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Potent Inhibition of Estrogen Sulfotransferase by Hydroxylated PCB Metabolites: A Novel Pathway Explaining the Estrogenic Activity of PCBs

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ABSTRACT. Polychlorinated biphenyls (PCBs) are persistent environmental pollutants which exert a variety of toxic effects in animals, including disturbances of sexual development and reproductive function. The estrogenic effects of PCBs may be mediated in part by hydroxylated PCB metabolites (PCB-OHs), but the mechanisms by which they are brought about are not understood. PCBs as well as PCB-OHs show low affinities for both α and ß estrogen receptor isoforms. In the present study we demonstrate that various environmentally relevant PCB-OHs are extremely potent inhibitors of human estrogen sulfotransferase, strongly suggesting that they indirectly induce estrogenic activity by increasing estradiol bioavailability in target tissues.

The endocrine-disrupting effects of PCBs have received much attention recently, in particular their estrogenic activity which is thought to play an important role in the impaired sexual differentiation and reproductive dysfunction observed in exposed birds, fish, reptiles and mammals (1-5). Also in humans, an increase has been observed over the last 50 years in the incidence of testicular cancer and of abnormal male reproductive tract development in some developed countries (4). Decreasing trends in semen quality and sperm counts have also been reported, but this may not be universal (4). Since similar abnormalities in sexual differentiation and reproductive function have been encountered in male offspring of women treated during pregnancy with the potent estrogen diethylstilbestrol (DES) to prevent miscarriage (6), it has been hypothesized that increased exposure to estrogenic and other endocrine-active chemicals, in particular during fetal and neonatal life, may contribute to the above-mentioned defects. This hypothesis is supported by laboratory animal studies showing disruption of endocrine pathways in the adult animal after *in utero* or early postnatal exposure to a variety of environmental contaminants including PCBs, polychlorinated dibenzodioxins and dibenzofurans, pesticides such as 2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT), plastic additives such as bisphenol A, and detergent additives such as alkylphenols (3,4).

Specific PCB congeners exhibit estrogenic activities in experimental animals, whereas other congeners are associated with anti-estrogenic activities (1,2). There is evidence that the estrogenic (and anti-estrogenic) activities of PCBs are mediated at least in part through hydroxylated metabolites (1,2,7), but the mechanism by which PCB-OHs exert their effects has not been established. It has been shown previously for a large number of PCB-OHs that their affinity for both α and ß estrogen receptor subtypes is low (8,9), suggesting that they have little activity as estrogen receptor agonists. However, it is possible that PCBs or PCB-OHs indirectly exert estrogenic activity by inhibiting estradiol (E2) metabolism, thus enhancing cellular E2 bioavailability. Sulfation by estrogen sulfotransferase (EST) is an important pathway for E2 inactivation (10). In this study, we investigated the potential inhibition of human EST (hEST) by hydroxylated PCBs.

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Materials and Methods

Materials. [^{3}H]E2 (3.22 MBq/nmol) was obtained from Amersham (Amersham, UK); [^{35}S]PAPS (52.9 MBq/μmol) from NEN (Boston, MA); unlabeled E2 and PAPS from Sigma (St. Louis, MO). The sources of the various PCB-OHs have been described previously (8,11). Recombinant hEST (12) was expressed in *S. typhimurium* as previously described (13). Cytosolic preparations from these bacteria were used without further purification. EST accounted for 5-7% of the cytosolic proteins. Similar results were obtained with hEST expressed in *E. coli* and purified as previously described (14).

Sulfotransferase assays. Estrogen sulfotransferase activity was analyzed by incubation of 1 nM [^{3}H]E2 for 30 min at 37 C with recombinant hEST (0.1 μg protein/ml) in the absence (blank) or presence of 50 μM PAPS in 0.2 ml 0.1 M sodium phosphate (pH 7.2), 2 mM EDTA and 1 mM dithiothreitol. The reactions were stopped by addition of 2 ml ice-cold water, and the mixtures were extracted with 2 ml dichloromethane. Sulfate formation was quantified by counting 1 ml of the aqueous phase. Enzymatic sulfation was corrected for background radioactivity estimated in the blanks. Kinetic parameters were determined by Lineweaver-Burk analysis (15) of the sulfation of varying substrate concentrations. Apparent K_i values were calculated from the change in slope of the Lineweaver-Burk plot in the presence of inhibitor (15).

Results and Discussion

The effects of increasing concentrations (0.01-1000 nM) of various PCB-OHs were tested on the sulfation of 1 nM E2 by recombinant hEST. The compounds are numbered as explained in Table 1. The nonhydroxylated compound *1* (PCB77) did not affect EST activity even at the highest concentration tested (1000 nM). However, hydroxylation of one of the phenyl rings induced strong inhibitory activity that was dependent on the positions of the substituents in this ring. Figure 1A shows the results with PCB-OHs having the same 3',4'-dichloro-substituted nonphenolic ring. Although the *ortho*-hydroxylated compounds *28* and *30* were relatively weak inhibitors, increasing potencies were observed with the *meta*-hydroxylated compounds *23* and *26*, and even higher inhibitory activities were observed with *para*-hydroxylated compounds, in particular *13* and *18*. Concentrations as low as 0.1 nM of the latter PCB-OHs significantly inhibited EST activity. Also from the potencies of other PCB-OHs it is concluded that an OH group in the *para* position with two adjacent Cl substituents is required for maximum EST inhibitory potency (Table 1).

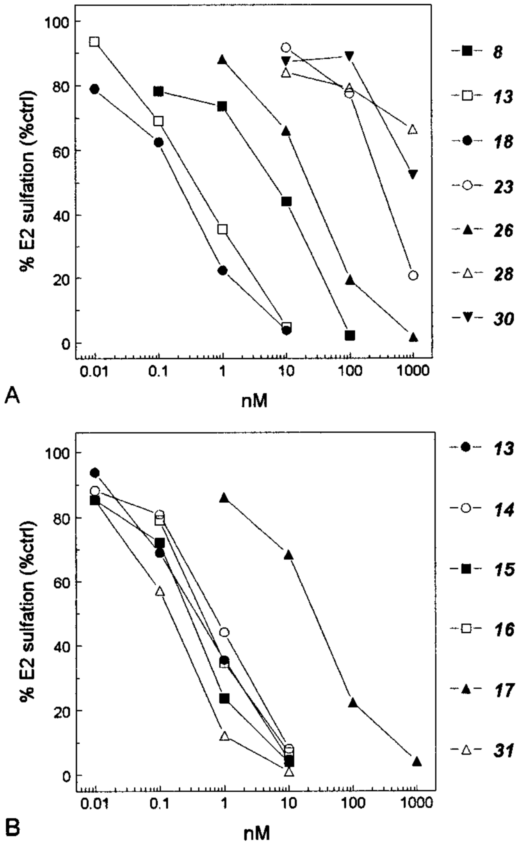

Fig. 1. Inhibition of the sulfation of 1 nM E2 by recombinant hEST by increasing concentrations of PCB-OHs with a 3',4'-dichloro-substituted nonphenolic ring (A) or with a 4-hydroxy-3,5-dichloro-substituted phenolic ring. Results are the means of 2-4 experiments.

Table 1. Potency of inhibition of hEST activity by PCB-OHs

Compound		IC$_{50}$ (nM)
1	3,4,3',4'-tetraCB	>1000
2	**4-OH-**2',4',6'-triCB	610-670
3	**4-OH-**2',3',4',5'-tetraCB	640-650
4	**4-OH-2**,2',4',6'-tetraCB	230-260
5	**4-OH-2**,2',3',4',5'-pentaCB	260-370
6	**4-OH-2**,2',3',4',6'-pentaCB	150-295
7	**4-OH-2**,2',3',5',6'-pentaCB	280-430
8	**4-OH-3**,3',4'-triCB	4.3-7.8
9	**4-OH-3**,2',4',6'-tetraCB	220-240
10	**4-OH-3**,2',3',4',5'-pentaCB	100-120
11	**4-OH-3**,2',3',4',6'-pentaCB	170-200
12	**4-OH-3**,2',3',5',6'-pentaCB	260-370
13	**4-OH-3,5**,3',4'-tetraCB	0.21-0.61
14	**4-OH-3,5**,3',5'-tetraCB	0.47-1.00
15	**4-OH-3,5**,2',3',4'-pentaCB	0.28-0.30
16	**4-OH-3,5**,3',4',5'-pentaCB	0.38-0.50
17	**4-OH-3,5**,2',3',4',5'-hexaCB	20-30
18	**4-OH-2,3,5**,3',4'-pentaCB	0.15-0.25
19	**4-OH-2,3,5**,2',3',4'-hexaCB	0.27-0.75
20	**4-OH-2,3,5**,2',4',5'-hexaCB	5.8-14
21	**4-OH-2,3,5**,2',3',4',5'-hexaCB	25-26
22	**4-OH-2,3,5,6**,2',4',5'-heptaCB	6.8-30
23	**3-OH-4,5**,3',4'-tetraCB	210-410
24	**3-OH-4,5**,2',3',4'-pentaCB	400-580
25	**3-OH-4,5**,3',4',5'-pentaCB	250-380
26	**3-OH-2,4,5**,3',4'-pentaCB	21-24
27	**3-OH-2,4,5**,2',3',4',5'-heptaCB	9.0-13
28	**2-OH-3,4**,3',4'-tetraCB	>1000
29	**2-OH-3,4**,2',3',4'-pentaCB	>1000
30	**2-OH-4,5**,3',4'-tetraCB	720->1000
31	**4,4'-(OH)$_2$-3,5,3',5'**-tetraCB	0.10-0.19
32	**3,3'-(OH)$_2$-4,4'**-diCB	35-52

The substitution pattern in the phenolic ring is indicated in bold. Data are presented as the range of values determined in 2-4 experiments.

Figure 1B compares the effects of PCB-OHs with an identical 4-hydroxy-3,5-dichloro-substituted phenolic ring. Potent inhibition was observed irrespective of whether the nonphenolic ring was substituted with two (3',4' or 3',5') or three (2',3',4' or 3',4',5') Cl atoms, but a marked reduction in inhibitory potency was observed with four (2',3',4',5') Cl substituents. Further analysis of other PCB-OHs indicated that in general the substitution of both *ortho* (2' and 6') positions or of two diametrically opposite (2' and 5') positions negatively affects EST inhibitory potency (Table 1). This suggests that binding of PCB-OHs to hEST is favored by a coplanar structure of the inhibitor and/or that there are steric constraints for accommodation of the substituted nonphenolic ring. However, other di-*ortho* (2,6 and 2,2') Cl substitutions did not decrease EST inhibitory potency, suggesting that the dimensions of the substituted nonphenolic ring are critical. From the concentration-inhibition relationships, IC_{50} values (concentrations producing 50% inhibition) were determined which are presented in Table 1. IC_{50} values for several PCB-OHs (*13*, *14*, *15*, *16*, *18* and *19*) are in the subnanomolar range. All these compounds are characterized by a 4-hydroxy-3,5-dichloro substitution pattern. Compound *31*, having such a pattern in both rings, is the most potent inhibitor identified in this study, with an IC_{50} value of 0.1 nM (Fig. 1B, Table 1).

To further appreciate the contributions of each phenolic and nonphenolic ring to the inhibitory activity of PCB-OHs towards hEST, the possible effects of a series of single-ring halogenated phenols were tested at a concentration of 1 μM. Figure 2 shows that phenol itself had little effect on EST activity, but halogenation resulted in the generation of marked inhibitory activity. In general, the potency of the halophenols increased with the number and size (I > Br > Cl > F) of the halogen substituents, suggesting that hydroxylated metabolites of polybromobiphenyls (16) may be even more potent inhibitors of hEST than the corresponding PCB-OHs. The inhibitory potency of 2,6-dichlorophenol is much lower than observed for PCB-OHs with identically substituted (4-hydroxy-3,5-dichloro) phenolic rings, indicating that the nonphenolic ring contributes importantly to the inhibitory effects of PCB-OHs on hEST. It should be noted that pentachlorophenol and other chlorophenols are also environmental pollutants resulting from their extensive use as preservatives in the wood and paper industry (17). Pentachlorophenol, which is also a major metabolite of the fungicide hexachlorobenzene, has been widely identified in human blood and urine (18,19). Although pentachlorophenol and other halogenated phenols exhibit lower EST inhibitory activity than several PCB-OHs, occupational exposure to these chemicals may be sufficiently high to contribute to endocrine-disrupting effects in exposed subjects.

The phenolic hydroxyl group in PCB-OHs is essential for potent inhibition of EST activity. Since EST catalyzes the sulfation of the phenolic 3-hydroxyl group of E2 (10), this suggests that PCB-OHs may also be substrates for this enzyme. To gain more insight in the mechanism of EST inhibition by PCB-OHs, the kinetics of this inhibition were studied by Lineweaver-Burk analysis (15) for compounds *8*, *16*, *18*, *26* and *31*) (Fig. 3). The double-reciprocal plots of the rate of E2 sulfation *versus* the E2 concentration in the absence or presence of a single concentration of different PCB-OHs (Fig. 3A) or different concentrations of a single PCB-OH (Fig. 3B) converged at approximately the same point on the *x*-axis. This indicates that these PCB-OHs are noncompetitive inhibitors of E2 sulfation and not competitive inhibitors which would be expected if they are also substrates for EST. The K_i values derived from these Lineweaver-Burk plots are in good agreement with the corresponding IC_{50} values for the different inhibitors. The noncompetitive type of inhibition can be explained by the presence of two substrate-binding sites on hEST, the active site as well as an allosteric site (20). Our results suggest that the potent inhibition of hEST by PCB-OHs is primarily due to binding of these inhibitors to the second, allosteric site.

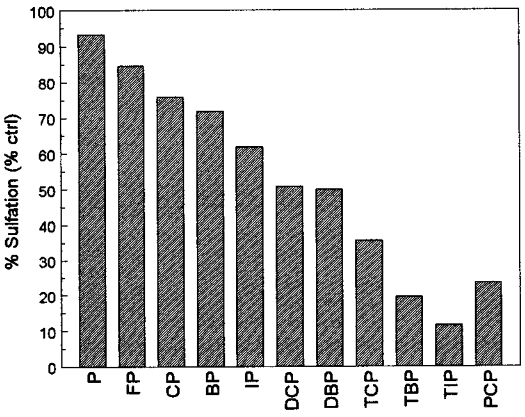

Fig. 2. Effects of different phenols on the sulfation of E2 by recombinant hEST. Sulfation of 1 nM E2 in the presence of 1 μM phenol is expressed as a percentage of that in the absence of inhibitor. P, phenol; FP, 2-fluorophenol; CP, 2-chlorophenol; BP, 2-bromophenol; IP, 2-iodophenol; DCP, 2,6-dichlorophenol; DBP, 2,6-tribromophenol; TCP, 2,4,6-trichlorophenol; TBP, 2,4,6-tribromophenol; TIP, 2,4,6-triiodophenol; PCP, pentachlorophenol. Results are the means of 2 experiments.

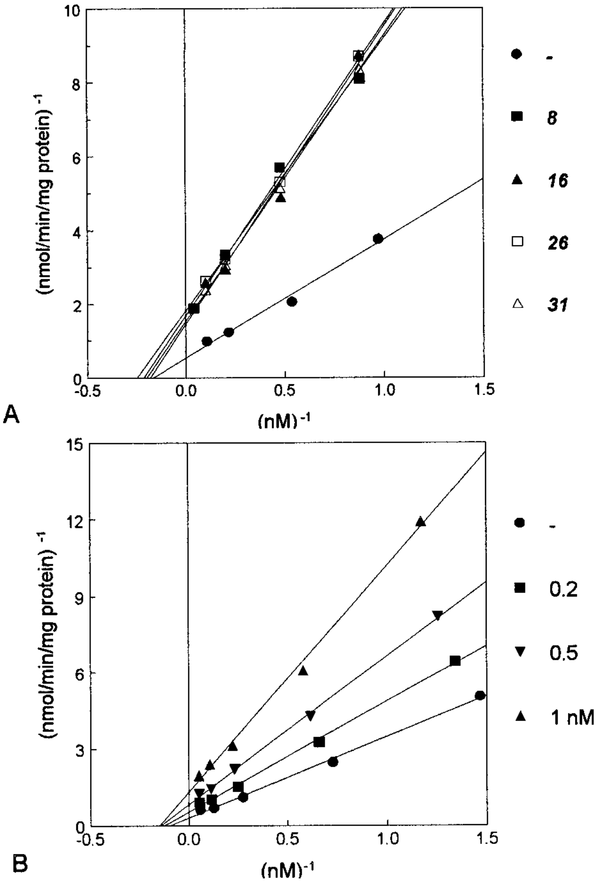

Fig. 3. Lineweaver-Burk plots of the sulfation of E2 by recombinant hEST in the absence or presence of (**A**) 6 nM *8*, 0.5 nM *16*, 22 nM *26* or 0.1 nM *31*, or (**B**) 0.2, 0.5 or 1 nM *18*. Results are representative for 2-4 experiments.

Binding of hydroxylated PCB metabolites to the estrogen receptor is an obvious mechanism by which these compounds could exert their estrogenic activity. However, previous studies have demonstrated that the affinity of PCB-OHs for both α and ß estrogen receptor subtypes is in general very low. Among the large number of PCB-OHs tested, compounds *2* and *3* showed by far the highest affinities for both estrogen receptors which were still >20-fold lower than the affinity of E2 itself (8,9). The results of our study provide a more attractive explanation for the estrogenic activity of PCB-OHs. Several congeners were found to be extremely potent inhibitors of hEST. The IC_{50} and K_i values of different PCB-OHs are up to 50-fold lower than the K_m value of E2 for hEST (4 nM) (21), indicating that these inhibitors have much higher affinity for the enzyme than its natural substrate. To our knowledge, inhibition of hEST is the most potent biological effect described to date regarding the endocrine-disrupting activity of PCBs or their metabolites. It is noteworthy that among the most potent EST inhibitors, ***18*** has been identified as one of the most abundant PCB-OHs in blood and tissues of animals and humans exposed to PCBs (22,23). By inhibiting the formation of inactive E2 sulfate, PCB-OHs can increase E2 bioavailability in target tissues, thereby exerting an indirect estrogenic effect. This may not necessarily be associated with significant changes in circulating levels of E2 and other estrogens but may take place locally in estrogen-sensitive tissues expressing EST, including testis (24), mammary gland (25) and endometrium (26).

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