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Effects of PCB 30 and its Hydroxylated Metabolites on Ecdysteroid-Mediated Gene Expression

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Disruption of endocrine function by environmental contaminants is a major topic of concern in environmental toxicology (Oberdorster *et al.* 1999). Active compounds have been termed hormonally active agents (HAAs) but are commonly called endocrine disrupters, environmental estrogens, or endocrine modulators. HAAs include bisphenol A, dieldrin, DDT (and metabolites), toxaphene, polychlorinated biphenyls (PCBs), dioxins, and phthalates. HAAs have been implicated in the decline of some wildlife populations (Maczka *et al.* 2000). Specifically, PCBs have been reported to cause adverse developmental, reproductive, neurological, and immunological effects in humans and wildlife (Maczka *et al.* 2000).

Invertebrates comprise 95% of the world's animal species, but vertebrate species have been the subject of most research in this area (Weiss 2000). Of invertebrate phyla, crustacean and insect endocrinology has received the most extensive study (Weiss 2000). While differences exist across species, in general, invertebrate endocrine systems have been highly conserved through evolution. As with vertebrates, steroids and peptides are important to the endocrine function of invertebrates.

Molting (ecdysis) is a critical endocrine-controlled process for normal growth and development of invertebrates (Zou and Fingerman 1997). The process is regulated by a multihormonal system with primary control by ecdysteroids, a class of steroid hormones. Ecdysteroids act through specific receptors by a mechanism consistent with that of steroid hormones in other species. Steroid hormones bind receptors within the target cell forming a hormone-receptor complex. The ecdysteroid receptor (EcR) complex forms heterodimers with ultraspiracle (USP) which binds ecdysteroid response elements in DNA. Associated response genes are activated leading to modification of transcription of mRNA and translation of proteins essential to ecdysis.

Because PCBs are widespread, persistent, and have the potential for harmful biological effects, they are of significant concern as organic environmental contaminants. PCBs have been shown to disrupt molting in

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some arthropods (Fingerman and Fingerman 1977; Zou and Fingerman 1997). Some PCBs have been shown to interact with steroid hormone receptor systems in vertebrates (Korach *et al.* 1987, Cheek *et al.* 1999; Anderson *et al.* 1999). Specifically, PCBs and/or their hydroxylated metabolites (OH-PCBs) are active at estrogen receptors (ER) and thyroid receptors (TR) which are analogous to EcRs in their mechanism of action. PCBs may be active at EcRs leading to life-threatening disruption of ecdysis.

Of the vertebrate steroid receptors, the thyroid receptor (TR) is closely comparable to the EcR in both structure and function (Oberdorster *et al.* 1999). Both form heterodimers, TR with the retinoic acid receptor (RXR) and EcR with USP. The ability of PCBs and OH-PCBs to bind TRs has been well documented (Cheek *et al.* 1999; Rickenbacher *et al.* 1986). Additionally, PCBs have been linked to numerous deleterious effects on thyroid function (Brower *et al.* 1998).

4-OH-PCB 30 has been shown to bind (Korach *et al.* 1987) and activate ERs (Anderson *et al.* 1999) *in vitro.* 4-OH PCB 30 also binds transport protein for the TR ligand, thyroxine (Rickenbacher *et al.* 1986). 3'-OH-2,4,6-trichlorobiphenyl and 4-OH-2,3,4,6-tetrachlorobiphenyl competitively bind TRs, thyroid-binding globulin, and the thyroxine transport protein, transthyretin, (Cheek *et al.* 1999). PCB 29 (2,4,5-trichlorobiphenyl) is structurally similar the parent compounds of these metabolites and has been shown to inhibit molting in the water flea, *Daphnia magna* (Zou and Fingerman 1997). Thus, PCB steroid receptor activity could extend to EcRs accounting for PCB-related molt disruption.

PCBs and OH-PCBs were tested for their effects on *in vitro* EcR-mediated activity in a reporter gene assay. PCB 30 (2,4,6-trichlorobiphenyl) and its hydroxylated metabolites 2'-OH-2,4,6-trichlorobiphenyl (2-OH-PCB 30), 3'-OH-2,4,6-trichlorobiphenyl (3-OH-PCB 30), and 4'-OH-2,4,6-trichlorobiphenyl (4-OH-PCB 30) were selected as models for this study.

MATERIALS AND METHODS

Ponasterone A (PonA) was purchased from Invitrogen, Carlsbad, CA; 20-OH-ecdysone (20-OH-E) was obtained from Sigma Aldrich, St. Louis, MO. Neat PCB standards were purchased from Accustandard, New Haven, CT.

The Ecdysone-Inducible Mammalian Expression System purchased from Invitrogen was used for determination of PCB and OH-PCB effects on EcR-mediated gene expression. Logarithmically growing cells (EcR-293, Invitrogen) were seeded at 2.5 x 10^5 cells/well in 24-well plates coated with poly-L-lysine and incubated overnight. The cells were transfected for 5 h with Lipofectamine 2000 Reagent (Invitrogen Life Technologies,

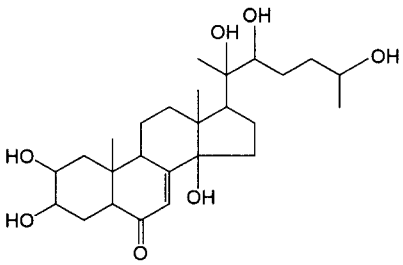
Carlsbad, CA) with the plasmid containing the ecdysone/glucocorticoid response elements linked to β-galactosidase (pIND/Hygro/lacZ; InVitrogen). Details of the procedures are described in the Life Technologies protocol, Form # 52630 (www.lifetech.com). After the 5-h transfection period, the cells were washed then dosed with PCB 30 and its 2-OH, 3-OH, and 4-OH metabolites over a concentration range of 0.01 to 1 ppm (0.04 to 4 μM); PonA and 20-OH-E were dosed in a separate experiment at 0.1 to 10 μM. Additionally, individual PCB 30 compounds were dosed at a constant level of 1 ppm (4 μM) over varying PonA concentrations. After a 16-h incubation, the cells were lysed and analyzed for β-galactosidase activity (substrate: ortho-nitrophenyl-β-galactoside (OPNG); absorbance: 405 nm) and protein (Bio-Rad assay, Bio-Rad Laboratories, Hercules, CA).

SigmaPlot 2001 (SPSS Inc., Chicago, IL) was used to calculate dose response curves by nonlinear regression. When comparing experimental treatments (PCBs plus PonA) to controls (PonA only), doses that showed no overlap of the 95% confidence interval of the respective regression curves were considered to be significantly different.

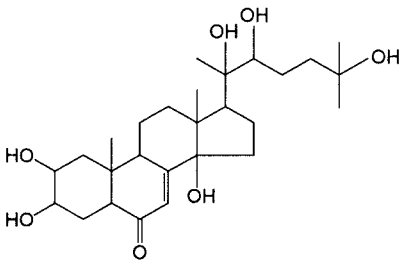
RESULTS AND DISCUSSION

Invitrogen's Ecdysone-Inducible Mammalian Expression System consists of mammalian cells transfected with genetic coding sequences for producing functional heterodimeric receptor subunits. The heterodimer consists of a *Drosophila*-derived EcR and a mammalian-derived RXR. Mammalian cells are not naturally responsive to ecdysteroids and do not contain EcRs. A hybrid response element (half glucocorticoid/half ecdysteroid) reduces cross-reactivity with endogenous mammalian steroid receptor dimers. The system has an ecdysteroid responsive promoter for expression of a reporter gene (β-galactosidase) that can be measured spectrophotometrically.

The assay's response to the principle natural ecdysteroid hormone, 20-OH-E, as well as the potent synthetic analog, PonA, was tested in transfected cells that were exposed to increasing concentrations of each ligand. Structures of these ecdysteroids are shown in Figure 1. Although 20-OH-E (n=1) elicited no response across the dose range (0-10 μM) tested, PonA (n=3) significantly increased β-galactosidase activity (Figure 2). Invitrogen recommends PonA as the ligand of choice for use in the assay; it is approximately 20 times more potent than 20-OH-E in other ecdysteroid-inducible *in vitro* systems (Hormann *et al.* 1999). For this reason, PonA was used as the reference ecdysteroid ligand for other experiments presented in this paper.



Ponasterone A



20-OH-Ecdysone

Figure 1. Structures of ecdysteroids used in this study

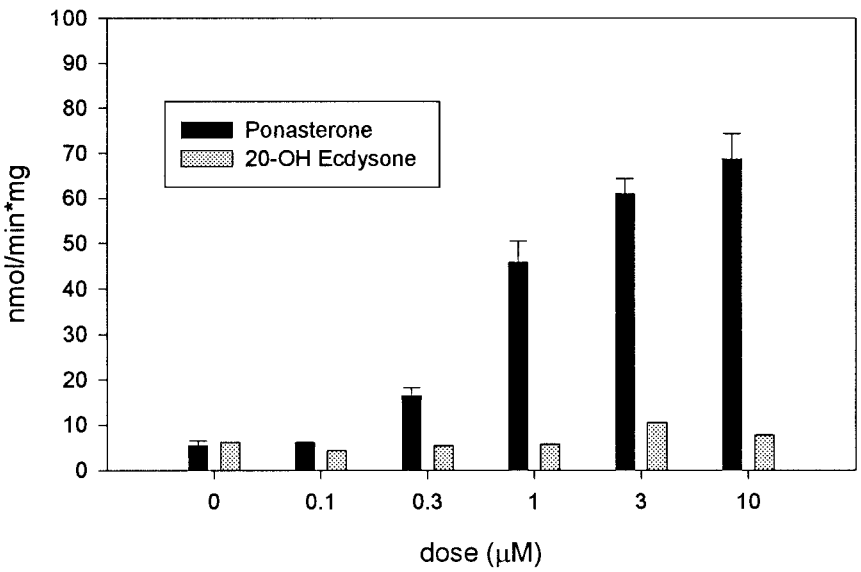


Figure 2. β-Galactosidase activity of ecdysteroid controls. Bars with PonA data indicate S.D.

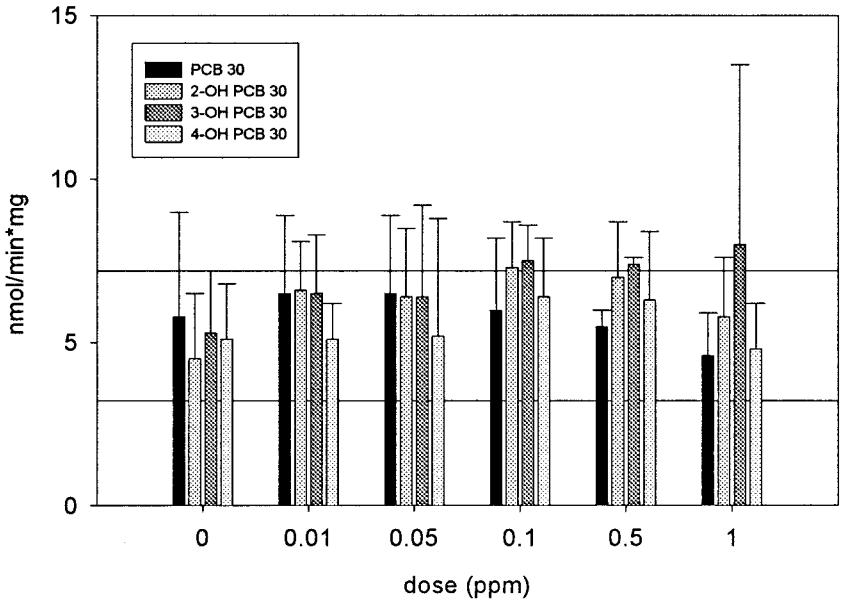


Figure 3. Mean (+/- S.D.) assay ecdysteroid activity of PCB compounds alone. Reference lines represent +/- S.D. of background level.

None of the PCB's tested produced a measurable increase in β-galactosidase activity (n=6) across the dose range (0 – 1 ppm) tested (Figure 3). The upper dose range was limited to 1 ppm due to the relatively low solubility of PCB's in aqueous systems. These results suggest that PCB 30 and its metabolites would not independently activate ecdysteroid receptor mediated responses *in vivo*.

PonA (n=5) was tested in the cell-based assay across a broad dose range (0-100μM) alone and in the presence of PCB 30 or its hydroxlyated metabolites (n=6) at 1 ppm (4 μM). It was demonstrated that 2-OH PCB 30 and 4-OH PCB 30 each significantly reduced maximal PonA-induced assay activity by approximately 30% (Figure 4B and 4D). PCB 30 gave a similar effect but the magnitude of suppression appeared to be minimal (Figure 4A). 3-OH PCB 30 appeared to minimally increase PonA-induced assay activity (Figure 4C). Because all changes in activity brought about by the addition of PCBs were manifested as alterations in maximal activity without an accompanying change in apparent EC_{50}, the likely mechanism is non-competitive inhibition.

The physiological significance of assay inhibition by 2-OH and 4-OH PCB 30 is unclear. 4-OH PCB 30 is predicted to be the favored product of

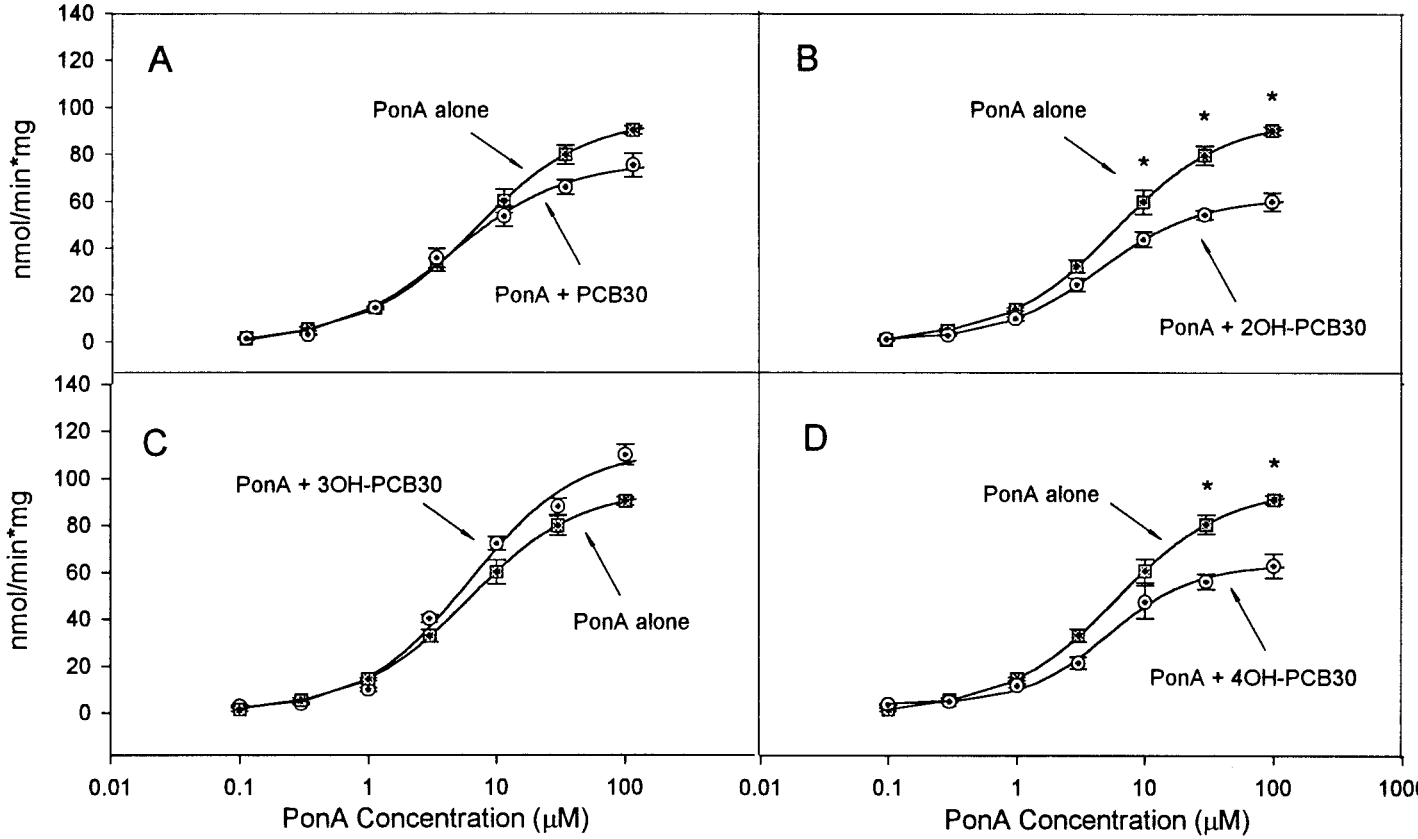


Figure 4. Effects of PCB 30 and metabolites on mean (+/- S.E.M) PonA-induced ecdysteroid activity (Asterisks indicate significant difference at doses where 95% C.I. of regression lines do not overlap)

Cytochrome P450 metabolism (Safe 1980), and it could be critically important with regard to potential *in vivo* ecdysteroid inhibition following exposure to PCB 30. If maximal receptor activity is required for an ecdysteroid-controlled function such as molting, then the observed inhibition could result in an adverse effect hindering the organism's ability to successfully complete and survive the process. The indicated non-competitive nature of the observed effect reveals that the precise site of action is somewhere other than the ecdysteroid ligand binding site. This observation suggests a key difference in 4-OH PCB 30 activity towards ecdysteroid receptors when compared to its activity with estrogen receptors where it competes directly with the steroid ligand for its binding site (Korach *et al.* 1987).

The relative lack of activity for 3-OH PCB 30 is noteworthy when considered along with inhibitory profiles exhibited by both 2-OH and 4-OH PCB 30. This observed structure activity relationship points to the critical nature of the hydroxyl group's substitution position in terms of its relative influence on ecdysteroid activity of these PCB 30 metabolites. Similar differences have been reported with other PCB structural isomers' activity toward estrogen receptors (Connor *et al.* 1997).

Results from these experiments indicate that PCB 30 and its hydroxylated metabolites do not produce *in vitro* ecdysteroid receptor mediated responses when administered individually. However, 4-OH and 2-OH PCB 30 appear to suppress PonA-induced ecdysteroid mediated gene expression in a manner consistent with non-competitive antagonism. 20-OH-E did not elicit the expected dose dependent response in the assay, but it is known to be much less potent than PonA for EcR activation.

The Invitrogen assay employed in this study has significant potential to provide useful data on ecdysteroid receptor activity of a wide variety of PCB congeners as well as other persistent environmental contaminants. Further work is needed to more clearly define the nature of observed effects of PCBs on ecdysteroid-mediated gene expression. Experiments addressing the effects of PCB 30 on invertebrate ecdysis should be conducted to determine the physiological significance of *in vitro* ecdysteroid inhibitory effects observed with 2-OH and 4-OH PCB 30. Future studies should also include screening an expanded list of PCBs to determine possible structure activity relationships for inhibition of ecdysteroid activity.

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