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Sulfation of Environmental Estrogen-like Chemicals by Human Cytosolic Sulfotransferases

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To investigate whether sulfation, a major Phase II detoxification pathway *in vivo,* can be employed as a means for the inactivation/disposal of environmental estrogens, recombinant human cytosolic sulfotransferases were prepared and tested for enzymatic activities with bisphenol A, diethylstilbestrol, 4-octylphenol, *p*-nonylphenol, and 17α-ethynylestradiol as substrates. Of the seven recombinant enzymes examined, only SULT1C sulfotransferase #1 showed no activities toward the environmental estrogens tested. Among the other six sulfotransferases, the simple phenol (P)-form phenol sulfotransferase and estrogen sulfotransferase appeared to be considerably more active toward environmental estrogens than the other four sulfotransferases. Metabolic labeling experiments revealed the sulfation of environmental estrogens and the release of their sulfated derivatives by HepG2 human hepatoma cells. Moreover, sulfated environmental estrogens appeared to be incapable of penetrating through the HepG2 cell membrane.

Sulfate conjugation represents a major pathway *in vivo* for the biotransformation and/or excretion of xenobiotics or endogenous compounds such as steroid and thyroid hormones, catecholamines, and bile acids (1–3). The responsible enzymes, the so-called "cytosolic sulfotransferases," catalyze the transfer of a sulfonate group from the active sulfate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), to a substrate compound containing either a hydroxyl or an amino group (4). It is generally believed that sulfation may increase the water-solubility of xenobiotic or endogenous compounds and facilitate their removal from the body (1–3).

Environmental estrogen-like chemicals have in recent years been increasingly recognized as a potential hazardous factor for human health (5). In general, they are able to bind to estrogen receptors and thereby mimic estrogenic actions. Some examples of this diverse group of compounds are diethylstilbestrol, bisphenol A, nonylphenol, polychlorinated biphenyls, and dichlorodiphenyltrichloroethane (6). These estrogen-like chemicals are becoming ubiquitous in the environment and making their way into the food chain. Among other adverse effects, the environmental estrogens have been implicated in the abnormal sexual development of reptiles and birds (7, 8), the decline in sperm quality of men (9, 10), and an increased incidence of human breast cancer (11, 12). Glucuronidation of an environmental estrogen, bisphenol A, by the rat liver UDP-glucuronosyltransferase has recently been reported (13). Considering that sulfation is extensively utilized in mammals for the removal of xenobiotics, we recently became interested in the possible occurrence of the sulfation of environmental estrogens.

We report in this communication a systematic study on the sulfation of representative environmental estrogens by human cytosolic sulfotransferases. Moreover, using HepG2 human hepatoma cells as a model, the metabolism of environmental estrogens through sulfation and the release of their sulfated derivatives were investigated.

MATERIALS AND METHODS

Materials. Dopamine, *p*-nitrophenol (pNP), aprotinin, thrombin, adenosine 5′-triphosphate (ATP), sodium dodecyl sulfate (SDS), Trizma base, isopropyl β-D-thiogalactopyranoside (IPTG), bisphenol A, diethylstilbestrol, 4-octylphenol, *p*-nonylphenol, and 17α-ethynylestradiol were obtained from Sigma Chemical Co. Cytosolic extract of Sf-9 insect cells infected with a baculovirus containing the cDNA encoding human estrogen (SULT1E) sulfotransferase (EST) and LA *Taq* DNA polymerase were purchased from PanVera Corp. Oligonucleotide primers were synthesized by Operon Technologies, Inc. pGEX-2TK glutathione *S*-transferase gene fusion vector, *E. coli* BL21, First-Strand cDNA Synthesis Kit, and glutathione Sepharose

Abbreviations used: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; RT-PCR, reverse-transcription polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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4B were products of Pharmacia Biotech. All restriction endonucleases were from New England Biolabs. cDNAs encoding human M-form (SULT1A3) and P-form (SULT1A1) phenol sulfotransferases (PSTs) and two SULT1C sulfotransferases (designated #1 and #2), packaged in pGEX-2TK vector, were prepared as previously described (14, 15). Carrier-free sodium [^{35}S]sulfate was from ICN Biomedicals. Chromatogram cellulose thin-layer chromatography (TLC) plates were from Eastman Kodak Co. [^{35}S]sulfated bisphenol A, diethylstilbestrol, 4-octylphenol, *p*-nonylphenol, and 17α-ethynylestradiol were isolated by thin-layer chromatography (16) from spent media of HepG2 cells labeled with [^{35}S]sulfate in the presence of, respectively, bisphenol A, diethylstilbestrol, 4-octylphenol, *p*-nonylphenol, and 17α-ethynylestradiol. HepG2 human hepatoma cell line (ATCC HB 8065) was from American Type Culture Collection. All other reagents were of the highest grades commercially available.

Molecular cloning, expression, and purification of human cytosolic sulfotransferases. We have previously cloned, expressed, and purified four human cytosolic sulfotransferases: the M-form (SULT1A3) and P-form (SULT1A1) phenol sulfotransferases (PSTs) and two SULT1C sulfotransferases (designated #1 and #2) (14, 15). To clone two additional human cytosolic sulfotransferases, the dehydroepiandrosterone (SULT2A1) sulfotransferase (DHEA ST) (17) and the thyroid hormone (SULT1B2) sulfotransferase (THST) (18), reverse-transcription polymerase chain reaction (RT-PCR) technique was employed. To amplify the human DHEA ST or THST sequence for subcloning into the pGEX-2TK expression vector, sense and antisense oligonucleotide primers (5′-GGCGAATTCATGTCGGACGATTTCTTA-3′ and 5′-GGCGAATTC-TTATTCCCATGGGAACAGCTC-3′ for DHEA ST and 5′-CGCGGA-TCCATGCTTTCCCCAAAAGATATTCTG-3′ and 5′-GGCGAATTC-TTAAATCTCTGTGCGGAATTGAA-3′ for THST), based on 5′- and 3′-regions of the nucleotide sequence encoding human THST or DHEA ST, were synthesized with *Eco*RI and/or *Bam*HI restriction sites (as underlined) incorporated at the ends. With these two sets of oligonucleotides as primers, PCR in 100-μl reaction mixtures were carried out under the action of LA *Taq* DNA polymerase using the human liver first-strand cDNA as the template. Amplification conditions were 27 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C. The final reaction mixtures were applied onto a 1.2% agarose gel and separated by electrophoresis. The discrete PCR product bands, visualized by ethidium bromide staining, were excised from the gel and the DNA fragments therein were isolated by spin filtration. After *Eco*RI or *Bam*HI/*Eco*RI digestion, the PCR products were subcloned into the *Eco*RI or *Bam*HI/*Eco*RI site of pGEX-2TK. To verify their authenticity, the cDNA inserts were subjected to nucleotide sequencing.

Competent *E. coli* BL21 cells were transformed with pGEX-2TK harboring the full-length cDNA encoding the human THST or DHEA ST. The transformed cells, grown to $OD_{600\,nm}$ = ~0.5 in 1 liter LB medium supplemented with 100 μg/ml ampicillin and induced with 0.1 mM IPTG overnight at room temperature, were collected by centrifugation and homogenized in 20 ml ice-cold STE (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) using an Aminco French press. The crude homogenate thus prepared was subjected to centrifugation at 10,000*g* for 30 min at 4°C. The supernatant collected was fractionated using 0.5 ml of glutathione–Sepharose, and the bound glutathione *S*-transferase fusion protein was treated with 2 ml of a thrombin digestion buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 2.5 mM $CaCl_2$) containing 5 unit/ml bovine thrombin. Following a 30-min incubation at room temperature with constant agitation, 5 μg of aprotinin was added to inactivate thrombin and the preparation was subjected to centrifugation. The recombinant enzyme present in the supernatant was analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and examined for enzymatic activities toward environmental estrogens.

Enzymatic assays using environmental estrogens as substrates. The sulfotransferase activities of the recombinant human cytosolic sulfotransferases were assayed using 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate (PAP[^{35}S]) as the sulfate donor. The standard assay mixture, with a final volume of 30 μl, contained 50 mM potassium phosphate buffer, pH 7.0, 14 μM PAP[^{35}S] (15 Ci/mmol), and 50 μM of the environmental estrogen tested. The reaction was started by the addition of the enzyme preparation, allowed to proceed for 15 min at 37°C, and terminated by heating at 100°C for 2 min. The precipitates formed were cleared by centrifugation, and the supernatant was subjected to the analysis of [^{35}S]sulfated product.

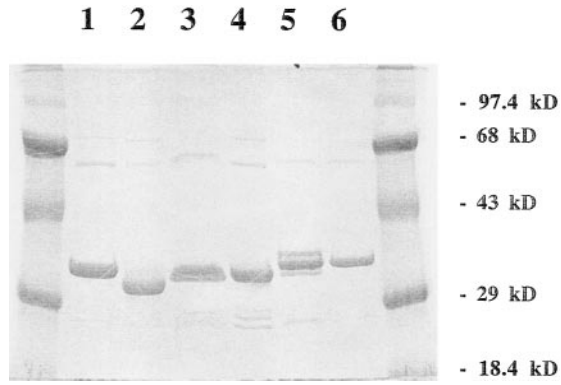

FIG. 1. SDS–gel electrophoretic patterns of purified recombinant human cytosolic sulfotransferases. Samples analyzed in lanes 1 through 6 were M-form PST, P-form PST, THST, SULT1C ST #1, SULT1C ST #2, and DHEA ST. SDS–PAGE was performed using a 12% polyacrylamide gel.

Metabolic labeling of HepG2 human hepatoma cells with [^{35}S]sulfate in the presence of environmental estrogens. HepG2 cells were routinely maintained, under a 5% CO_2 atmosphere at 37°C, in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, penicillin G (30 μg/ml) and streptomycin sulfate (50 μg/ml). Confluent HepG2 cells grown in individual wells of a 24-well culture plate, pre-incubated in sulfate-free (prepared by omitting streptomycin sulfate and replacing magnesium sulfate with magnesium chloride) MEM for 4 h, were labeled with 0.2-ml aliquots of the same medium containing [^{35}S]sulfate (0.3 mCi/ml), and different concentrations (from 1 to 100 μM) of bisphenol A, diethylstilbestrol, 4-octylphenol, *p*-nonylphenol, or 17α-ethynylestradiol. At the end of an 18-h labeling, the media were collected, spin-filtered, and subjected to the analysis of [^{35}S]sulfated environmental estrogens by TLC.

Miscellaneous methods. PAP[^{35}S] (15 Ci/mmol) was synthesized from ATP and [^{35}S]sulfate using the sulfate-activating enzymes, ATP sulfurylase and adenosine 5′-phosphosulfate kinase, from *Bacillus stearothermophilus* as described previously (19). Protein determination was based on the method of Bradford (20) with bovine serum albumin as the standard. The analysis of [^{35}S]sulfated products generated during the enzymatic assays was based on the thin-layer chromatography separation procedure using n-butanol/isopropanol/88% formic acid/water (3:1:1:1; by volume) as the solvent system (16). SDS–PAGE was performed using the method of Laemmli (21).

RESULTS AND DISCUSSION

As pointed out earlier, environmental estrogen-like chemicals are increasingly being recognized as a potential hazardous factor for human health. They have been implicated in, for example, the abnormal sexual development of reptiles and birds (7, 8), the decline in sperm quality of men (9, 10, and an increased incidence of human breast cancer (11, 12). An important issue

TABLE 1

Specific Activities of Human Cytosolic Sulfotransferases Toward Environmental Estrogens and Their Typical Substrates[a]

	P-PST	M-PST	THST	SULT1C ST #1	SULT1C ST #2	DHEA ST	EST
Bisphenol A	965 ± 206	N.D.[b]	3.9 ± 0.2	N.D.	173 ± 12	19.9 ± 3.5	51.9 ± 11.0
4-Octylphenol	1920 ± 152	675 ± 81	17.1 ± 8.7	N.D.	1920 ± 103	18.3 ± 5.8	475 ± 34
p-Nonylphenol	1969 ± 117	39.7 ± 1.7	52.7 ± 4.3	N.D.	268 ± 63	39.9 ± 12.2	221 ± 18
Diethylstilbestrol	1538 ± 233	19.0 ± 7.9	3.2 ± 0.1	N.D.	296 ± 45	11.3 ± 7.1	188 ± 27
17α-Ethynylestradiol	1200 ± 79	N.D.	1.6 ± 0.1	N.D.	N.D.	26.9 ± 10.1	243 ± 57
p-Nitrophenol	1627 ± 110	454 ± 401	12.8 ± 1.0	0.7 ± 0.2	709 ± 137	35.9 ± 3.3	N.D.
Dopamine	407 ± 122	2851 ± 126	N.D.	N.D.	N.D.	N.D.	N.D.
Dehydroepiandrosterone	N.D.	N.D.	N.D.	N.D.	N.D.	489 ± 113	54.1 ± 13.4
Estrone	190 ± 11	18.4 ± 6.4	N.D.	N.D.	59 ± 7	22.6 ± 7.9	58.5 ± 9.1

[a] Specific activity refers to pmol substrate sulfated/min/mg purified enzyme or pmol substrate sulfated/min/mg protein (in the case of unpurified EST commercially obtained). Data represent means ± SD derived from three experiments.

[b] Activity not detected.

therefore is whether the detoxification mechanisms operated *in vivo* may function to fend off these environmental estrogen-like chemicals. Glucuronidation of an environmental estrogen, bisphenol A, by the rat liver UDP-glucuronosyltransferase has recently been reported (13). Considering that sulfation, a major Phase II detoxification pathway, is known to be utilized *in vivo* for the removal of xenobiotics, we recently became interested in the possible occurrence of the sulfation of environmental estrogens. The underlying hypothesis for the studies presented below is that sulfation may be employed as an important means *in vivo* for the inactivation/disposal of environmental estrogens.

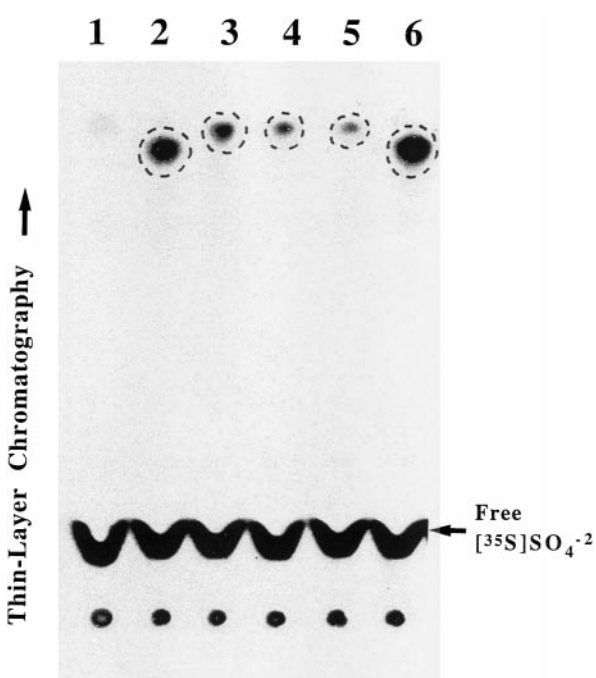

FIG. 2. Analysis of [^{35}S]sulfated environmental estrogens generated and released by HepG2 cells labeled with [^{35}S]sulfate in the presence of environmental estrogens. The compounds tested were bisphenol A (lane 2), diethylstilbestrol (lane 3), 4-octylphenol (lane 4), *p*-nonylphenol (lane 5), and 17α-ethynylestradiol (lane 6). Lane 1 shows the control medium without added environmental estrogen. Dashed line circles indicate the positions of synthetic sulfated environmental estrogen standards detected by UV irradiation of the TLC plate used for the analysis.

Molecular cloning, expression, purification, and characterization of human cytosolic sulfotransferases. We have previously cloned, expressed, and purified human simple phenol (P)-form (SULT1A1) and monoamine (M)-form (SULT1A3) phenol sulfotransferases (PSTs) (14), as well as two human SULT1C sulfotransferases (designated #1 and #2) (15). Using oligonucleotide pairs designed based on 5′- and 3′-regions of reported sequences of human dehydroepiandrosterone (SULT2A1) sulfotransferase (DHEA ST) (17) and thyroid hormone (SULT1B2) sulfotransferase (THST) (18), we have RT-PCR-cloned their coding cDNAs and expressed these two human cytosolic sulfotransferases in the present study. As shown in Fig. 1, purified DHEA ST and THST, as well as the other four human cytosolic sulfotransferases prepared previously, appeared to be highly homogeneous, migrating as major bands of molecular masses between 32 and 35 kDa.

The six human cytosolic sulfotransferases prepared as described above, plus a cytosolic extract of EST-expressing Sf-9 insect cells obtained commercially, were assayed for enzymatic activities toward their typical substrates, as well as representative environmental estrogens including bisphenol A, diethylstilbestrol, 4-octylphenol, *p*-nonylphenol, and 17α-ethynylestradiol. Results compiled in Table 1 showed that, except for SULT1C sulfotransferase #1, all the other six human cytosolic sulfotransferases could catalyze the sulfation of, at least some, environmental estrogens tested. Among these six sulfotransferases, P-form PST and EST (considering that an unpurified cytosolic extract, instead of pu-

TABLE 2

Time-Course Study on the Uptake of [^{35}S]Sulfated Environmental Estrogens by HepG2 Human Hepatoma Cells

[^{35}S]Sulfated environmental estrogen tested	Time point of sample collection during the incubation period (h)					
	0	1	3	6	15	30
	(cpm/25 μl incubation medium)					
BPA[^{35}S]	6,688 ± 29	6,492 ± 22	6,601 ± 166	7,228 ± 376	6,982 ± 46	6,762 ± 53
DES[^{35}S]	3,367 ± 39	3,086 ± 22	3,300 ± 21	3,420 ± 42	3,480 ± 16	3,456 ± 62
4-OP[^{35}S]	2,292 ± 16	2,383 ± 33	2,476 ± 44	2,493 ± 32	2,507 ± 17	2,489 ± 93
P-NonP[^{35}S]	1,501 ± 90	1,362 ± 22	1,382 ± 13	1,455 ± 91	1,611 ± 235	1,490 ± 89
EES[^{35}S]	10,886 ± 67	10,575 ± 75	10,697 ± 129	11,184 ± 311	11,353 ± 34	11,282 ± 65

[a] BPA[^{35}S], DES[^{35}S], 4-OP[^{35}S], P-NonP[^{35}S], and EES[^{35}S] stand for [^{35}S]sulfated bisphenol A, diethylstilbestrol, 4-octylphenol, *p*-nonylphenol, and 17α-ethynylestradiol.

[b] Data shown represent means ± SD derived from five experiments.

rified enzyme, was used) appeared to be considerably more active toward environmental estrogens than the other four. These results demonstrated for the first time the sulfation of environmental estrogens catalyzed by human cytosolic sulfotransferases. It should be pointed out, however, that these activity data were obtained based on assays performed under a single set of conditions (50 mM potassium phosphate buffer, pH 7.0, and 50 μM substrate concentration). More conditions with respect to buffers at different pHs, substrate concentrations, added divalent cations, etc. will need to be tested in order to reveal in greater detail the differential activities of different human cytosolic sulfotransferases toward environmental estrogens.

Generation and release of [^{35}S]sulfated environmental estrogens by HepG2 human hepatoma cells metabolically labeled with [^{35}S]sulfate. HepG2 cells were used as a model for investigating whether the sulfation of environmental estrogens occurs *in vivo.* Confluent HepG2 cells grown in individual wells of a 24-well plate, preincubated in sulfate-free MEM, were labeled with 0.2-ml aliquots of the same medium containing [^{35}S]sulfate (0.25 mCi/ml) and different concentrations (ranging from 1 to 100 μM) of environmental estrogens including bisphenol A, diethylstilbestrol, 4-octylphenol, *p*-nonylphenol, and 17α-ethynylestradiol. At the end of an 18-h labeling, the media were collected for the analysis of [^{35}S]sulfated forms of the environmental estrogens tested. As shown in Fig. 2, analysis using thin-layer chromatography revealed indeed the presence of sulfated environmental estrogens in the medium samples containing as low as 10 μM environmental estrogens. These results demonstrated clearly the occurrence of the sulfation of environmental estrogens in HepG2 cells and the release of [^{35}S]sulfated environmental estrogens into the culture media.

To investigate whether the sulfated environmental estrogens are capable of penetrating through the cell membrane, the [^{35}S]sulfated forms of the environmental estrogens were purified and added to the media of HepG2 cells cultured in individual wells of a 24-well plate. At 0, 1, 3, 6, 15, and 30 h after the addition of [^{35}S]sulfated environmental estrogens, aliquots of the incubation media were collected and counted for [^{35}S]-radioactivity. Results compiled in Table 2 revealed virtually no change in the amount of [^{35}S]-radioactivity for samples of the five sets of incubation media collected at different time points. Moreover, upon washing, the lysates prepared from the cells were found to contain virtually no [^{35}S]-radioactivity. These results indicate strongly that the sulfated forms of environmental estrogens may not be able to penetrate through the cell membrane.

In summary, the results obtained in the present study showed clearly the occurrence of the sulfation of environmental estrogens. That the sulfated environmental estrogens failed to penetrate through the HepG2 cell membrane may imply sulfation as a useful mechanism for the removal of these hazardous compounds. More studies are warranted in order to fully appreciate the involvement of different sulfotransferases in the inactivation/removal of environmental estrogens *in vivo.*

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