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A Role for Sulfation-Desulfation in the Uptake of Bisphenol A into Breast Tumor Cells

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Summary

Bisphenol A (BPA) is a widely used plasticizer whose estrogenic properties may impact hormone-responsive disorders and fetal development. In vivo, BPA appears to have greater activity than is suggested by its estrogen receptor (ER) binding affinity. This may be a result of BPA sulfation/desulfation providing a pathway for selective uptake into hormone-responsive cells. BPA is a substrate for estrogen sulfotransferase, and bisphenol A sulfate (BPAS) and disulfate are substrates for estrone sulfatase. Although the sulfated xenobiotics bind poorly to the ER, both stimulated the growth of receptor-positive breast tumor cells. Treatment of MCF-7 cells with BPAS leads to desulfation and uptake of BPA. No BPAS is found inside the cells. These findings suggest a mechanism for the selective uptake of BPA into cells expressing estrone sulfatase. Therefore, sulfation may increase the estrogenic potential of xenobiotics.

Introduction

The role of estrogenic xenobiotics in hormone-responsive neoplastic disease and disruption of normal endocrine function remains a source of considerable controversy [1–6]. The xenoestrogen bisphenol A (BPA, **1**, Figure 1) has achieved particular notoriety in this regard. BPA is a widely used plasticizer that may be found in food packaging and dental sealants [7, 8]. BPA has been implicated in a number of endocrine-related disorders, from recurrent miscarriages to developmental disruptions associated with in utero exposure. Because of its widespread use, there is a potential for continuous low-level exposure, which may be of specific concern for pregnant women and those with hormone-related disorders. A recent study of the concentration of BPA in urine samples from 394 adults showed that 95% of the samples contained BPA [9]. BPA binds with modest affinity to the estrogen receptor (ER) and exhibits estrogenic properties in tissue culture [10–13]. Because of its modest binding affinity to the ER, its role in stimulating hormonal responses in humans is controversial [14–16]. Some of the questions that remain are (1) whether or not the results in animal models are accurate and (2) whether or not the results from animal models can be extrapolated to humans. While the answers to these questions are still being debated, there seems to be a consensus that there is a potential for deleterious effects from exposure to BPA, as well as other xenoestrogens, and further study is needed (see references cited above). There has yet to be a determination of whether BPA reaches sufficient concentration in target tissues to drive binding to ERs. It is therefore critical to examine how in vivo biochemical modifications of BPA and other estrogenic xenobiotics affect their bioavailability and potential to bind to relevant receptors.

Compounds such as BPA are subject to the activity of phenol-sulfotransferases, a group of relatively promiscuous enzymes that mediate the sulfation of molecules containing a phenol moiety (Figure 2) [17, 18]. While the primary pathway for modification of ingested phenols at high concentrations is glucuronidation, when present at low concentrations sulfation is the more likely modification [19, 20]. BPA is readily sulfated in tissue culture by liver cells (HepG2) [21], and it has been reported that estrogenic phenols such as BPA and octylphenol are substrates for estrogen sulfotransferase [21]. This enzyme efficiently mediates the sulfation of steroids, which increases their water solubility and ability to circulate through the body [22]. The ability of sulfotransferases to sulfate BPA is of critical importance in evaluating the potential of this molecule to function as an estrogen in vivo. Sulfation of phenols drastically decreases their membrane permeability, which should have a chemoprotective effect. Experiments with HepG2 cells show that BPA is taken up by these cells, sulfated, and actively transported out of the cells. Once sulfated, the bisphenol A sulfate (BPAS, **2**, Figure 1) is not capable of reentering the liver cells [21]. Sulfation may also reduce inherent binding to the ER, which may further block its estrogenic effect and reduce the stimulation of breast tumor cell growth [23]. These results suggest that sulfation does play a role in chemoprotection against the effects of xenobiotics such as BPA.

Sulfatases are a group of enzymes that catalyze the hydrolysis of sulfate esters [24]. The opposing activity of sulfatases and sulfotransferases determines the sulfation state of a variety of molecules, including steroids, neurotransmitters, carbohydrates, and, possibly, proteins (Figure 2) [25]. There is mounting evidence that estrone sulfatase, also known as arylsulfatase C, facilitates the uptake of estrogens into breast cancer cells by mediating the desulfation of estrone sulfate, the dominant circulating estrogen in women [22, 26–28]. There is both chemical and immunochemical evidence that

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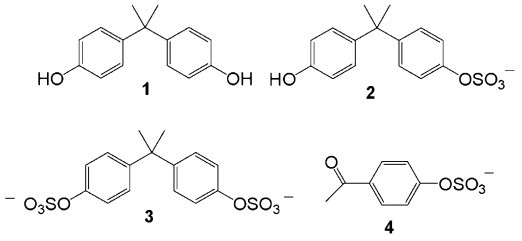

Figure 1. Structures of Relevant Compounds

Structure of bisphenol A (**1**), bisphenol A sulfate (**2**), bisphenol A disufate (**3**), and *p*-acetylphenyl sulfate (**4**), a chromogenic substrate for estrone sulfatase.

this enzyme is located in the outer leaflet of the cell membrane and is thus exquisitely poised to facilitate the conversion of charged, impermeant molecules, such as steroid sulfates, into neutral molecules capable of entering the cell by passive diffusion [29, 30]. If this sulfatase were to catalyze the hydrolysis of sulfated xenobiotics such as BPA, it might also facilitate the uptake of these molecules into tumor cells. In this case, sulfation would not result in chemoprotection, but it might provide a mechanism for selective uptake of phenolic xenoestrogens into cells expressing sulfatases. Given this possibility, we sought to discover whether the relevant human enzymes are capable of carrying out the sulfation and desulfation of BPA.

Results and Discussion

Estrogen Sulfotransferase Activity

In vivo, the sulfation of estrone is carried out by estrogen sulfotransferase. This sulfation results in an ionic molecule that can be transported in the blood more easily. Therefore, if BPA is sulfated via this pathway, it would have to be a substrate for estrogen sulfotransferase. The activity of estrogen sulfotransferase with BPA was determined by using an in vitro assay similar to one that has been reported previously [31, 32]. BPA was incubated with enzymatically synthesized [^{35}S]3′phosphoadenosine 5′-phosphosulfate (PAPS) and estrogen sulfotransferase; the amount of sulfate transfer was determined by separation of the products on a silica gel TLC plate, followed by quantitation with phosphorimaging. BPA is indeed a good substrate for estrogen sulfotransferase. The average K_M for BPA with estrogen sulfotransferase is 17.9 μM. Figure 3 shows a typical Lineweaver-Burk plot for estrogen sulfotransferase activity.

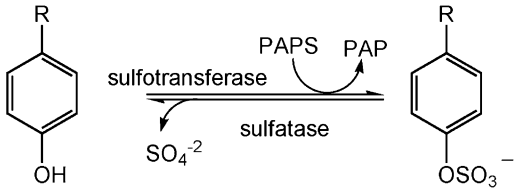

Figure 2. Pathway for the Interconversion of Phenols and Their Sulfate Esters

The sulfation is carried out by a sulfotransferase with the conversion of 3′phosphoadenosine 5′-phosphosulfate (PAPS) to 3′phosphoadenosine 5′-phosphate (PAP).

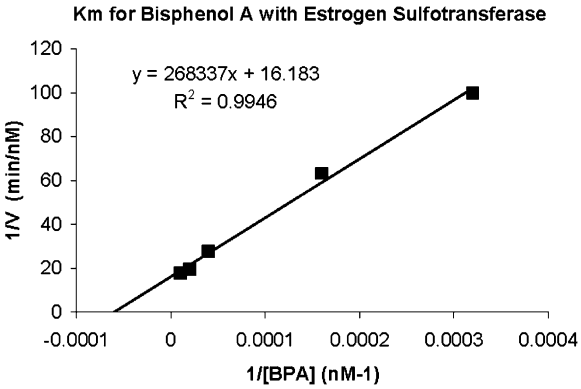

Figure 3. Representative Lineweaver-Burk Plot for the Sulfation of Bisphenol A by Estrogen Sulfotransferase

Estrogen Receptor Binding

It has been reported that the ER binds BPA with an affinity that is approximately 2000- to 5000-fold less than the affinity for estradiol [10]. To evaluate the role of sulfation in BPA binding to hormone receptors, we assayed BPAS and bisphenol A disulfate (BPADS, **3**, Figure 1) for ER binding. A competitive binding assay was performed by using cytosolic ER derived from human breast cancer cells (MCF-7). The percentages of ^{3}H-estradiol still bound to the ER after treatment with increasing concentrations of BPA (■), BPAS (▲), and BPADS (●) are graphed in Figure 4. The amount of estradiol bound to the ER decreases linearly with increasing concentrations of BPA. The amount of estradiol bound to the ER decreases in a nonlinear manner with both BPAS and BPADS. It takes about 10 times (BPAS) or 100 times (BPADS) as much of the sulfated forms of BPA to displace the same amount of estradiol from the ER as BPA. Therefore, the affinities for the sulfated forms of BPA are ~10- and 100-fold less than that for BPA. This suggests that sulfation of BPA may actually function as a protective measure against ER binding if the cells do not have a way to remove the sulfate group.

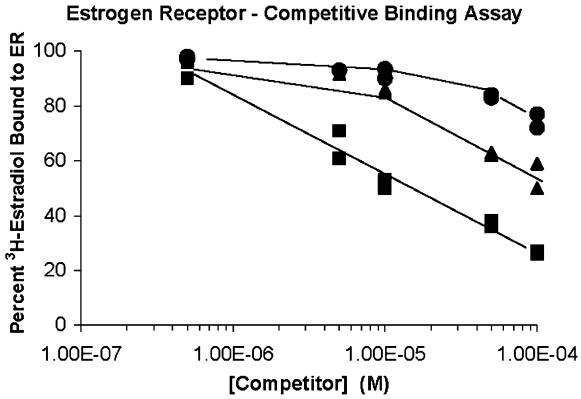

Figure 4. Competitive Binding for the Estrogen Receptor

Competitive binding assay with bisphenol A (■), bisphenol A sulfate (▲), and bisphenol A disulfate (●) to displace labeled estradiol from the estrogen receptor.

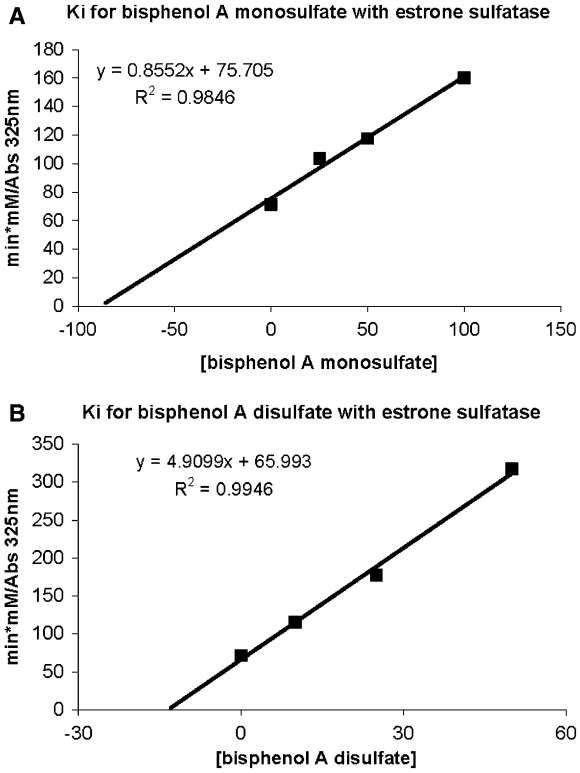

Figure 5. Binding Affinity for Estrone Sulfatase

(A and B) Secondary replots of slopes from Lineweaver-Burk plots for (A) bisphenol A monosulfate and (B) bisphenol A disulfate.

Estrone Sulfatase Activity

We have shown that BPA is a substrate for estrogen sulfotransferase. Therefore, BPA can be sulfated by the same enzyme as estrone. The question of whether the sulfated forms can be desulfated by enzymes that desulfate estrone sulfate remains. Estrone sulfatase, the enzyme responsible for desulfating estrone sulfate, is overexpressed in MCF-7 cells, which are ER-positive breast tumor cells, and in placental cells. We therefore tested BPAS and BPADS as substrates for this enzyme. Because it is difficult to directly assay these molecules as substrates for estrone sulfatase, the binding of these molecules was determined indirectly by monitoring their ability to inhibit the hydrolysis of *p*-acetylphenyl sulfate (**4**, Figure 1) (The K_i for a competitive substrate is equal to the K_M for the substrate [33].). Both the monosulfate and disulfate bind with micromolar affinity, 88.5 μM and 13.4 μM, respectively, to estrone sulfatase. Figure 5 shows the replots of the slopes of the Lineweaver-Burk plots that were used to determine the K_i. The ability of these compounds to serve as substrates for the enzyme was then confirmed with NMR by following the enzyme-catalyzed conversion of the disulfate to the monosulfate, and the monosulfate to BPA (data not shown). These experiments show that BPA can be sulfated and desulfated in vitro by the same enzymes that sulfate and desulfate estrone in vivo. It should be noted that estrone sulfatase may be only one of several enzymes that mediate the desulfation of arylsulfates in and around breast tumor cells, but it is likely that BPAS and BPADS would be substrates for these enzymes as well [34].

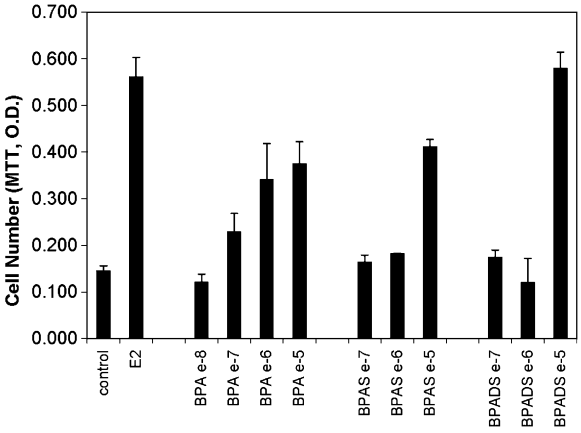

Figure 6. Stimulation of MCF-7 Cell Growth

Stimulation of MCF-7 cell growth by estradiol (E_2, 1 nM) and different molar concentrations of bisphenol A (BPA, 10 nM–10 μM), bisphenol A sulfate (BPAS, 100 nM–10 μM), and bisphenol A disulfate (BPADS, 100 nM–10 μM). Error bars were determined from four replicative samples of each treatment.

MCF-7 Cell Growth Stimulation

It has been suggested that the sulfation of BPA is protective because sulfated BPA cannot penetrate the cell membrane in liver cells. We wanted to determine if the same protection would be seen in breast cancer cells, which are known to overexpress estrone sulfatase. Shimizu et al. showed that levels of BPAS as high as 100 nM do not promote the growth of MCF-7 cells [23]. However, these concentrations of BPAS would not be high enough to saturate estrone sulfatase, and, consequently, the desulfation process would likely be very slow. We therefore assayed BPA, BPAS, and BPADS at higher concentrations for the ability to promote growth of ER-positive breast cancer cells. In spite of the fact that both sulfated molecules have much lower affinity for the ER than BPA, they do stimulate the growth of MCF-7 cells. Figure 6 shows the growth of MCF-7 cells after treatment with estradiol and varying concentrations of BPA, BPAS, and BPADS. As would be expected, treatment with estradiol increased the growth of cells by about 3.5 times what was seen in the untreated control. At the lowest concentration of BPA, the cell growth was similar to that of the control. However, increasing the concentration of BPA increased the cell growth over that of the control. Treatment with concentrations of BPAS and BPADS up to 1 μM resulted in little or no increase in cell growth over the control, which was similar to the results reported by Shimizu et al. However, treatment with a concentration of 10 μM BPAS or BPADS increased cell growth by the same amount or more than treatment with the same concentration of BPA. It is unlikely that the binding of BPADS to the ER is causing the growth, because even at 10 μM BPADS only about 10% of bound estradiol was displaced from the ER. It is more likely that the increase in cell growth at 10 μM BPAS or BPADS is the result of BPA binding to the ER for the following reason. At concentrations up to 1 μM BPAS or BPADS, the enzymatic desulfation would be slow; therefore, the growth of the cells would also be slow relative to the assay time. At higher concentrations, the desulfation would be faster, and the rate of cell growth would also be faster. It is

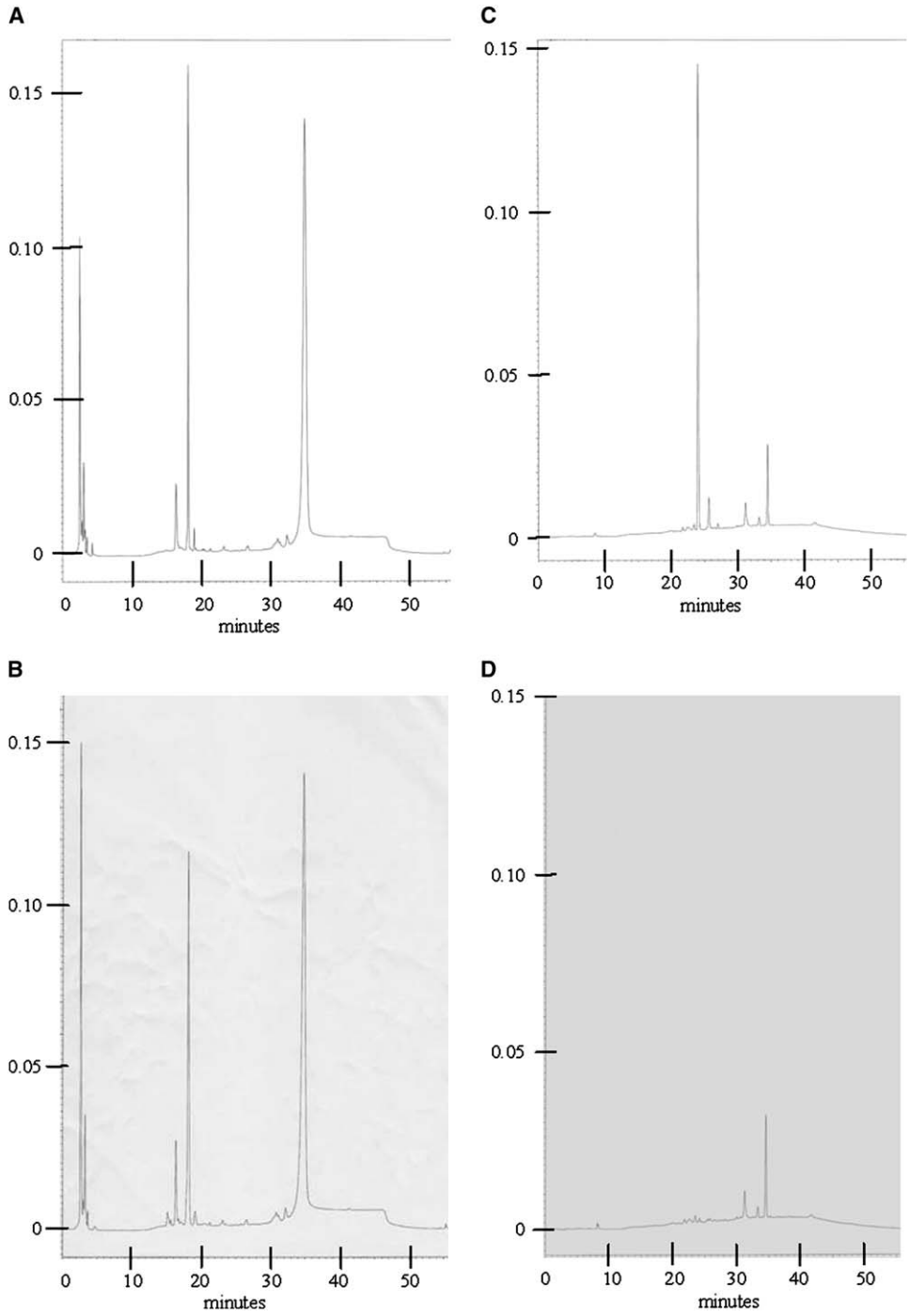

Figure 7. HPLC Analysis of Cell Extracts

(A–D) HPLC traces of (A) the media at t = 0 hr after treating cells with bisphenol A sulfate (BPAS), (B) the media at t = 36 hr after treating cells with BPAS, (C) the final ether extract of the NaOH wash of cells treated with BPAS, and (D) the final ether extract of the NaOH wash for control cells.

also possible that the binding of BPAS to the ER is responsible for the increased growth. It was therefore critical to determine which form of BPA was present in the tumor cells.

Determination of the Bioactive Form of BPA

To determine whether BPAS can penetrate cells expressing estrone sulfatase, and to see whether removal of the sulfate group is required for uptake, MCF-7 cells were treated with ^{3}H-BPAS. After incubation, the media were collected and the cells were harvested and lysed. HPLC analysis and scintillation counting showed that the media contained some of the labeled BPAS, and that the rest of the radiolabel was associated with the cell debris (data not shown). An initial control was performed by using vesicles constructed of 1,2-distearoyl-sn-glycero-3-phosphocholine to determine whether BPA could be extracted from a membrane-like environment

and whether the extraction conditions would desulfate BPAS. The vesicles were treated with a solution containing a mixture of BPA and BPAS that was shaken gently overnight. The vesicles were then washed with 1 N NaOH. The NaOH solution was extracted with ether, neutralized to ~pH 7, and again extracted with ether. The final ether extract contained both BPA and BPAS (results not shown). This indicated that the extraction conditions would not desulfate BPAS and that both BPA and BPAS could be extracted from vesicles, and presumably membranes, by using this technique. In a subsequent assay, MCF-7 cells were treated with BPAS, the media and cells were separated, and the cells were washed with 1 M NaOH. The NaOH solution was extracted as described above. The media and the resulting ether extracts were submitted to HPLC analysis (see Figure 7). HPLC traces resulting from injections of aliquots of the media, shown in Figures 7A and 7B, show a decrease in the concentration of BPAS (retention time = 19 min) outside the cell over time. However, there was no increase in BPA (retention time = 24 min) in the media. Traces resulting from the final ether extract for cells treated with BPAS show only one large peak with a retention time of 24 min. This peak coeluted with BPA when the sample was spiked with pure compound. Ether extracts of control cells not treated with BPAS did not have a peak at 24 min. These results clearly show that BPA, not BPAS, is absorbed by MCF-7 cells treated with BPAS. Therefore, the estrogenic response in MCF-7 cells must be the result of BPA, not BPAS, binding to the ER. It is important to note that there was no BPA found in the media, and that all of the BPA produced was found inside the cells. This apparent coupling of desulfation and uptake of BPA suggests a pathway for increasing the concentration of BPA inside cells capable of desulfating BPAS and BPADS, such as receptor-positive breast tumor cells and placental cells. Previously, sulfation was thought to facilitate the excretion of xenobiotics [21]. In light of these data, it must be considered that sulfation of xenobiotics may actually enhance their estrogenic activity in cells that have the ability to hydrolyze the sulfate group.

Significance

In vivo studies reveal a higher estrogenic activity for BPA than can be accommodated by simple receptor binding data, which may be the result of the existence of an unknown BPA metabolite [6]. One of the main pathways for metabolizing xenobiotics is the addition of a sulfate group. Estrogen sulfation promotes increased estrogen uptake into cells that express sulfatases [35]. Similarly, it is possible that the potency of BPA may be enhanced as a consequence of sulfation, which leads to selective uptake in tissues that express sulfatases on their exterior. We have confirmed that BPA is sulfated and desulfated by the same enzymes as estrone, and that sulfated versions of BPA stimulate breast cancer cell growth. We have also shown that when MCF-7 cells are treated with BPAS, the concentration of BPAS outside the cell decreases over time with the concurrent appearance of BPA inside the cells. There is no BPAS associated with the cells. Taken together, these findings demonstrate that an enzymatic pathway for sulfation and desulfation of BPA by human enzymes exists in vitro and suggests that the pathway may also be active in vivo. It should be stressed that none of the data presented here address the existence of an in vivo pathway. However, the presence of these enzymes in humans and the selective overexpression of estrone sulfatase in various tissues raise important questions about the pathway of xenoestrogen uptake into these cells. This, in turn, suggests that more attention needs to be focused on determining whether the sulfated metabolites of BPA, and other xenoestrogens, may be implicated in the low-dose biological effects of these compounds.

Experimental Procedures

Estrogen Sulfotransferase Activity

Estrogen sulfotransferase (EST) activity was measured in 50 μl 20 mM Tris-HCl (pH 7.4), containing 1 mM EDTA, by using 20 μg EST and 0.098–100 μM bisphenol A (BPA) for K_M determination. The reactions were initiated by the addition of 2.5 nM enzymatically synthesized ^{35}S-PAPS [36], and they were quenched after a 10 min incubation at room temperature with 50 μl methanol. Half of the quenched reaction (50 μl) was applied to the loading lane of Whatman 10 × 20 silica plates and was eluted with 8:2:1 butanol:ethanol:H_2O (3 hr). The plates were air dried, and the amount of radiolabel incorporated into bisphenol A sulfate (BPAS) was quantified by phosphorimaging. This method is similar to a method for screening against several other sulfotransferases [31, 32].

Synthesis of Bisphenol A Sulfate and Bisphenol A Disulfate

BPAS and bisphenol A disulfate (BPADS) were synthesized from BSA (Aldrich) and sulfur trioxide pyridinium complex (Aldrich) as follows.

BPAS

BSA and 1.1 equivalents of sulfur trioxide were stirred in dry pyridine under argon for 36 hr. The solvent was removed under reduced pressure, and the crude reaction mixture was purified by column chromatography (10% methanol in CH_2Cl_2). The pyridinium salt was dissolved in water and passed down a Dowex 50W X-8 cation exchange column, sodium form. ^{1}H-NMR (CD_3OD, 300 MHz): δ7.18 (s, 4H), 7.02 (2H, d, J = 8.8 Hz), 6.67 (2H, d, J = 8.8 Hz), 1.6 (6H, s), ^{13}C-NMR (CD_3OD, 101 MHz): δ 156.2, 151.5, 149.2, 142.7, 126.7, 128.4, 121.9, 115.6, 42.8, 31.5. ESIMS (negative ion mode) 291 (BPAS monoanion).

BPADS

BSA and 3.8 equivalents of sulfur trioxide were stirred in dry pyridine for 24 hr. The solvent was removed under reduced pressure. The remaining mixture was dissolved in ddH_2O, washed five times with CH_3Cl, washed three times with ether, and passed down a Dowex 50W X-8 cation exchange column, sodium form. ^{1}H-NMR (D_2O, 400 MHz): δ 7.13 (2H, d, J = 7.7 Hz), 7.02 (2H, d, J = 7.7 Hz), 1.48 (s, 6H), ^{13}C-NMR (CD_3OD, 101 MHz): δ 148.9, 148.6, 128.1, 120.9, 41.9, 29.9. ESIMS (negative ion mode) 386.9 (BPADS monoanion).

Relative Estrogen Receptor Binding

Estrogen receptor (ER) binding was performed as described previously [37]. A human breast cell cytosol was prepared from MCF-7 cells and diluted to 1 mg protein/ml. Each assay tube contained 100 μl diluted cytosol and 2.5 nM [^{3}H]estradiol (E_2) (Amhersham, Arlington, IL) plus test compound (BPA or sulfated BPAs) at 10^{-6}–10^{-4} M. The tubes were incubated for 18 hr at 4°C, and then a suspension of dextran-coated charcoal was added to remove free [^{3}H]E_2. Bound [^{3}H]E_2 was extracted from the supernatant with ethanol, and the radioactivity was determined by liquid scintillation. The protein bound radiolabel was expressed as a percentage of maximum binding (in the absence of competitor) and was plotted against the concentration of competitor.

Breast Cancer Cell Growth Assays

MCF-7 breast cancer cells were propagated in DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 6 μg/ml insulin (Sigma Chemical Co., St. Louis, MO). To test

the growth response, MCF-7 cells were plated at 15,000 cells per well in 4-well plates (well diameter: 18 mm) (Nunc, Naperville, IL) in phenol red-free medium (PRF-DMEM) containing 3% charcoal-stripped FBS (Hyclone) plus 6 μg/ml insulin. Two days later, medium was replaced with fresh medium containing treatments. Each well of individual plates was treated with test compound: 10^{-9} M E_2, or 10^{-8}–10^{-5} M BPA, BPAS, or BPADS. The medium was replaced every 2 days until the eighth day of treatment. The number of live cells present in each culture well was determined by using a colorimetric assay based on the biochemical reduction of a tetrazolium salt, MTT (Sigma), to form a blue formazan product [38]. Briefly, medium was removed from the cells, and a solution of MTT (1 mg/ml in PRF-DMEM) was added. The cells were incubated in the MTT solution for 3 hr at 37°C and then developed by the addition of acidic isopropanol (0.04 N HCl in isopropanol). The optical density (OD) of the isopropanol solution was read at 570 nm. Thus, treatment effects were estimated by comparing the mean OD of control cultures against the mean OD of four replicate samples of each treatment.

HPLC Analysis

MCF-7 cells were prepared as described for the cell growth assays, with the exception of using six-well plates (well diameter: 35 mm). The cells were treated with either water or enough BPAS in water to make a final concentration of 250 μM BPAS. After incubation for 36 hr, the media were collected and reserved for HPLC analysis. The cells were washed with water to remove any remaining media. The water was collected and reserved for HPLC analysis. The cells were washed with 1 M NaOH. The NaOH solution was collected, extracted with ether, neutralized with 1 M HCl, and extracted with ether again. The ether solution was collected and reserved for HPLC analysis. All HPLC analysis was carried out by using a 300 mm × 3.9 mm alphaBond-C_{18} 125A analytical column (Alltech Associates Inc., Deerfield, IL), eluted with a mobile phase of water/acetonitrile, both containing 1% acetic acid, at a flow rate of 1 ml/min. The total run time was 55 min. The mobile phase gradient was run with 95% water and 5% acetonitrile for 5 min, then with 5% water and 95% acetonitrile over 25 min. These conditions were maintained for 5 min, until the column was brought back to initial conditions over 15 min. The HPLC system used throughout this work was composed of a 600E HPLC pump (Waters). The column effluent was monitored in tandem with a UV-visible detector (Waters 2487) at a wavelength of 270 nm.

Estrone Sulfatase Activity

K_is were determined for both BPAS and BPADS by measuring the inhibition of the hydrolysis of *p*-acetylphenyl sulfate (Aldrich). Enzyme assays were carried out in 1.5 ml polypropylene microcentrifuge tubes. Estrone sulfatase was purified from fresh human placenta as previously reported [39]. Estrone sulfatase, 15.7 units, was preincubated for 10 min at 37°C with 520 μl 20 mM Tris-Cl (Promega) buffer (pH 7.4) containing varying concentrations of either BPAS or BPADS. A total of 140 μl buffer containing *p*-acetylphenyl sulfate at varying concentrations (preequilibrated to 37°C) was added to the enzyme mixture. Aliquots (100 μl) were removed at T = 2, 4, 6, 8, and 10 min and were quenched into 600 μl 1.4 N NaOH. Absorbance readings for *p*-acetylphenoxide were measured at 325 nm (ε = 21,000 M^{-1} cm^{-1}). The K_i for each inhibitor was determined from Lineweaver-Burk plots and secondary replots of the slopes of Lineweaver-Burk plots versus the corresponding inhibitor concentration.

NMR Assays for Enzyme-Catalyzed Conversion

Enzyme assays were carried out in 15 ml polypropylene conical tubes. A total of 2.4 ml estrone sulfatase in 30 mM glycine buffer (pH 7.4) containing 0.1% (w/v) Triton X-100 (Promega) and 0.02% (w/v) sodium azide was incubated with 8.1 ml 4 mM BPAS or BPADS in 30 mM glycine buffer (pH 7.4) containing 0.02% (w/v) Triton X-100 at 37°C. At T = 17, 51, and 68 hr, 3.5 ml aliquots were removed, frozen, and lyophilized to remove water. The lyophilized powder was dissolved in DMSO-d6, and proton nuclear magnetic resonance spectra were obtained by using a Varian-400 (400 MHz). The chemical shifts were determined in parts per million relative to the solvent reference. These spectra were compared to spectra of the expected products under the same conditions.

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