activating enzyme, interactions between 3′-OH-PCB180 and BaP was also studied. The metabolite amplified the DNA damage signaling response to BaP. In conclusion, metabolism of PCB180 to its hydroxyl metabolite and the subsequent induction of CYP1A1 seem important for DNA damage induced by PCB180 *in vivo*. Amplification of the response with estradiol may explain why DNA damage was only seen in female rats.

1. Introduction

Polychlorinated biphenyls (PCBs) are man-made products that were used in technical and commercial applications from 1929 and onwards. Production was terminated in many countries around the globe during the 1970s. The PCBs became and remain one of the most serious and ubiquitous environmental organic pollutants known [33,27,14,13]. PCB180 is among the most abundant PCB congeners found in humans and wildlife and is one of the seven indicator PCB congeners (PCBs 28, 52, 101, 118, 138, 153 and 180) used to quantify total dietary exposure [43].

PCBs are commonly divided into dioxin-like (DL) and non-dioxin-like (NDL) PCBs. The planar DL-PCBs mediate biochemical activity via binding to the aryl hydrocarbon receptor (AhR) and, subsequently induce a number of xenobiotic metabolizing enzymes, such as cytochrome P450 (CYP) enzymes 1A1, 1A2 and 1B1. The NDL-PCBs mediate biochemical reactions via activation of constitutive active (androstane) receptor (CAR) and the pregnane X receptor (PXR) [24]. These receptors activate several xenobiotic metabolizing enzymes including CYP2B1 and CYP3A1 [39].

The oxidative metabolism of PCBs to hydroxyl metabolites represents a major pathway for the removal of these compounds from humans and wildlife [22,36,34]. High levels of OH-PCBs have been detected in wildlife, laboratory animals and humans [7,28]. In human blood, approximately 40 different OH-PCBs have been identified [21]. The toxicological impact of hydroxyl metabolites of PCBs is poorly understood, but several studies indicate that they

Abbreviations: PCBs, polychlorinated biphenyls; PAHs, polycyclic aromatic hydrocarbons; BaP, benzo[*a*]pyrene; DL-PCBs, dioxin-like PCBs; NDL-PCBs, non-dioxin-like PCBs; HepG2, human hepatocellular carcinoma cell line; ANF, α-Naphthoflavone; Est, 17β-estradiol; NAC, N-Acyetyl-L-Cysteine; OH-PCB180, 2,2′,3,4,4′,5,5′-heptachloro-3′-biphenylol [3′-OH-PCB180]; PCB180, 2,2′,3,4,4′,5,5′-heptachlorobiphenyl.

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may have adverse effects in humans; for example, thyroid hormone disruption and possibly neurodevelopmental effects [44].

In a recent *in vivo* study on the NDL PCB180 we documented a dose-related DNA damage response in liver samples from female rats, but not in livers from male rats [42]. In the present study this response has been studied further. We employed Chk1 phosphorylation (Ser317) and γH2AX as markers of DNA damage signaling and of genotoxic stress. Chk1 is phosphorylated by ATR in response to single-stranded DNA regions [30] and may have a role as "signal spreader" and should have the potential to be a sensitive marker of genotoxic stress [6]. γH2AX was used to indicate double strand breaks (DSBs) [37], which are highly mutagenic DNA damages [31]. We relate the pChk1 and γH2AX responses to the presence of the 3′-OH-PCB180 metabolite in liver samples and show that 3′-OH-PCB180 induces CYP1A1 mRNA in human HepG2 cells. We also show that the pChk1 and γH2AX responses were completely inhibited by antioxidants.

2. Materials and methods

2.1. In vivo study

The *in vivo* study was conducted at the laboratory animal unit of the National Institute for Health and Welfare in Kuopio, Finland (for more details see [32,42]). Briefly, the study protocol was approved by the National Animal Experiment Board (license No. ESLH-2006-07965/Ym23). The experimental protocol followed the OECD 407 Guideline on Repeated Dose 28-day Oral Toxicity Study in Rodents. A total of 80 Sprague–Dawley rats were used at the age of 6 weeks; the mean body weight (±SD) of males was 186.3 ± 14.1 g and 136.3 ± 6.8 g for females. They were kept in stainless steel, wire-bottomed cages holding 5 rats per cage (45 × 38 × 19 cm) and given standard pelleted R36 feed (Lactamin, Sweden), and tap water *ad libitum*. The animals were free of typical rodent pathogens and were housed under standard conditions.

In order to achieve rapidly the kinetic steady state, the total dose was divided into 6 daily loading doses and 3 weekly maintenance doses, which were calculated according to the formula of Gibaldi and Perrier [16]. PCB 180 dissolved in corn oil or corn oil alone (control) was administered by oral gavage at 4 ml/kg using a metal cannula with a ball tip. Loading doses were administered on days 0–5, and maintenance doses on days 10, 17 and 24 of the study. At the end of the treatment period rats were anesthetized with CO_2/O_2 (70/30%), and killed by exsanguination. Liver samples were snap frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.2. Detection of liver tissue PCBs levels

For the analysis of unchanged PCB180 and OH-PCBs liver tissue from five individuals from each dose was pooled and extracted according to Jensen [23]. Briefly, the OH-PCBs were isolated using potassium hydroxide (0.5 M) in 50% ethanol, and the fraction containing the OH-PCBs was derivatized with diazomethane as described elsewhere [20]. In both fractions lipids were removed by concentrated sulfuric acid treatment. Further clean-up of both fractions was done on an activated silica gel (0.1 g) with 0.9 g activated silica gel impregnated with sulfuric acid (2:1, w/w) on top of the column. Neutrals, i.e. in this case PCB180, was eluted with 10 mL of cyclohexane:dichloromethane (1:1, v/v) and phenolic compounds were eluted with 15 mL of the same solvent. For the phenolic fraction (OH-PCBs) a second column packed with 0.7 g of activated silica was used, a first fraction with 4 mL of cyclohexane was applied to remove possible interferences and the OH-PCBs were then eluted with dichloromethane (10 mL). 2,2′,3,3′,4,5,6,6′-Octachlorobiphenyl (PCB200) and 2,3,3′,4′,5,5′,6-heptachloro-4-biphenylol (4-OH-PCB193) was used as internal standards for quantification purposes for PCB180 and OH-PCBs, respectively. The analysis of PCB180 and related OH-PCBs was performed using a Varian 450 GC equipped with a Varian CP-8400 autosampler and an electron capture detector (ECD) maintained at 360 °C. Injections (0.8 μl) were done on a split/splitless injection operated in the splitless mode at a temperature of 260 °C. The column used was a non-polar Varian CP-Sil 8 CB (25 m × 0.15 mm × 0.12 μm) column (Middleburg, the Netherlands) with hydrogen as carrier gas and nitrogen as make-up gas. The GC temperature program used for OH-PCBs was 80 °C (2 min), 20 °C/min to 230 °C (0 min), 2 °C/min to 250 °C (0 min) and finally 30 °C/min to 320 °C (held for 5 min). For PCB180 the program was 80 °C (2 min), 20 °C/min to 300 °C (5 min).

2.3. Cell culture

The human hepatocellular carcinoma cell line (HepG2), known to metabolize xenobiotics, was purchased from the American Type Culture Collection. The cells were grown in a minimal essential medium with Earle's salts and L-glutamine, and supplemented with sodium pyruvate (1 mM), non-essential amino acids, (100 IU/100 mg/ml) penicillin/streptomycin, and 10% inactivated fetal bovine serum. Media components were purchased from Gibco (Gibco® BRL, Life Technologies, Täby, Sweden). HepG2 cells were seeded (0.3×10^6/ml) 72 h prior to exposure to PCBs (1 μM) and/or benzo(a)pyrene (BaP) (5 μM). 10nM 17β-estradiol (Est), 1 μM α-Naphthoflavone (ANF) or 1 mM N-Acyetyl-L-Cysteine (NAC) were added 1 h before PCB or BaP exposure.

2.4. Chemicals

Ultrapure PCB180 and 3′-OH-CB180 used in this study were obtained from the ATHON project [38] (for more details about the purification of PCBs see [1,2]). PCB 180 was bought from Chiron, Trondheim, Norway and 3′-OH-PCB 180 from Accustandard, New Haven, USA. An additional check for impurities in the 3′-OH-PCB180 metabolite batch was performed, and the level of polychlorinated dibenzo-*p*-dioxins, -furans and DL-PCB impurities was 2.12 ng WHO-TEQ/g 3′-OH-PCB180 as determined using the protocol described by [11]. The value is well below the limit values on impurities of dioxin-like compounds as set for ATHON and should not have any appreciable impact on e.g. CYP1A1 levels. Benzo(a)pyrene (BaP), α-Naphthoflavone (ANF), 17β-estradiol (Est), 2′,7′-Dichlorofluorescein diacetate and N-Acyetyl-L-Cysteine (NAC) were purchased from Sigma–Aldrich (Sigma–Aldrich Sweden AB, Stockholm, Sweden). Other chemicals and solutions used in this study were obtained from international sources, unless otherwise noted.

2.5. Western blotting

The cells were washed with phosphate-buffered saline and lysed in IPB.7 (1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM NaF, 1 mM Na_3VO_4, 0.1 mg/ml trypsin inhibitor, and 1 mg/ml aprotinin). The protein was quantified by using Coomassie Plus™ Protein Assay Reagent (Pierce, Täby, Sweden), and the samples were subjected to SDS–PAGE. The separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) and, thereafter, blocked in non-fatty milk for 1 h. Subsequently, the protein bands were probed overnight with antibodies at −4 °C.

Primary antibodies used were phospho-γH2AX-(Ser319), phospho-p53-(Ser-15) and phospho-Chk1-(Ser317) (Cell Signaling Technology). The loading control Cdk2 (M2) sc-163 was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Secondary antibodies used were goat anti-rabbit IgG sc-2004, and goat anti-mouse