Effects of polychlorinated biphenyls on the hepatic transformation of estradiol-17beta into catechol- and methoxy-estrogens in prepubertal female Sprague Dawley rats

Bio Aikawa[1], Karen Leingartner[1], Guillaume Pelletier[1], Jiping Zhu[1], Saswati Sen[1], Make Wade[1], Gong-hua Xiao[1], Wayne J Browers[1], Nanqin Li[1], Gerard Cooke[1], Ih Chu[1], Daniel Desaulniers[1]

[1]Health Canada

Introduction

In rats, acute or short term exposure to high doses of organochlorines and pesticides increase the expression of cytochrome P450 enzymes and the production of the toxic catecholestrogens (CE) (estrogens hydroxylated in position 2 and 4).[1, 2, 3, 4] Methylation of the CE by the enzyme catechol-*o*-methyltransferase (COMT) is a major mechanism for deactivating CE. Aroclor 1254 exposure in the adult rat generates metabolites of polychlorinated biphenyls (PCB) suspected of inhibiting the deactivation of CE by COMT.[5] It has been shown that postnatal exposure to a mixture of aryl-hydrocarbon receptor agonists decreased mRNA expression of COMT. Collectively, these observations suggest that exposure to xenobiotics could lead to tissue specific accumulation of toxic estrogen metabolites.[6] Change in the hepatic metabolism of estrogens was shown to be a persistent effect[7], and thus might lead to adverse outcomes. Changes in estrogen metabolism and signaling affect hypertension, lipid metabolism, brain development, numerous organs (ovaries, uterus, placenta, testicles, breast, bones), systems (vascular, immune), and cancers (breast, endometrium, ovary, prostate, liver, kidney, brain).[8, 9, 10, 11]

This paper describes the preliminary results of an experiment examining the effects of PCB treatment on the hepatic transformation of estradiol-17β (E2) into CE and methoxyestrogens (MeO-E) in prepubertal female Sprague Dawley rats. Metabolism of ^{14}C-estradiol-17β (^{14}C-E2) to known and unknown radioactive metabolites was assessed following high performance thin-layer chromatography (HP-TLC) separation and phosphorimaging detection to determine the relative abundance of metabolites produced. Gas chromatography/mass spectrometry (GC/MS) with negative ion chemical ionization (NCI) was used to quantify 4 estrogen metabolites that were formed: 2OH- and 4OH-E2, and 2MeO- and 4MeO-E2.

Materials and Methods

Pregnant rats were dosed from gestation day 1 to postnatal day (PND) 21 with a mixture of PCBs prepared based on the profile found in Arctic human populations. The mixture was prepared and validated by others.[12] According to body weight, a measured volume of the PCB mixture dissolved in corn oil was applied onto a small cookie that was consumed by the dams each day. The control rats received corn oil only, while the treated rats received 1.1 mg/kg/day of the PCB mixture. The PCB congeners and their amount (expressed as a percentage of the total) were: PCB 28-0.65%, 52-1.4%, 99- 8.85%, 101-1.32%, 105-1.5%, 118-6.61%, 128-0.65%, 138-19.52%, 153-28.9%, 156-2.64%, 170-5.11%, 180-13.85%, 183-1.76%, and 187-7.23%. Female rats were sacrificed at PND 21 and liver was collected and stored at –80ºC for analysis.

Liver samples (approximately 1g) were homogenized in Buffer-1 (0.05 M Tris, 0.15 M KCl, pH 7.4, 5 mM ascorbic acid, 0.1 mM EDTA) and centrifuged at 9000g (20 minutes, 4^{o}C). The supernatant was further centrifuged (176 000g_{max}, 1 hour, 4^{o}C) to separate the cytosol (supernatant) and microsomes (pellet). For the negative control, heat inactivated samples were prepared by boiling control microsomes and cytosol for 5 minutes. The complete hepatic metabolism reaction (1ml, 37^{o}C) was 50 min long. During the first 30 min, 1 mg of microsome was incubated in Buffer-1, with 50 µM E2 containing 0.1µCi of [4-^{14}C-estradiol] (American Radiolabeled Chemicals, St. Louis, MO) and 0.25 mM NADPH. To assess cytosolic COMT activity, the reaction was continued for another 20 minutes after the addition of 1 mg of cytosolic protein from the same liver sample and 0.9 ml of supplemented Buffer-1 (pH 8.5, 0.6 mM L-adenosyl-S-methionine, 2 mM $MgCl_2$, 2 mM dithiothreitol). COMT activity was inhibited in some samples by adding pyrogallol (1mM) during the second incubation. The metabolism reaction was terminated by transferring the tubes to

an ice water bath and adding 2 ml of ice-cold methanol containing 5 mM ascorbic acid and 10 estrogen standards (1 µg/ml of each). The tubes were vortexed, centrifuged (3000g, 15 minutes, 4^{o}C), and the supernatants were applied to conditioned Sep-Pak columns. Samples were eluted with 2 ml of methanol and evaporated using a SpeedVac system.

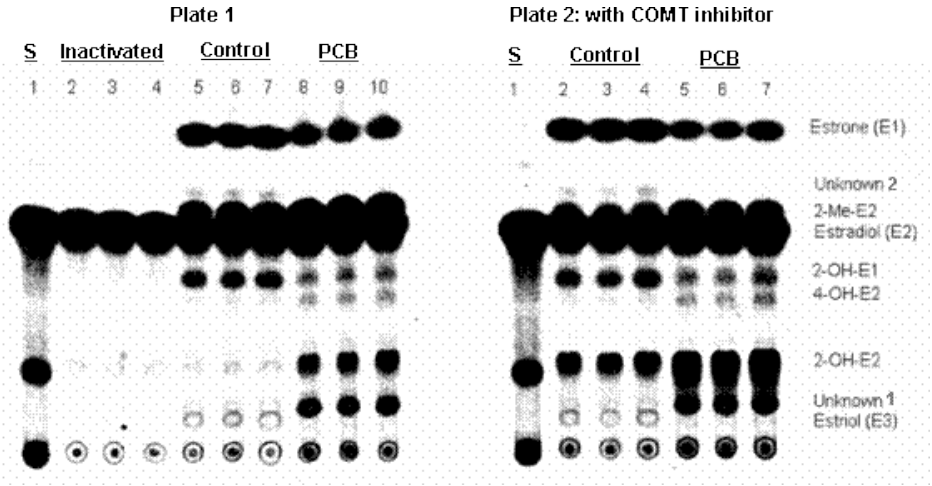
For HP-TLC separation of the estrogen metabolites, the dried samples were re-suspended in 20µl of chloroform-methanol (1:1 v/v) and applied to TLC plates (Merck HP-TLC Silica Gel 60). Plates were developed in a pre-equilibrated chamber using 8:2 chloroform-acetone as the mobile phase, and allowed to dry. To detect the radioactive metabolites, the TLC plates were placed in an exposure cassette along with a storage phosphor GP screen (Amersham) for 48 hours, and the screen was scanned using a Storm 860 scanner (Molecular Dynamics). Iodine vapour exposure was used to visualize the estrogen standards.

For samples analyzed by GC-MS, the metabolism reaction was slightly modified and did not include the [4-^{14}C-estradiol], pyrogallol inhibitor, or estrogen standards. 100µl of pentafluoropropionic acid anhydride (PFAA): acetonitrile (3:7, v/v) was added to the dried extract, and heated at 50^{o}C for 30 min. The mixture was evaporated to dryness under a stream of nitrogen, re-dissolved in 100µl of hexane, and injected to an Agilent 6800N gas chromatograph (GC) coupled to an Agilent 5973N quadrupole mass spectrometer (MS). The carrier gas was helium and a DB-5MS column (30m x 0.25mm x 0.25µm, J&W Scientific) was used. Injection volume was 1 µl in a splitless mode. The temperature program was started at 60^{o}C holding for 5 min, then increased by 25^{o}C/min to 190^{o}C, by 2^{o}C/min to 230^{o}C, by 5^{o}C/min to 250^{o}C, and by 50^{o}C/min to 290^{o}C and holding for 2 min. The total analysis time was 31.4 min. Injection port temperature and auxiliary temperature were 290^{o}C and 250^{o}C, respectively. Methane was used as reagent gas for NCI. Quadrupole and MS source temperatures were 150^{o}C. Analysis was done by selected ion monitoring using one target ion and one qualifier ion for each analyte.

Results and Discussion

HP-TLC analyses (Figure 1) demonstrated that the PCB treatment induced a major increase in the formation of 2OH-E2, and of an unknown metabolite (unknown 1). The abundance of 4OH-E2 was also increased, but that of estrone (E1), 2OH-E1 and unknown 2, were decreased. The amount of 2MeO-E2 also appeared to increase with the PCB treatment, however the large abundance of substrate (E2) and the presence of 4MeO-E2 (not shown here) located between 2MeO-E2 and E2, made it difficult to demonstrate effects on methoxy-E2. The difference in levels of 2OH-E2 and 4OH-E2 demonstrated that the 2OH-E2 formation is a predominant metabolic pathway. The comparison of plate 1 (P1) with plate 2 (P2) provides an indirect demonstration of CE via methylation by COMT. The control samples with an active COMT system (P1, L5-7) showed very little

Figure 1: Phosphorimager imprints of radioactive metabolites separated by HP-TLC



S: substrate, ^{14}C-estradiol-17b. P1, lane (L)2-4: heat inactivated microsomes. P1 L5-7 and P2 L2-4: triplicate of the same control rat. P1 L8-10 and P2 L5-7: triplicate of the same PCB treated rat. On P2, all samples were incubated

with the COMT inhibitor pyrogallol.

2OH-E2, in contrast, in the absence of COMT activity (P2, L2-4) there is a clear accumulation of 2OH-E2. This effect is also evident with the samples of the PCB treated rat (P1, L8-10 versus P2, L5-7). These results strongly support that COMT is involved in the detoxification of 2OH-E2. In the case of methoxy-E2, the resolution potential of the HP-TLC technique is limited and does not permit the detection of COMT effects on 4MeO-E2. The quantitative GC/MS-NCI technique is being validated and should allow us to confirm these findings. At this early stage, GC/MS-NCI analysis revealed that the PCB treatment increased the global transformation of E2 by 20%, the abundance of 2OH-E2, 4OH-E2, 2MeO-E2, and 4MeO-E2, by 214%, 133%, 292%, and 760%, respectively. This confirms the results for 2OH-E2, 4OH-E2 and 2MeO-E2 obtained with the previous method, and revealed the increase in 4MeO-E2 that was not detectable from the HP-TLC technique.

This work is novel in its HP-TLC technique, and in demonstrating major effects on estrogen metabolism in weanling female rats following *in utero* and lactational exposure to a PCB mixture representative of the profile found in Arctic human population. Following further refinement of the methods, this approach will be used to study tissue specific effects, at lower dose of exposure, and following exposure to different chemicals.

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[1] Badawi, A. F., Cavalieri, E. L., and Rogan, E. G. (2000). *Carcinogenesis* **21**, 1593-1599.

[2] Graham, M. J., Lucier, G. W., Linko, P., et al. (1988). *Carcinogenesis* **8**, 1935-1941.

[3] Painter, J.T., Pinnella, K.D., Tessari, J.D., et al. (2001). In *PCBs:Recent Advances In Environmental Toxicology And Health Effects* (L.W.Robertson and L.G.Hansen, Eds.), pp. 277-279. The University Press of Kentucky, Lexington, USA.

[4] Suchar, L. A., Chang, R. L., Thomas, P. E., et al. (1996). *Endocrinology* **137**, 663-676.

[5] Garner, C. E., Burka, L. T., Etheridge, A. E., et al. (2000). *Toxicol Appl.Pharmacol.* **162**, 115-123.

[6] Desaulniers, D., Xiao, G. H., Leingartner, K., et al. (2005). *Toxicol.Sci.* (In press).

[7] Desaulniers, D., Cooke, G. M., Leingartner, K., et al. (2005). *Int.J.Toxicol.* (In press).

[8] Cavalieri, E. L., Devanesan, P., Bosland, et al. (2002). *Carcinogenesis* **23**, 329-333.

[9] McEwen, B. S. and Alves, S. E. (1999). *Endocr.Rev.* **20**, 279-307.

[10] Rogan, E. G., Badawi, A. F., Devanesan, P. D., et al. (2003). *Carcinogenesis* **24**, 697-702.

[11] Zhu, B. T. and Conney, A. H. (1998). *Carcinogenesis* **19**, 1-27.

[12] Chu,I., Bowers,W., Nakai,J., et al. (2004). In *Synopsis of Research Conducted Under the 2003-2004 Northern Contaminants Program* (S.Smith, J.Stow, and F.Carrillo, Eds.), pp. 37-45. Minister of Indian Affairs and Northern Development, Ottawa, ON.