0013-7227/02/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 87(3):1142–1150

Potent Inhibition of Estrogen Sulfotransferase by Hydroxylated Metabolites of Polyhalogenated Aromatic Hydrocarbons Reveals Alternative Mechanism for Estrogenic Activity of Endocrine Disrupters

MONIQUE H. A. KESTER, SEMA BULDUK, HANS VAN TOOR, DICK TIBBOEL, WALTER MEINL, HANSRUEDI GLATT, CHARLES N. FALANY, MICHAEL W. H. COUGHTRIE, A. GERLIENKE SCHUUR, ABRAHAM BROUWER, AND THEO J. VISSER

Departments of Internal Medicine (M.H.A.K., S.B., H.v.T., T.J.V.) and Pediatric Surgery (M.H.A.K., D.T.), Erasmus University Medical Center, 3015 GJ Rotterdam, The Netherlands; Department of Toxicology, German Institute of Human Nutrition (W.M., H.G.), D-14558 Potsdam-Rehbrucke, Germany; Department of Pharmacology and Toxicology, University of Alabama (C.N.F.), Birmingham, Alabama 35294; Department of Molecular and Cellular Pathology, University of Dundee (M.W.H.C.), Dundee DD1 9SY, United Kingdom; Toxicology Group, Department of Agrotechnology and Food Sciences, Wageningen University (A.G.S.), 6700 EA Wageningen, The Netherlands; and Institute for Environmental Studies, Vrije Universiteit (A.B.), 1081 HV Amsterdam, The Netherlands

Polyhalogenated aromatic hydrocarbons (PHAHs), such as polychlorinated dibenzo-*p*-dioxins and dibenzofurans, polybrominated diphenylethers, and bisphenol A derivatives are persistent environmental pollutants, which are capable of interfering with reproductive and endocrine function in birds, fish, reptiles, and mammals. PHAHs exert estrogenic effects that may be mediated in part by their hydroxylated metabolites (PHAH-OHs), the mechanisms of which remain to be identified. PHAH-OHs show low affinity for the ER. Alternatively, they may exert their estrogenic effects by inhibiting E2 metabolism. As sulfation of E2 by estrogen sulfotransferase (SULT1E1) is an important pathway for E2 inactivation, inhibition of SULT1E1 may lead to an increased bioavailability of estrogens in tissues expressing this enzyme. Therefore, we studied the possible inhibition of human SULT1E1 by hydroxylated PHAH metabolites and the sulfation of the different compounds by SULT1E1. We found marked inhibition of SULT1E1 by various PHAH-OHs, in particular by compounds with two adjacent halogen substituents around the hydroxyl group that were effective at (sub)nanomolar concentrations. Depending on the structure, the inhibition is primarily competitive or noncompetitive. Most PHAH-OHs are also sulfated by SULT1E1. We also investigated the inhibitory effects of the various PHAH-OHs on E2 sulfation by human liver cytosol and found that the effects were strongly correlated with their inhibitions of recombinant SULT1E1 ($r = 0.922$). Based on these results, we hypothesize that hydroxylated PHAHs exert their estrogenic effects at least in part by inhibiting SULT1E1-catalyzed E2 sulfation. (*J Clin Endocrinol Metab* 87: 1142–1150, 2002)

RECENTLY, MANY STUDIES have been performed on the interaction of environmental chemicals with the endocrine system, which results in reproductive and developmental anomalies in various organisms (1–4). Endocrine-disrupting effects have been observed in adult animals that were exposed *in utero* to polyhalogenated aromatic hydrocarbons (PHAHs) such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) (5–7). Hilakivi-Clarke *et al.* (8) demonstrated in rats that exposure to natural estrogens *in utero* advanced puberty onset and increased breast cancer risk in the offspring; this may also apply to environmental estrogens. In humans, abnormalities in the development of the reproductive tract, reduced sperm counts (3, 9), and increased incidence of germ cell cancer (10) have been related to exposure to endocrine disrupters present in the environment. Furthermore, several studies have reported increased levels of PCBs, 2,2-bis-(4-chlorophenyl)-1,1,1-trichloroethane and its metabolite 2,2-bis-(4-chlorophenyl)-1,1-dichloroethylene in breast cancer patients (11, 12). However, recent epidemiological research does not support the hypothesis that women exposed to organochlorines such as PCBs, PCDDs, 2,2-bis-(4-chlorophenyl)-1,1,1-trichloroethane, and 2,2-bis-(4-chlorophenyl)-1,1-dichloroethylene have an increased breast cancer risk (13–15).

Hydroxylated metabolites of the PHAHs (PHAH-OHs) may contribute to the aforementioned effects. Hydroxylated metabolites of PCBs (PCB-OHs), PCDDs (PCDD-OHs), and PCDFs (PCDF-OHs) and other organohalogens have been identified in blood, bile, and urine of animals treated with the parent compound, but also in wildlife samples as well as in environmentally exposed human subjects (16–20). Hydroxylation of PCDDs and PCDFs in mammals preferentially occurs on the lateral (2, 3, 7, 8) positions (18–20). For PCBs, it has been determined that hydroxylated metabolites are partially responsible for the endocrine-disrupting effects. For instance, Crews *et al.* (21) showed that different hydroxylated PCBs altered the sexual differentiation of the turtle.

Abbreviations: BPA, Bisphenol A; -OH, hydroxylated metabolite of; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PBDE, polybrominated diphenylether; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzo-*p*-dioxin; PCDF, polychlorinated dibenzofuran; PHAH, polyhalogenated aromatic hydrocarbon; SULT1E1, estrogen sulfotransferase.

The mechanisms by which PHAH-OHs exert their estrogenic effects are still not understood. Binding affinities for ERα and ERβ are relatively low (22, 23). It is possible, however, that PHAH-OHs exert part of their estrogenic effects by increasing the bioavailability of E2 through inhibition of E2 inactivation in target tissues. Sulfation by the specific estrogen sulfotransferase SULT1E1 is an important pathway for the inactivation of E2 (24). The human enzyme has a low K_m value of 4 nM for E2 and is expressed in target tissues such as the endometrium, mammary gland, and testis as well as in the liver (25–29). Recently, we demonstrated potent inhibition of SULT1E1 by hydroxylated PCB metabolites (30), in particular compounds with two adjacent chlorine substituents around the hydroxyl group. Here, we have investigated the potency and type of inhibition of human SULT1E1 by other important PHAH-OHs, such as PCDD-OHs, PCDF-OHs, and hydroxylated polybromodiphenylethers (PBDE-OHs), and by halogenated bisphenol A (BPA) derivatives as well as their sulfation by SULT1E1. To determine the relevance of our findings using recombinant enzyme, we also studied the inhibition of E2 sulfation by native SULT1E1 in human liver cytosol (29). Furthermore, we analyzed the sulfation of the various PHAH-OHs by SULT1E1 and SULT1A1, another phenol sulfotransferase abundantly expressed in human liver (31, 32).

Materials and Methods

Materials

E2 and 3′-phosphoadenosine-5′-phosphosulfate (PAPS) were obtained from Sigma (St. Louis, MO), [^{3}H]E2 (3.22 MBq/nmol) was obtained from Amersham Pharmacia Biotech (Little Chalfont, UK), and [^{35}S]PAPS (52.9 MBq/μmol) was purchased from NEN Life Science Products (Boston, MA). The sources of the various hydroxylated organohalogens have been described previously (23, 33, 34). Human SULT1E1 (35) and SULT1A1 (31) were expressed in *Salmonella typhimurium* as previously described (36, 37). Cytosolic fractions were prepared and used without further purification (36, 37). SULT1E1 accounted for 5–7% of the cytosolic proteins. Similar results were obtained using recombinant SULT1E1 expressed in *Escherichia coli* and purified as previously described (38). Normal human liver was obtained at surgical resection of liver tumors, and cytosol was prepared as previously described (39). Approval was obtained from the medical ethical committee of Erasmus University Medical Center.

Estrogen sulfotransferase assay

Estrogen sulfotransferase activity was analyzed by measuring the formation of water-soluble [^{3}H]E2 sulfate after incubation of 1 nM [^{3}H]E2 for 30 min at 37 C with recombinant SULT1E1 (0.1 μg total cytosolic protein/ml) in the presence or absence (blanks) of 50 μM of the cofactor 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 the addition of 2 ml ice-cold water, and unreacted [^{3}H]E2 was removed by extraction with 2 ml dichloromethane. Sulfate formation was quantified by liquid scintillation counting of 1 ml of the aqueous phase. Enzymatic sulfation was corrected for background radioactivity estimated in the blanks. Inhibition of E2 sulfation by PHAH-OHs was assessed by addition of 0.01 nM to 10 μM of these compounds to the reaction mixtures. Kinetic parameters for E2 sulfation were determined by Lineweaver-Burk analysis (40) of the sulfation of varying substrate concentrations. Apparent K_i values for inhibitors were calculated from the change in slope of the Lineweaver-Burk plot in the presence of a fixed inhibitor concentration (40).

Sulfation of PHAH-OHs

The above assay of estrogen sulfotransferase activity is based on the sulfation of limited concentrations of radioactive E2 by excess unlabeled PAPS. As radioactive PHAH-OHs are not available, their sulfation was analyzed in comparison with E2 by determining the transfer of $^{35}SO_3^-$ from [^{35}S]PAPS to an excess of unlabeled substrate. Sulfation of PHAH-OHs by SULT1E1 was compared with their sulfation by the human phenol sulfotransferase SULT1A1. Assay mixtures contained 1 μM PHAH-OH or E2, 0.3 μM [^{35}S]PAPS, and 15 μg (recombinant SULT1E1) or 100 μg (recombinant SULT1A1) of total cytosolic protein/ml in a total volume of 150 μl 10 mM potassium phosphate (pH 7.4) and were incubated for 30 min at 37 C. The formation of sulfated products was analyzed using the $BaSO_4$ precipitation method of Foldes and Meek (41) as well as by HPLC. In the former method, unreacted [^{35}S]PAPS and protein were precipitated by successive addition of 200 μl 0.1 M barium acetate, 200 μl 0.1 M barium hydroxide, and 200 μl 0.1 M zinc sulfate. The presumably soluble sulfated products were quantified by liquid scintillation counting of 500 μl of the supernatant. Sulfation was corrected for background radioactivity determined in blanks without substrate. For HPLC analysis, the reactions were stopped by the addition of 150 μl ice-cold methanol. After centrifugation, 100 μl of the supernatant were injected onto a 4.6 × 250-mm Symmetry C_{18} column connected to an Alliance HPLC system (Waters Chromatography BV, Etten-Leur, The Netherlands), and eluted with a gradient of acetonitrile in 50 mM triethyl ammonium acetate (pH 6.8) at a flow of 1.0 ml/min. The proportion of acetonitrile was increased linearly from 35% to 65% in 15 min and further to 90% in an additional 10 min. The radioactivity in the eluate was determined using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT).

Results

The effects of 0.01 nM to 10 μM of different classes of PHAH-OHs on the sulfation of 1 nM E2 by SULT1E1 were tested. Table 1 lists the exact structural formulas of the tested compounds, and Figs. 1-4 show their core structures. The concentration-dependent inhibition of SULT1E1 activity by subsets of various types of PHAH-OHs is presented in Fig. 1 (PCDD-OHs), Fig. 2 (PCDF-OHs), Fig. 3 (PBDE-OHs), and Fig. 4 (BPA derivatives). From these concentration-inhibition curves, the concentrations causing 50% inhibition (IC_{50} values) were determined. Table 1 presents the IC_{50} values and relative potencies compared with the cognate substrate E2 for all compounds tested.

Figure 1 shows the results with PCDD-OHs having the hydroxyl group in position 2, and the same 7,8-dichloro substitution pattern in the nonphenolic ring. Increasing potencies were observed as the number of chlorine substituents surrounding the hydroxyl group increased from 0 to 2, with mean IC_{50} values of 300 nM for 2-OH-7,8-DiCDD, 30 nM for 2-OH-3,7,8-TrCDD, and 4 nM for 2-OH-1,3,7,8-TeCDD (Table 1).

Figure 2 presents the concentration-inhibition relationships for PCDF-OHs with the hydroxyl group in the 2 or 3 position, *para* or *meta* to the furan oxygen, respectively. Again, the potency increased with increasing number of chlorine substituents adjacent to the hydroxyl group. Furthermore, 3-OH-PCDFs appeared to be slightly more potent inhibitors than 2-OH-PCDFs possessing comparable chlorine substitution patterns. Of all the PCDF-OHs tested, 3-OH-2,4,7,8,9-PeCDF was the most potent inhibitor of E2 sulfation. With a mean IC_{50} value of as low as 0.18 nM, this compound has a more than 30-fold higher affinity for SULT1E1 than the natural substrate E2 (Table 1).

As demonstrated in Fig. 3, all PBDE-OHs tested were

TABLE 1. Potency of inhibition of human SULT1E1 activity by PHAH-OHs

Compound	Code	IC_{50} (nM)	Relative potency
E2		3.8–7.1	1
PCDD-OH			
2-Hydroxy-7,8-dichlorodibenzo-p-dioxin	2-OH-7,8-DiCDD	200–390	0.02
2-Hydroxy-3,7,8-trichlorodibenzo-p-dioxin	2-OH-3,7,8-TrCDD	28–40	0.17
2-Hydroxy-1,3,7,8-tetrachlorodibenzo-p-dioxin	2-OH-1,3,7,8-TeCDD	2.4–6.1	1.4
PCDF-OH			
4-Hydroxy-1,3,6,7-tetrachlorodibenzofuran	4-OH-1,3,6,7-TeCDF	6.6–6.7	0.84
3-Hydroxy-2,6,7,8-tetrachlorodibenzofuran	3-OH-2,6,7,8-TeCDF	5.6–9.1	0.76
3-Hydroxy-2,4,7,8-tetrachlorodibenzofuran	3-OH-2,4,7,8-TeCDF	0.68–2.2	4.0
3-Hydroxy-2,4,7,8,9-pentachlorodibenzofuran	3-OH-2,4,7,8,9-PeCDF	0.16–0.20	31
2-Hydroxy-7,8-dichlorodibenzofuran	2-OH-7,8-DiCDF	230–560	0.02
2-Hydroxy-6,7,8-trichlorodibenzofuran	2-OH-6,7,8-TrCDF	350–800	0.01
2-Hydroxy-1,3,7,8-tetrachlorodibenzofuran	2-OH-1,3,7,8-TeCDF	5.6–6.2	0.97
1-Hydroxy-2,4,7,8-tetrachlorodibenzofuran	1-OH-2,4,7,8-TeCDF	240–280	0.02
PHDE-OH			
4-Hydroxy-2′,4′,6′-tribromodiphenylether	4-OH-2′,4′,6′-TrBDE	780 to >1,000	<0.01
4-Hydroxy-2′,3,4′,6′-tetrabromodiphenylether	4-OH-3,2′,4′,6′-TeBDE	>1,000	<0.01
4-Hydroxy-2′,3,4′,5,6′-pentabromodiphenylether	4-OH-3,5,2′,4′,6′-PeBDE	200–240	0.03
2-Hydroxy-2′,4,4′-trichlorodiphenylether	2-OH-4,2′,4′-TrCDE	850 to >1,000	<0.01
BPA			
4,4′-Isopropylidenediphenol (bisphenol A)	BPA	>10,000	<0.001
3,3′,5,5′-Tetrachlorobisphenol A	3,5,3′,5′-TeCBPA	29–53	0.15
3,3′,5,5′-Tetrabromobisphenol A	3,5,3′,5′-TeBBPA	12–33	0.30

IC_{50} values are presented as the range of values from two to four experiments. Relative potencies are calculated as ratio of the IC_{50} value of E2 over that of inhibitor.

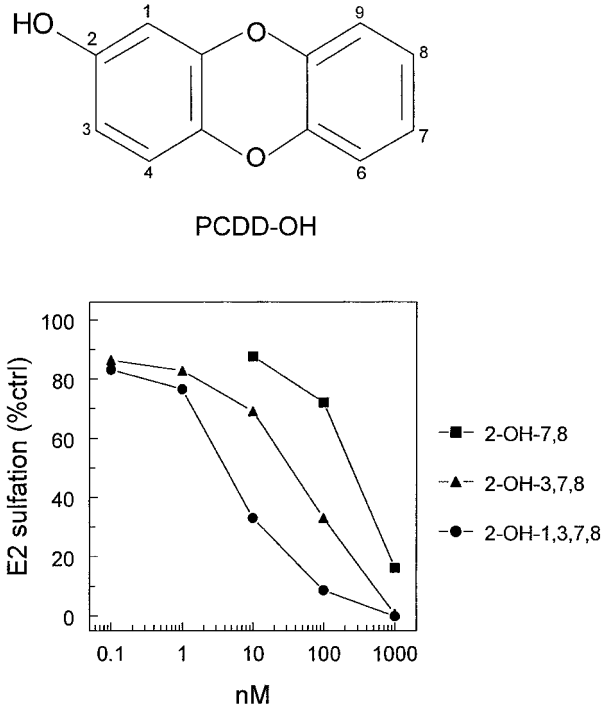


FIG. 1. *Upper part*, Core structure of PCDD-OH. Chlorine substituents are not indicated. *Lower part*, Inhibition of E2 sulfation by recombinant human SULT1E1 by 0.1–1000 nM PCDD-OHs. Reaction conditions: 1 nM [^{3}H]E2, 0.1 μg total cytosolic protein/ml, 50 μM PAPS, and 30-min incubation. Results are the means of two to four experiments; the coefficient of variation was less than 20%.

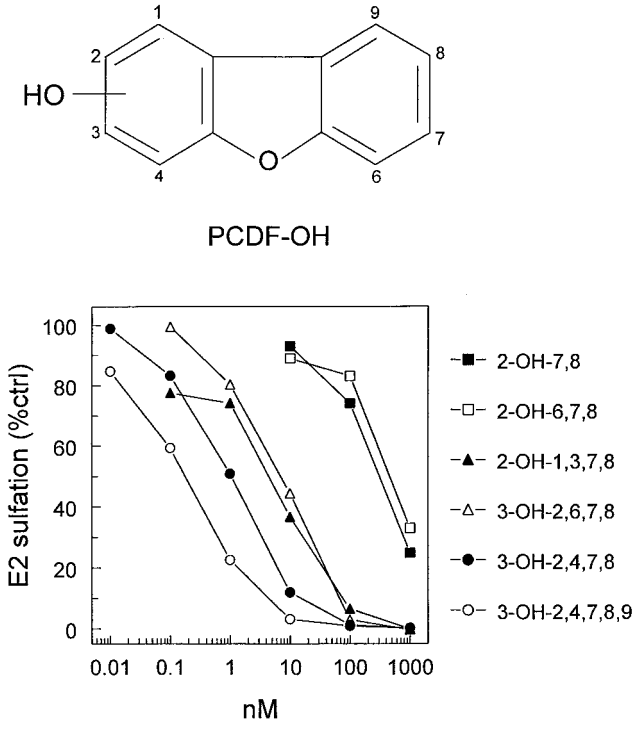
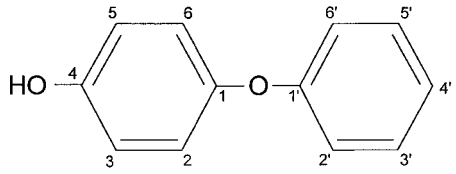
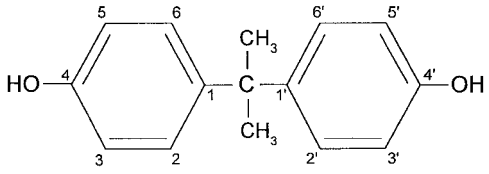


FIG. 2. *Upper part*, Core structure of PCDF-OH. The position of the hydroxyl group varies between the different PCDF-OHs. Chlorine substituents are not indicated. *Lower part*, Inhibition of E2 sulfation by recombinant human SULT1E1 by 0.01–1000 nM PCDF-OHs. Reaction conditions: 1 nM [^{3}H]E2, 0.1 μg total cytosolic protein/ml, 50 μM PAPS, and 30-min incubation. Results are the means of two to four experiments; the coefficient of variation was less than 20%.

relatively weak inhibitors of E2 sulfation by SULT1E1, with IC_{50} values greater than 200 nM (Table 1). Also, the one hydroxylated, polychlorodiphenylether tested, 2-OH-4,2′,4′-TrCDE, only inhibited SULT1E1 activity at micromolar concentrations (Table 1). BPA did not affect E2 sulfation at concentrations less than 1 μM, whereas its derivatives having halogens in all positions adjacent to the two hydroxyl groups,



PBDE-OH



BPA

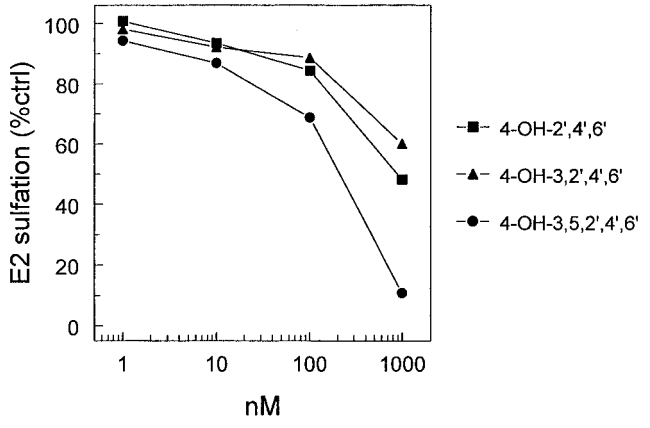


FIG. 3. *Upper part*, Core structure of PBDE-OH. Bromine substituents are not indicated. *Lower part*, Inhibition of E2 sulfation by recombinant human SULT1E1 by 1–1000 nM PBDE-OHs. Reaction conditions: 1 nM [^{3}H]E2, 0.1 μg total cytosolic protein/ml, 50 μM PAPS, and 30-min incubation. Results are the means of two to four experiments; the coefficient of variation was less than 20%.

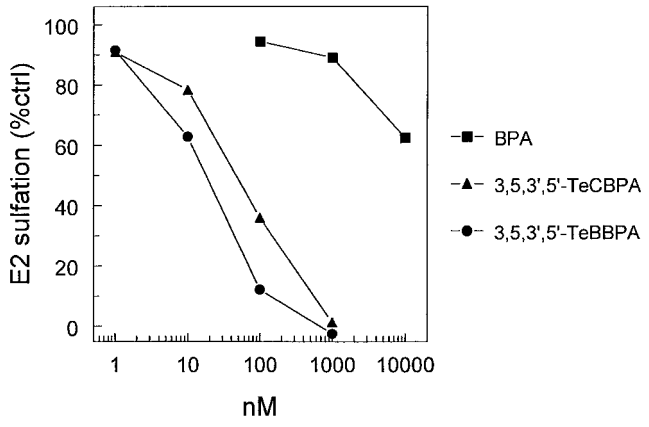


FIG. 4. *Upper part*, Core structure of BPA derivatives. Halogen substituents are not indicated. *Lower part*, Inhibition of E2 sulfation by recombinant human SULT1E1 by 1–10000 nM BPA or halogenated BPA. Reaction conditions: 1 nM [^{3}H]E2, 0.1 μg total cytosolic protein/ml, 50 μM PAPS, and 30-min incubation. Results are the means of two to four experiments; the coefficient of variation was less than 20%.

3,5,3′,5′-TeCBPA and 3,5,3′,5′-TeBBPA, were relatively potent inhibitors, with mean IC_{50} values of 40 and 20 nM, respectively (Fig. 4 and Table 1).

The type of inhibition of E2 sulfation by the different PHAH-OHs was studied by Lineweaver-Burk analysis (Fig. 5). Depending on the structure, different types of inhibition were observed. Addition of 2-OH-7,8-DiCDD (Fig. 5A) or 2-OH-7,8-DiCDF (Fig. 5B) changed the slope, but had little effect on the *y*-axis intercept of these plots, indicative of competitive inhibition. However, 2-OH-1,3,7,8-TeCDD (Fig. 5A) and 3-OH-2,4,7,8,9-PeCDF (Fig. 5B) affected both the slope and the *y*-axis intercept, and the plots converged at about the same point on the *x*-axis, indicating primarily noncompetitive inhibition by these potent inhibitors. The Lineweaver-Burk analyses of the effects of BPA and 3,5,3′,5′-TeCBPA (Fig. 5C) and of 4-OH-3,5,2′,4′,6′-PeBDE (Fig. 5D) indicate that these compounds inhibit E2 sulfation primarily in a noncompetitive manner. The K_i values derived from these plots are in good agreement with the corresponding IC_{50} values for the different inhibitors, amounting to 60 nM for 2-OH-7,8-DiCDD, 2 nM for 2-OH-1,3,7,8-TeCDD, 270 nM for 2-OH-7,8-DiCDF, 0.15 nM for 3-OH-2,4,7,8,9-PeCDF, 150 nM for 4-OH-3,5,2′,4′,6′-PeBDE, 14 μM for BPA, and 35 nM for 3,5,3′,5′-TeCBPA.

Binding of PHAH-OHs to the active site of SULT1E1 is likely to result in their sulfation, which was tested directly by incubating 1 μM of the various compounds with 0.3 μM [^{35}S]PAPS and SULT1E1. These experiments also included different PCB-OHs that have previously been shown to inhibit E2 sulfation by SULT1E1 (30). Product formation was analyzed by the method of Foldes and Meek (41), which involves precipitation of the remaining [^{35}S]PAPS with $BaSO_4$, presumably leaving the radioactive sulfated products in solution. The results of this established procedure were compared with a method developed in our laboratory based on the separation of labeled PAPS and sulfated products by HPLC. Figure 6A shows the HPLC analysis of the sulfation of 2-OH-7,8-DiCDF as a representative example, demonstrating the clear separation between remaining [^{35}S]PAPS and ^{35}S-labeled sulfated product. Figure 6B shows that there was reasonable agreement between the results of the two methods regarding the sulfation of most compounds, although in several instances the $BaSO_4$ precipitation method significantly underestimated the formation of sulfated products in comparison with HPLC analysis. This was especially the case with sulfated compounds that were strongly retarded on the C_{18} column, suggesting that relatively nonpolar sulfates are partially lost in the $BaSO_4$ precipitation method.

Table 2 shows the results of the HPLC measurements of the sulfation of the various PHAH-OHs by SULT1E1 in comparison with the sulfation of these compounds by the human phenol sulfotransferase SULT1A1. We found that most PHAH-OHs tested in this study were sulfated by SULT1E1 as well as by SULT1A1 (Table 2).

We also studied the inhibition of E2 sulfation by various PHAH-OHs using human liver cytosol as a source of native human SULT1E1. Figure 7 compares the effects of different PHAH-OHs (0.1 or 1 μM) on E2 sulfation by SULT1E1 and

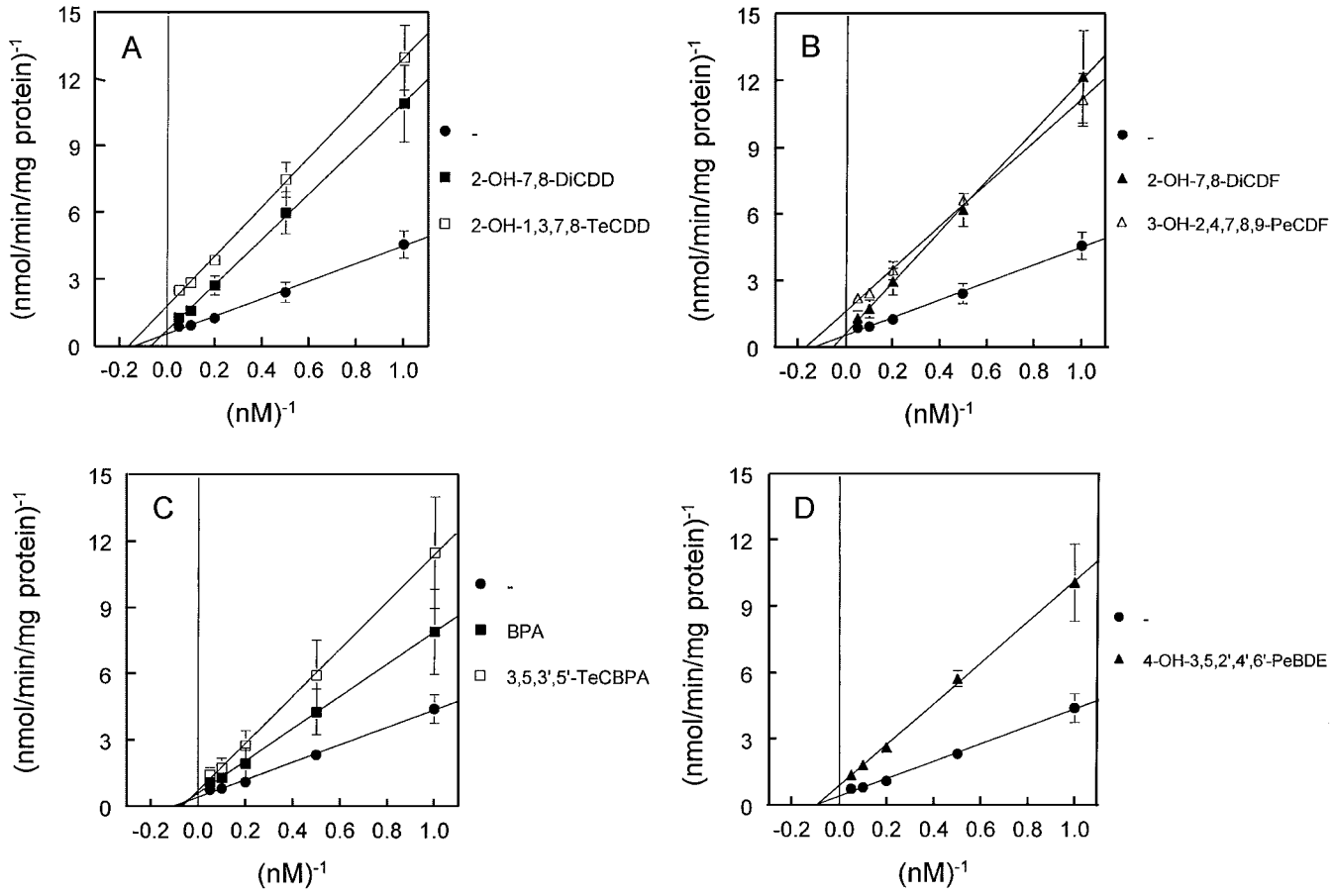


FIG. 5. Lineweaver-Burk analysis of the inhibition of the sulfation of 1–20 nM E2 by 0.1 μg/ml recombinant human SULT1E1 by 250 nM 2-OH-7,8-DiCDD or 4 nM 2-OH-1,3,7,8-TeCDD (A), by 500 nM 2-OH-7,8-DiCDF or 0.2 nM 3-OH-2,4,7,8,9-PeCDF (B), by 12 μM BPA or 60 nM 3,5,3′,5′-TeCBPA (C), or by 200 nM 4-OH-3,5,2′,4′,6′-PeBDE (D). Results are the mean ± SD of two to four experiments. At points where no error bar is shown the SD is smaller than the symbol.

human liver cytosol, showing a strong correlation between the inhibitions of the recombinant and native enzymes ($r = 0.922$). In general, human liver enzyme was less potently inhibited than recombinant human SULT1E1.

Discussion

Structure-activity relationship of SULT1E1 inhibition by PHAH-OHs

We previously showed that various PCB-OHs potently inhibit E2 sulfation by human SULT1E1 (30). This finding suggested that endocrine-disrupting chemicals may act by increasing the bioavailability of hormones through inhibition of hormone-conjugating enzymes in target tissues (30, 42). In this study we tested the effects of other classes of PHAH-OHs on E2 sulfation by SULT1E1. The results demonstrate that in all classes of PHAH-OHs tested, those with two adjacent halogen substituents around the OH group are the most potent inhibitors of E2 sulfation by SULT1E1, which is in agreement with the structure-activity relationship found for SULT1E1 inhibition by single ring halogenated phenols and hydroxylated PCBs (30). A possible explanation for the increase in potency by adjacent halogen substitutions is the increased dissociation of the OH group. The potency of inhibitors with this substitution pattern decreases in the order PCDF-OH > PCDD-OH > BPA > PBDE-OH derivatives. The planar structures of PCDD-OHs and PCDF-OHs *vs.* the nonplanar structures of BPAs and PBDE-OHs may play a role in this, because the patterns for inhibition of SULT1E1 by PCB-OHs suggested preferred binding of coplanar compounds to the enzyme (30). This finding fits with the primarily planar structure of the natural ligand E2. Recently, the crystal structure of the mouse estrogen sulfotransferase has been elucidated (43). Mouse and human SULT1E1 show 77% amino acid sequence identity; both orthologous enzymes have K_m values in the nanomolar range (25, 44). The modeling of hydroxylated PHAHs in E2-binding sites of mouse SULT1E1 or human SULT1E1, when its crystal structure also becomes available, should further our understanding of the structural requirements for inhibition of SULT1E1.

Regarding the varying potencies of the different groups of (hydroxylated) PHAHs, it should be noted that levels of exposure are also different for the various PHAHs. The plasticizer BPA is commonly used in the food-packaging industry and in dentistry. Microgram amounts were found in the liquid from vegetable cans with plastic linings and in the saliva of patients with dental sealings (45). TBBPA, TCBPA,

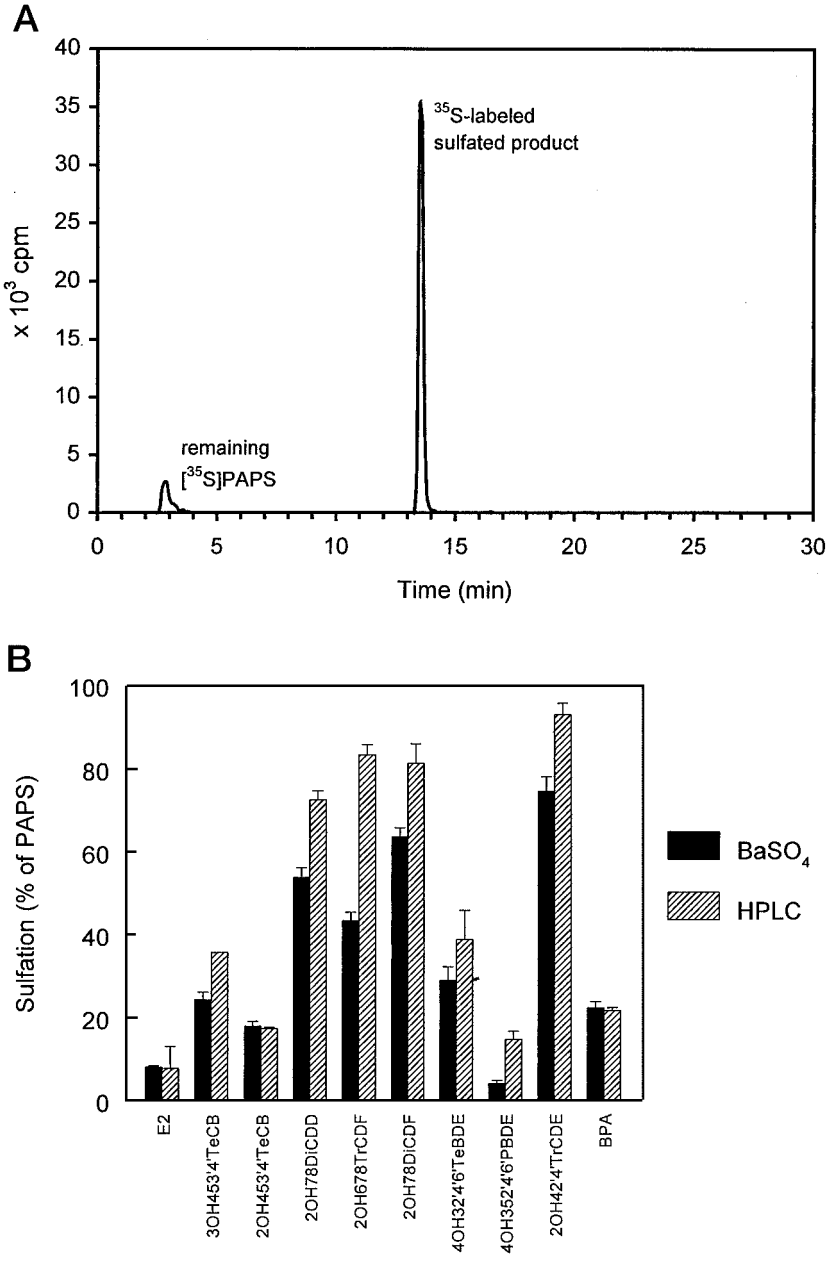


FIG. 6. A, HPLC analysis of the sulfation of 2-OH-7,8-DiCDF by SULT1E1 using [^{35}S]PAPS. B, Sulfation of PHAH-OHs by SULT1E1 measured by the $BaSO_4$ precipitation method and by HPLC analysis. Reaction conditions: 1 μM substrate, 15 μg total cytosolic protein/ml, 0.3 μM [^{35}S]PAPS, and 30-min incubation. Results are the mean ± SD of triplicate ($BaSO_4$) or duplicate (HPLC) determinations from a representative experiment.

and polybrominated diphenylethers are high production volume chemicals that are widely used in consumer electronics and many other products as flame retardants; the production volume of TBBPA in 1995 was higher than 50,000 tons/yr (46). The background exposure of humans to PCDDs and PCDFs is much lower. Only trace levels (parts per trillion) of PCDDs and PCDFs have been detected in normal human blood and tissues (47). However, as in our studies IC_{50} values for some PCDD-OHs and PCDF-OHs were less than 1 nM, and 1 nM equals approximately 20 parts per trillion, the *in vitro* effects of the most potent PCDD-OHs and PCDF-OHs were observed at concentrations that may actually be in the same range that they are present in human tissues.

Depending on the structure, different types of inhibition were found with different PHAH-OHs. SULT1E1 is known to have two substrate-binding sites, the active site as well as an allosteric site (24, 48). Binding of E2 to the latter site is thought to be largely responsible for the phenomenon of substrate inhibition that is observed at increasing E2 concentrations. The primarily competitive or noncompetitive nature by which the different PHAH-OHs inhibit the sulfation of E2 by SULT1E1 may thus be explained by their preferential affinity for the active or the allosteric site, respectively.

Sulfation of PHAH-OHs by SULT1E1 and SULT1A1

Estrogenic chemicals such as alkylphenols, diethylstilbestrol, and BPA have recently been shown to be substrates for human SULT1A1 (49, 50). In this study we compared the sulfation of PHAH-OHs by SULT1E1 and SULT1A1. Most of

TABLE 2. Sulfation of PHAH-OHs by human SULT1E1 and SULT1A1

Compound	Sulfation (% of PAPS added)	
	SULT1E1	SULT1A1
PCB-OH		
4-OH-3,3′,4′-TrCB	9.9 ± 1.0	19.3 ± 1.8
4-OH-2,3,5,3′,4′-PeCB	0.6 ± 0.8	0.7 ± 1.0
4-OH-3,2′,3′,4′,5′-PeCB	17.6 ± 2.0	6.6 ± 3.1
3-OH-4,5,3′,4′-TeCB	33.4 ± 3.3	10.5 ± 3.2
2-OH-4,5,3′,4′-TeCB	19.4 ± 2.8	19.0 ± 4.7
4,4′-$(OH)_2$-3,5,3′,5′-TeCB	1.0 ± 1.1	18.3 ± 2.8
PCDD-OH		
2-OH-7,8-DiCDD	70.6 ± 2.7	74.1 ± 10.1
2-OH-3,7,8-TrCDD	22.7 ± 9.5	11.4 ± 1.4
2-OH-1,3,7,8-TeCDD	28.2 ± 3.5	31.7 ± 6.9
PCDF-OH		
4-OH-1,3,6,7-TeCDF	6.9 ± 0.3	10.7 ± 1.4
3-OH-2,6,7,8-TeCDF	7.9 ± 1.1	7.3 ± 4.7
3-OH-2,4,7,8-TeCDF	2.5 ± 0.2	3.1 ± 1.1
3-OH-2,4,7,8,9-PeCDF	1.0 ± 0.0	1.0 ± 0.3
2-OH-7,8-DiCDF	82.4 ± 1.7	31.6 ± 4.4
2-OH-6,7,8-TrCDF	81.1 ± 3.1	55.2 ± 8.7
2-OH-1,3,7,8-TeCDF	7.0 ± 1.4	3.3 ± 2.3
1-OH-2,4,7,8-TeCDF	0.0 ± 0.0	24.8 ± 3.4
PHDE-OH		
4-OH-2′,4′,6′-TrBDE	2.5 ± 1.4	10.7 ± 0.9
4-OH-3,2′,4′,6′-TeBDE	43.1 ± 6.1	30.4 ± 6.2
4-OH-3,5,2′,4′,6′-PeBDE	13.3 ± 1.9	0.7 ± 0.7
2-OH-4,2′,4′-TrCDE	89.2 ± 5.1	80.5 ± 7.6
BPA	23.6 ± 2.9	62.0 ± 3.2
3,5,3′,5′-TeCBPA	7.7 ± 2.5	26.2 ± 1.1
3,5,3′,5′-TeBBPA	5.8 ± 0.8	15.9 ± 0.7

Reaction conditions: 1 μM substrate, 15 (SULT1E1) or 100 (SULT1A1) μg total cytosolic protein/ml, 0.3 μM [^{35}S]PAPS, and 30-min incubation. Data are presented as the mean ± SD from two to three experiments.

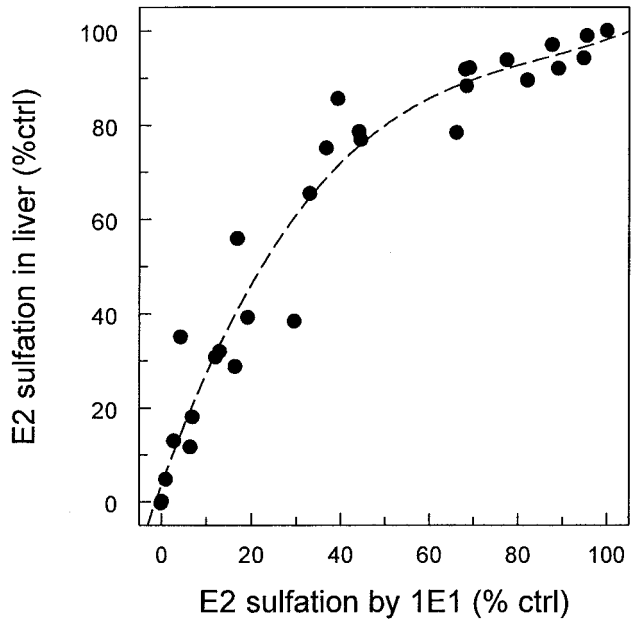


FIG. 7. Sulfation of E2 by human liver cytosol *vs.* SULT1E1 in the presence of different PHAH-OHs. Reaction conditions: 1 nM [^{3}H]E2, 0.1 or 1 μM PHAH-OH, 0.1 (SULT1E1) or 10 (human liver) μg total cytosolic protein/ml, 50 μM PAPS, and 30-min incubation. The PHAH-OHs tested were the PCDD-OHs and PCDF-OHs listed in Table 1 as well as 12 different PCB-OHs (compounds 5, 8, 10, 14, 16, 17, 23, 24, 26, 28, 31, and 32 from Ref. 30). Results are expressed as a percentage of control E2 sulfation in the absence of inhibitor and are presented as the means of two to four experiments (SULT1E1) or as the means of triplicate determinations from a representative experiment (human liver cytosol).

the compounds tested were sulfated by SULT1E1 as well as by SULT1A1. It should be noted, however, that these incubations had to be performed with a limited PAPS concentration and excess substrate, which is very different from the conditions used to test the effects of PHAH-OHs on the sulfation of E2 by SULT1E1. Sulfation of the different PHAH-OHs was tested at a single substrate concentration of 1 μM, although the IC_{50} and K_i values for their inhibition of E2 sulfation ranged from less than 1 nM to more than 10 μM. Therefore, the findings presented in Table 2 are not representative of the rate of sulfation of the different PHAH-OHs by SULT1E1 under the conditions where they were tested as inhibitors of E2 sulfation. Also, in view of the large variation in saturation of the low K_m SULT1E1 and the high K_m SULT1A1 at 1 μM of the various substrates, the data reported in Table 2 are not representative of the substrate preferences of these isoenzymes at lower, more relevant PHAH-OH concentrations. The *in vivo* significance of sulfation of hydroxylated PHAHs in PHAH metabolism remains to be established.

Effects of PHAH-OHs on E2 sulfation by human liver cytosol

The effects of PHAH-OHs on E2 sulfation by SULT1E1 and by human liver cytosol were highly correlated. However, the relationship between the rates of E2 sulfation by the different enzyme preparations was nonlinear. Although SULT1E1 is the better enzyme for E2 sulfation, with a K_m value of approximately 4 nM, SULT1A1 also catalyzes E2 sulfation, with a K_m value of 2–5 μM (51). The potencies of inhibition of SULT1E1 differ by orders of magnitude from those by which the different PHAH-OHs inhibit human SULT1A1 activity, which is characterized by apparent K_i values in the micromolar range (33). The nonlinear relationship between the rates of E2 sulfation by recombinant SULT1E1 and human liver cytosol in the presence of various PHAH-OHs may be explained by the presence of SULT1A1 in human liver. However, at nanomolar concentrations, E2 is predominantly sulfated in human liver by SULT1E1 (29), suggesting that the nonlinear relationship is not due to significant sulfation of E2 in human liver by SULT1A1. A more likely explanation is suggested by our findings that the PHAH-OHs are sulfated by SULT1E1 as well as by SULT1A1. Therefore, compared with recombinant SULT1E1, larger amounts of the PHAH-OHs will be metabolized in human liver cytosol, decreasing their inhibitory effects on native SULT1E1. An additional explanation may be that, compared with recombinant SULT1E1, human liver cytosol contains more proteins to which PHAH-OHs bind, diminishing their availability for native SULT1E1.

Concluding remarks

In this study we have demonstrated that E2 sulfation catalyzed by recombinant human SULT1E1 and that catalyzed by human liver sulfotransferase are both potently inhib-

ited by different classes of PHAH-OHs, with IC_{50} values for recombinant SULT1E1 inhibition in the low or even subnanomolar range. The potent inhibitors 2-OH-3,7,8-TrCDD, 2-OH-1,3,7,8-TeCDD, 3-OH-2,4,7,8-TeCDF, and 3-OH-2,4,7,8,9-PeCDF, with IC_{50} values of 34, 4.1, 1.4, and 0.18 nM, respectively, have been identified in mammalian species (20). The most potent inhibitors have an even higher affinity for human SULT1E1 than its cognate substrate E2, which has a K_m value of 4 nM for the enzyme (25). Therefore, we hypothesize that part of the estrogenic activity of PHAHs is explained by an increase in E2 bioavailability through inhibition of human SULT1E1 by hydroxylated PHAH metabolites. Such a mechanism for the pseudoestrogenic activity of PHAH-OHs is particularly relevant for estrogen-responsive tissues that express SULT1E1, such as the endometrium, mammary gland, and testis (26–28). The effects of PHAH-OHs on the regulation of local estrogen levels in these tissues will depend on a variety of factors, such as the supply or local generation of the various PHAH metabolites, their potency in inhibiting SULT1E1, their rate of inactivation by SULT1E1 and other isoenzymes such as SULT1A1, their urinary or biliary excretion rates, and also on the reversal of the sulfation of E2 and the inhibitors by local estrogen sulfatase expression (52).

PHAHs are known also to affect the thyroid hormone system. In laboratory animals, plasma T_4 is markedly decreased as a result of competitive binding of the hydroxylated metabolites to the plasma carrier transthyretin and induction of hepatic UDP-glucuronyltransferases by the PHAHs themselves (53). PHAH-OHs have also been reported to inhibit the *in vitro* deiodination of thyroid hormone by the type I iodothyronine deiodinase as well as the sulfation of the hormone by human SULT1A1 (33). During human fetal development, sulfation is an important pathway of thyroid hormone inactivation (54). We have recently demonstrated that human SULT1E1 also efficiently catalyzes the sulfation of iodothyronines, among which are the prohormone T_4 and the active hormone T_3 (25). Inhibition of SULT1E1 may thus also have thyroid hormone-disrupting effects during fetal development. Further studies should determine to what extent estrogen and possibly thyroid hormone levels are disrupted by hydroxylated PHAHs through inhibition of estrogen sulfotransferase.

Acknowledgments

We thank Drs. S. H. Safe, A. Bergman, and H. Kuroki for kindly providing hydroxylated PHAH metabolites.

Received July 9, 2001. Accepted November 28, 2001.

Address all correspondence and requests for reprints to: Dr. Theo J. Visser, Department of Internal Medicine, Erasmus University Medical Center, Room Bd 234, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: visser@inw3.azr.nl.

This work was supported by the Sophia Foundation for Medical Research (Project 211), the Commission of the European Communities (BMH1-CT92-0097 and QL93-2000-00930), Deutsche Forschungsgemeinschaft (INK 26), and Tenovus Scotland (Tayside).

References

1. **Cheek AO, Vonier PM, Oberdorster E, Burrow BC, McLachlan JA** 1998 Environmental signaling: a biological context for endocrine disruption. Environ Health Perspect 106(Suppl 1):5–10
2. **Cooper RL, Kavlock RJ** 1997 Endocrine disruptors and reproductive development: a weight-of-evidence overview. J Endocrinol 152:159–166
3. **Neubert D** 1997 Vulnerability of the endocrine system to xenobiotic influence. Regul Toxicol Pharmacol 26:9–29
4. **Ahlborg UG, Brouwer A, Fingerhut MA, Jacobson JL, Jacobson SW, Kennedy SW, Kettrup AAF, Koeman JH, Poiger H, Rappe C, Safe SH, Seegal RF, Tuomisto J, Van den Berg M** 1992 Impact of polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls on human and environmental health, with special emphasis on application of the toxic equivalency factor concept. Eur J Pharmacol 228:179–199
5. **Safe S, Astroff M, Harris M, Zacharewski T, Dickerson R, Romkes M, Biegel L** 1991 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds as antiestrogens: characterization and mechanism of action. Pharmacol Toxicol 69:400–409
6. **Peterson RE, Theobald HM, Kimmel GL** 1993 Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons. Crit Rev Toxicol 23:283–335
7. **Safe SH** 1994 Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. Crit Rev Toxicol 24:87–149
8. **Hilakivi-Clarke L, Clarke R, Onojafe I, Raygada M, Cho E, Lippman MA** 1997 A maternal diet high in n–6 polyunsaturated fats alters mammary gland development, puberty onset, and breast cancer risk among female rat offspring. Proc Natl Acad Sci USA 94:9372–9377
9. **Auger J, Kunstmann JM, Czyglik F, Jouannet P** 1995 Decline in semen quality among fertile men in Paris during the past 20 years. N Engl J Med 332:281–285
10. **Skakkebaek NE, Rajpert-de Meyts E, Jørgensen N, Carlsen E, Petersen PM, Giwercman A, Andersen AG, Jensen TK, Andersson AM, Muller J** 1998 Germ cell cancer and disorders of spermatogenesis: an environmental connection? APMIS 106:3–11
11. **Wolff MS, Tonioli PG, Lee EW, Rivera M, Dubin N** 1993 Blood levels of organochlorine residues and risk of breast cancer. J Natl Cancer Inst 85: 648–652
12. **Hoyer AP, Jorgensen T, Brock JW, Grandjean P** 2000 Organochlorine exposure and breast cancer survival. J Clin Epidemiol 53:323–330
13. **Dorgan JF, Brock JW, Rothman N, Needham LL, Miller R, Stephenson HE Jr, Schussler N, Taylor PR** 1999 Serum organochlorine pesticides and PCBs and breast cancer risk: results from a prospective analysis (USA). Cancer Causes Control 10:1–11
14. **Adami HO, Lipworth L, Titus-Ernstoff L, Hsieh CC, Hanberg A, Ahlborg U, Baron J, Trichopoulos D** 1995 Organochlorine compounds and estrogen-related cancers in women. Cancer Causes Control 6:551–566
15. **Safe S** 2000 Endocrine disruptors and human health–is there a problem? An update. Environ Health Perspect 108:487–493
16. **Bergman A, Klasson-Wehler E, Kuroki H** 1994 Selective retention of hydroxylated PCB metabolites in blood. Environ Health Perspect 102:464–469
17. **Morse DC, Klasson-Wehler E, Wesseling W, Koeman JH, Brouwer A** 1996 Alterations in rat brain thyroid hormone status following pre- and postnatal exposure to polychlorinated biphenyls (Aroclor 1254). Toxicol Appl Pharmacol 136:269–279
18. **Wroblewski VJ, Olson JR** 1985 Hepatic metabolism of 2,3,7,8-tetradibenzo-p-dioxin (TCDD) in the rat and guinea pig. Toxicol Appl Pharmacol 81:231–240
19. **Pluess N, Poiger H, Schlatter C, Buser HR** 1987 The metabolism of some pentachlorodibenzofurans in the rat. Xenobiotica 17:209–216
20. **Hu K, Bunce NJ** 1999 Metabolism of polychlorinated dibenzo-p-dioxins and related dioxin-like compounds. J Toxicol Environ Health 2:183–210
21. **Crews D, Bergeron JM, McLachlan JA** 1995 The role of estrogen in sex turtle determination and the effect of PCBs. Environ Health Perspect 103(Suppl 7):73–77
22. **Bergeron RM, Thompson TB, Leonard LS, Pluta L, Gaido KW** 1999 Estrogenicity of bisphenol A in a human endometrial carcinoma cell line. Mol Cell Endocrinol 150:179–187
23. **Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, Van Der Saag PT, Van der Burg B, Gustafsson JA** 1998 Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 139:4252–4263
24. **Strott CA** 1996 Steroid sulfotransferases. Endocr Rev 17:670–697
25. **Kester MHA, Van Dijk CH, Tibboel D, Hood AM, Rose NJM, Meinl W, Pabel U, Glatt H, Falany CN, Coughtrie MWH, Visser TJ** 1999 Sulfation of thyroid hormone by estrogen sulfotransferase. J Clin Endocrinol Metab 84: 2577–2580
26. **Falany JL, Azziz R, Falany CN** 1998 Identification and characterization of cytosolic sulfotransferases in normal human endometrium. Chem Biol Interact 109:329–339
27. **Lewis AJ, Walle UK, King RS, Kadlubar FF, Falany CN, Walle T** 1998 Bioactivation of the cooked food mutagen *N*-hydroxy-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by estrogen sulfotransferase in human mammary epithelial cells. Carcinogenesis 19:2049–2053
28. **Qian VM, Song WC** 1999 Regulation of estrogen sulfotransferase expression in Leydig cells by cyclic adenosine 3′,5′-monophosphate and androgen. Endocrinology 140:1048–1053
29. **Song WC, Qian Y, Li AP** 1998 Estrogen sulfotransferase expression in the

human liver: marked interindividual variation and lack of gender specificity. J Pharmacol Exp Ther 284:1197–1202
30. **Kester MHA, Bulduk S, Tibboel D, Meinl W, Glatt H, Falany CN, Coughtrie MWH, Bergman A, Safe SH, Kuiper GGJM, Schuur AG, Brouwer A, Visser TJ** 2000 Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: a novel pathway explaining the estrogenic activity of PCBs. Endocrinology 141:1897–1900
31. **Wilborn TW, Comer KA, Dooley TP, Reardon IM, Heinrikson IL, Falany CN** 1993 Sequence analysis and expression of the cDNA for the phenol-sulfating form of human liver phenol sulfotransferase. Mol Pharmacol 43:70–77
32. **Falany CN** 1997 Enzymology of human cytosolic sulfotransferases. FASEB J 11:211–218
33. **Schuur AG, Legger FF, Van Meeteren ME, Moonen MJ, Van Leeuwen-Bol I, Bergman A, Visser TJ, Brouwer A** 1998 In vitro inhibition of thyroid hormone sulfation by hydroxylated metabolites of halogenated aromatic hydrocarbons. Chem Res Toxicol 111:1075–1081
34. **Meerts IATM, Van Zanden JJ, Luijks EAC, Van Leeuwen-Bol I, Marsh G, Jakobsson E, Bergman A, Brouwer A** 2000 Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin *in vitro*. Toxicol Sci 56:95–104
35. **Falany CN, Krashnykh V, Falany J** 1995 Bacterial expression and characterization of a cDNA for human liver estrogen sulfotransferase. J Steroid Biochem Mol Biol 52:529–539
36. **Hagen M, Pabel U, Landsiedel R, Bartsch I, Falany CN, Glatt H** 1998 Expression of human estrogen sulfotransferase in Salmonella typhimurium: differences between hHST and hEST in the enantioselective activation of 1-hydroxyethylpyrene to a mutagen. Chem Biol Interact 109:249–253
37. **Glatt H, Engelke CEH, Pabel U, Teubner W, Jones AL, Coughtrie MWH, Andrae U, Falany CN, Meinl W** 2000 Sulfotransferases: genetics and role in toxicology. Toxicol Lett 112–113:341–348
38. **Rubin GL, Harrold AJ, Mills JA, Falany CN, Coughtrie MWH** 1999 Regulation of sulphotransferase expression in the endometrium during the menstrual cycle, by oral contraceptives and during early pregnancy. Mol Hum Reprod 5:995–1002
39. **Visser TJ, Kaptein E, Terpstra OT, Krenning EP** 1988 Deiodination of thyroid hormone by human liver. J Clin Endocrinol Metab 67:17–24
40. **Ainsworth S** 1977 Michaelis-Menten kinetics. In: Ainsworth S, ed. Steady-state enzyme kinetics. London: MacMillan; 43–73
41. **Foldes A, Meek JL** 1973 Rat brain phenolsulfotransferase: partial purification and some properties. Biochim Biophys Acta 327:365–374
42. **Song WC, Melner MH** 2000 Editorial: steroid transformation enzymes as critical regulators of steroid action *in vivo*. Endocrinology 141:1587–1589
43. **Kakuta Y, Pedersen LG, Carter CW, Negishi M, Pedersen LC** 1997 Crystal structure of estrogen sulfotransferase. Nat Struct Biol 4:904–908
44. **Kakuta Y, Pedersen LP, Chae K, Song WC, Leblanc D, London R, Carter CW, Negishi M** 1998 Mouse steroid sulfotransferases: substrate specificity and preliminary x-ray crystallographic analysis. Biochem Pharmacol 55:313–317
45. **Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N** 1995 Xenoestrogens released from lacquer coatings in food cans. Environ Health Perspect 103:608–612
46. **WHO** 1995 International Programme on Chemical Safety: tetrabromobisphenol A and derivatives. Geneva: WHO; Environ Health Criteria 172
47. **Larsen J** 1995 Levels of pollutants and their metabolites: exposures to organic substances. Toxicology 101:11–27
48. **Zhang H, Varmalova O, Vargas FM, Falany CN, Leyh TS** 1998 Sulfuryl transfer: the catalytic mechanism of human estrogen sulfotransferase. J Biol Chem 273:10888–10892
49. **Harris RM, Waring RH, Kirk CJ, Hughes PJ** 2000 Sulfation of "estrogenic" alkylphenols and 17β-estradiol by human platelet phenol sulfotransferases. J Biol Chem 275:159–166
50. **Suiko M, Sakakibara Y, Liu MC** 2000 Sulfation of environmental estrogen-like chemicals by human cytosolic sulfotransferases. Biochem Biophys Res Commun 267:80–84
51. **Falany CN, Wheeler J, Oh TS, Falany JL** 1994 Steroid sulfation by expressed human cytosolic sulfotransferases. J Steroid Biochem Mol Biol 48:369–375
52. **Coughtrie MWH, Sharp S, Maxwell K, Innes NP** 1998 Biology and function of the reversible sulfation pathway catalysed by human sulfotransferases and sulfatases. Chem Biol Interact 109:3–27
53. **Brouwer A, Morse DC, Lans MC, Schuur AG, Murk AJ, Klasson-Wehler E, Bergman A, Visser TJ** 1998 Interactions of persistant environmental organohalogens with the thyroid hormone system: mechanisms and possible consequences for animal and human health. Toxicol Industr Health 14:59–84
54. **Visser TJ** 1994 Role of sulfation in thyroid hormone metabolism. Chem Biol Interact 92:293–303