Effects of Endocrine Disruptors on Dehydroepiandrosterone Sulfotransferase and Enzymes Involved in PAPS Synthesis: Genomic and Nongenomic Pathways

Robert Harris,[1] Nahid Turan,[2] Christopher Kirk,[1] David Ramsden,[2] and Rosemary Waring[1]

[1]School of Biosciences, The University of Birmingham, Edgbaston, Birmingham, United Kingdom; [2]Department of Medicine, Division of Medical Sciences, The University of Birmingham, Queen Elizabeth Hospital, Birmingham, United Kingdom

BACKGROUND: Sulfation plays an important role both in detoxification and in the control of steroid activity. Studies in rodents have shown that the conversion of dehydroepiandrosterone (DHEA) to DHEA-sulfate is involved in learning and the memory process.

METHODS: The effects of a range of plasticizers and related compounds commonly encountered in the environment were evaluated kinetically against human DHEA sulfotransferase (SULT 2A1) and by reverse transcriptase-polymerase chain reaction (RT-PCR) against several enzymes involved in the synthesis of the sulfotransferase cofactor adenosine 3′-phosphate 5′-phosphosulfate (PAPS).

RESULTS: We found that several of the chemicals acted as competitive inhibitors of SULT 2A1 (K_i for 4-*tert*-octylphenol is 2.8 μM). Additionally, after treatment of TE 671 cells with 0.005–0.5 μM 4-*n*-octylphenol, bis(2-ethylhexyl)phthalate, and diisodecyl phthalate, real-time RT-PCR showed dose-dependent decreases in the steady-state mRNA levels of cysteine dioxygenase type I, sulfite oxidase, and 3′-phosphate 5′-phosphosulfate synthase I.

CONCLUSIONS: These data suggest that environmental contaminants may exert effects on neuronal function both by direct inhibition of sulfotransferase enzymes and by interrupting the supply of PAPS, which has wider implications for endocrine disruption and xenobiotic metabolism.

KEY WORDS: DHEA, endocrine disruptor, nongenomic, PAPS, plasticizers, sulfotransferase. *Environ Health Perspect* 115(suppl 1):51–54 (2007). doi:10.1289/ehp.9365 available via *http://dx.doi.org/* [Online 8 June 2007]

Endocrine disruptors (EDs) have been studied quite extensively in environmental biology (Toppari et al. 1996); however, their exact effects on humans are uncertain. Rising rates of hormone-dependent cancers, such as breast, ovary, and testicular cancer, appear to be linked to rising levels of EDs contaminating the environment (Weir et al. 2000), although a causal link between observed abnormalities and chemical exposure has not been established (Baker 2001). These compounds may modulate both the endocrine, neuronal, and immune systems resulting in alteration of homeostasis, reproduction, development, and behavior (Amaral Mendes 2002).

EDs are not classical poisons or carcinogens (Colborn et al. 1996); the detailed mechanisms by which EDs exert their effects are gradually being elucidated (Tapiero et al. 2002). They can directly initiate or inhibit actions mediated by members of the superfamily of zinc finger nuclear receptors, such as estrogen, androgen, and triiodothyronine receptors, although their chemical structures may not resemble those of steroids or related hormones. Alternatively, they may exert indirect genomic effects by modulating the metabolism of hormones. In this context, sulfation plays a key role because the cellular availability of steroid and thyroid hormones is modulated by their sulfation and desulfation by sulfotransferases and sulfatases, respectively. Sulfation of hydroxyl residues in these hormones renders them inactive and speeds exit from the cell, whereas desulfation regenerates their endocrine potential. A decrease in the sulfation/desulfation ratio could therefore lead to inappropriately high levels of hormones within cells (Falany et al. 2002; Kirk et al. 2001; Qian et al. 1998).

Dehydroepiandrosterone (DHEA) and its sulfate-ester (DHEAS) are neurosteroids secreted mainly by the adrenal cortex and gonads, although synthesis of DHEAS can also take place in the central nervous system (CNS). In humans, plasma concentrations of DHEAS peak during the teenage years and thereafter decline by about 10% per decade (Ravaglia et al. 1996). It has been shown that any disruption of sulfation of DHEA or pregnenolone can block memory processes in rodents (Vallée et al. 2001), probably because these neurosteroids modulate acetylcholine release and γ-aminobutyric acid (GABA) and *N*-methyl-D-aspartate (NMDA) receptor action in the cortex and hippocampus (Dubrovsky 2005; Mayo et al. 2003). Results from studies in humans have been rather less conclusive because *in vivo* experiments are not possible. The situation is confounded because the brain can synthesize DHEA independently of the adrenal gland; therefore, measurement of DHEA and DHEAS in the plasma may not give a true indication of levels in the CNS (Racchi et al. 2003). Reduced levels of both plasma DHEAS and pregnenolone sulfate have been linked with decreased cognitive function (Armanini et al. 2003; Mayo et al. 2003). However, there is evidence that DHEA levels may exert subtle effects, particularly in Alzheimer's disease (Vallée et al. 2001). DHEA sulfation is catalyzed by SULT 2A1, a member of the SULT 2 subfamily of the sulfotransferases, which mainly acts on endogenous hydroxysteroids, including pregnenolone, but can also sulfate various xenoestrogens and drugs. SULT 2A1 is unusual in that, although it has a K_m for DHEA of about 2 μM (Chang et al. 2001), the physiologic concentration of DHEA ranges from 1–20 nM (Heuser et al. 1998).

The essential co-factor in sulfation reactions is PAPS, which is synthesized *in vivo* by oxidation of sulfur-containing amino acids (Do and Tappaz 1996; Griffith 1987). Four enzymes play a key role in the synthesis of PAPS from cysteine. These are cysteine dioxygenase type I (CDO1), sulfite oxidase (SUOX), and the bifunctional enzymes 3′-phosphoadenosine 5′-phosphosulfate synthase 1 and 2 (PAPSS1 and PAPSS2). In humans, the supply of PAPS appears to be rate limiting for sulfation of both endogenous and exogenous compounds.

Chemicals produced from the plastics and detergent industries, such as alkylphenols and bisphenol A, have been discovered to be estrogenic (Jobling et al. 1995). Plasticizers are ubiquitous in the environment at low levels and may be present in mixtures that result in additive, antagonistic, and/or synergistic effects (Guenther et al. 2002). In this article we show that a range of plasticizers commonly encountered in the environment act as competitive inhibitors of SULT 2A1 when sulfating physiologic concentrations of DHEA. In addition, they reduce steady-state mRNA levels of enzymes involved in PAPS synthesis.

This article is part of the monograph "Endocrine Disruptors—Exposure Assessment, Novel End Points, and Low-Dose and Mixture Effects."

Address correspondence to R. Harris, School of Biosciences, The University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK. Telephone: 44 (0)121 414 3393. Fax: 44 (0)121 414 5925. E-mail: R.M.Harris@bham.ac.uk

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Materials and Methods

Materials. We used the following EDs in this study: bis(2-ethylhexyl)adipate, bis(2-ethylhexyl)phthalate, 4-*n*-nonylphenol, and 2,4-dichlorophenol from Lancaster Synthesis (Morecambe, UK); resorcinol from Riedel-de Haën Fine Chemicals (Seelze, Germany); dioctyl phthalate and diisodecyl phthalate from Fluka (CH-9471 Buchs SG, Switzerland); benzyl butyl phthalate, bisphenol A, bisphenol A dimethacrylate, 4-chloro-3-methylphenol, diisononyl phthalate, 4-*n*-octylphenol, 4-*tert*-octylphenol, and 2-phenylphenol from Aldrich (Gillingham, UK); and dibutyl phthalate, 17β-estradiol (E_2), and diethylstilbestrol (DES) from Sigma (Poole, UK).

All cell culture reagents were purchased from PAA Laboratories Ltd. (Yeovil, UK). TE 671 cells were obtained from the European Collection of Cell Cultures (Porton Down, UK). RNA-Bee was from AMS Biotechnology (Europe) Ltd. (Abingdon, UK). We purchased RNAsecure Reagent from Ambion (Huntingdon, UK), primers from Alta Bioscience (Birmingham, UK), and SYBR Green PCR Master Mix from Applied Biosystems (Cheshire, UK). Agarose and all other RT-PCR reagents were purchased from Bioline (London, UK), and all other chemicals and reagents were from Sigma.

SULT 2A1 activity. We screened 16 putative endocrine-disrupting chemicals for activity against the sulfation of 10 nM DHEA at a saturating concentration or 100 µM, whichever was greater. Human liver cytosol was used as a source of SULT 2A1. Liver tissue was obtained either from fully informed consenting patients undergoing transplantation for end-stage liver disease or from normal donor liver, surplus to surgical requirements. Ethical approval for the use of these tissues for research purposes was granted by South Birmingham Health Authority Local Research Ethics Committee (Reference CA/5192). The sulfation of physiologically relevant concentrations of DHEA was assayed using tritiated DHEA in a buffer of sodium phosphate (20 mM), magnesium acetate (5 mM), and disodium edetate (0.1 mM) at pH 7.0 by a method developed for determining E_2 sulfation, as previously described (Harris et al. 2004). The uninhibited cytosol metabolized < 10% of the DHEA, and the sulfation of DHEA concentrations as low as 0.25 nM could reliably be measured.

Cell culture. Human medulloblastoma-derived TE 671 cells were routinely cultured in Dulbecco's modified Eagle medium (DMEM) with 10% (vol/vol) heat-inactivated fetal calf serum, 2.5 µg/mL amphotericin B, penicillin (100 U/mL)/streptomycin (100 µg/mL) and 2 mM L-glutamine. Cells were incubated at 37°C in 5% carbon dioxide.

For dosing experiments, cells were grown in phenol red–free DMEM, supplemented as before, for 72 hr prior to splitting, counting, and plating the cells in dosing media [phenol red–free DMEM supplemented as before, except with 10% (vol/vol) heat-inactivated dialyzed fetal calf serum]. Cells were counted using a Fuchs-Rosenthal Hemocytometer (Fisher Scientific, Loughborough, UK) and trypan blue (0.4% wt/vol).

TE 671 cells were plated at 4×10^4 cells/well in 24-well cell culture plates for gene expression experiments. The plates were incubated for 24 hr to equilibrate prior to dosing with 0.005–0.5 µM of the EDs. E_2 and DES (0.1–10 nM) were used as positive controls. Dimethyl sulfoxide (DMSO) was used as the solvent carrier.

RT-PCR primers. The gene sequences of interest were obtained from GenBank (2006). We used Oligo (MedProbe, Oslo, Norway) to design oligonucleotide primers for *CDO1* (GenBank accession no. NM_001801), *SUOX* (GenBank accession no. BC065193), *PAPSS1* (GenBank accession no. Y10387), and *HPRT1* (GenBank accession no. M26434) (Table 1). Primer pairs were designed to cross exon–intron boundaries to minimize genomic DNA amplification. We selected hypoxanthine phosphoribosyltransferase 1 (HPRT1) for the internal standard because its expression was relatively low compared with other standards and it is thought to be unaffected by estrogens (Pernas-Alonso et al. 1999).

RNA isolation. Total RNA was extracted using RNA-Bee, following the manufacturer's guidelines; RNA was extracted from TE 671 cells dosed for 24 hr with 0.005–0.5 µM EDs, 0.1–10 nM, E_2 or DES (in quadruplicate for each dose). RNA pellets were resuspended in 25 µL of 1× RNAsecure Reagent and samples were heated at 60°C for 10 min. Aliquots were taken for agarose gel electrophoresis, RNA spectrophotometric quantification, and the reverse transcription (RT) reaction.

RNA quantification. RNA was quantified using the GeneQuant II RNA/DNA calculator (Pharmacia Biotech, Amersham Biosciences UK Ltd., Buckinghamshire, UK) with a capillary cell and quartz capillary tubes. The purity of RNA was determined from the A260/280 ratio and the concentration determined from the absorption at 260 nm.

Real time RT-PCR. RT products were produced by heating 10 µL (1 µg) RNA and 2 µL (0.5 µg) first strand (poly T) primers for 5 min at 70°C. dNTP mix (4 µL of 10 mM, 2.5 mM of each base), 4 µL reaction buffer (5×), and 0.5 µL BIOSCRIPT (MMLV-reverse transcriptase RNase H minus) (200 U/µL) were added and samples incubated for 60 min at 42°C. Reactions were stopped by heating for 10 min at 70°C.

Real-time PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Reaction mixtures (per well) contained: 0.4 µL (10 µM) forward primer; 0.4 µL (10 µM) reverse primer; 10 µL SYBR Green PCR Master Mix; 8.2 µL nuclease-free water, and 1 µL RT product. No-template controls were also prepared. The annealing temperature selected was 60°C, and the program was run for 44 cycles. A dissociation protocol was also run, and the melting curve distinguished any nonspecific product and primer-dimers.

The expression of CDO1, SUOX, and PAPSS1 mRNA was investigated in TE 671 cells treated for 24 hr with EDs as above. The expression of the internal standard HPRT was also assayed for each sample. Relative gene expression levels were obtained using the $\Delta\Delta C_t$ method (Winer et al. 1999). All samples were run in quadruplicate.

Statistical analysis. The results are presented as means ± SE. Statistical significance of the differences between the undosed control groups and test groups were assessed by

Table 1. Primers for real-time RT-PCR.

Primer	Sequence (5′→3′)	No. of base pairs	cDNA size (bp)	Oligo annealing temperature (°C)
HPRT1				
Forward	CCTGCTGGATTACATTAAAGCACTG	25	282	56.1
Reverse	CTTCGTGGGGTCCTTTTCACCAGC	24		63.5
CDO1				
Forward	GGGTGAAGGACATGGCAGCAG	21	360	59
Reverse	AGCGAGCCCGAAGTTGCATTT	21		59.7
SUOX				
Forward	AGCACCCATCCCTCCTACCCC	21	494	59.2
Reverse	TCCTGAGCAGCCCTACACCGA	21		59.2
PAPSS1				
Forward	GATGTATGAGGGCCGCCGTGT	21	667	60.5
Reverse	AGGGGCCATCGTCAGCACTTT	21		59.4

Table 2. K_i values for the inhibition of DHEA sulfation by putative endocrine-disrupting chemicals.

Compound	K_i (µM)
4-*n*-Nonylphenol	10.5 ± 0.6
4-*n*-Octylphenol	13.7 ± 0.2
4-*tert*-Octylphenol	2.8 ± 0.1
Bisphenol A	16.3 ± 0.8
Benzyl butyl phthalate	7.0 ± 0.8
Dibutyl phthalate	21.5 ± 1.4

Values are mean ± SE; $n = 4$. Only results for those compounds causing > 50% inhibition of enzyme activity at 100 µM are shown.

one-way analysis of variance (ANOVA) followed by Dunnet multiple comparisons test, using GraphPad Instat Software (GraphPad Software, Inc., San Diego, CA, USA). ANOVA was also used to test linear trends between column means and column numbers. All values with $p < 0.05$ were considered statistically significant.

Results

SULT 2A1 activity. Of the plasticizers tested, only six caused > 50% inhibition of SULT 2A1 enzyme activity at a concentration of 100 µM; crude IC_{50} (concentration inhibited by 50%) values for these compounds were obtained from a second set of assays. Using these data, we performed duplicate experiments in which the sulfation of DHEA, at concentrations of 2.5, 5, 10, and 20 nM, was measured with the test compounds at nine concentrations ranging from 0 to approximately twice the IC_{50} to determine the nature of the inhibition. We evaluated the results graphically by standard kinetic procedures (Cornish-Bowden 2004). All six compounds were competitive inhibitors, with no evidence of interaction at any allosteric site. The values for the inhibition constants (K_i) are shown in Table 2.

Enzymes of PAPS synthesis. The real-time RT-PCR results were expressed as relative gene expression levels compared with undosed controls using the $\Delta\Delta C_t$ method. Treatment with 0.005–0.5 µM 4-*n*-octylphenol (Figure 1) showed significant dose-dependent decreases in SUOX ($p < 0.02$) and PAPSS1 ($p < 0.001$) steady-state mRNA levels. CDO1 mRNA expression also showed a dose-dependent decreasing trend. Cells treated with 4-*n*-nonylphenol showed a very significant dose-dependent decrease in PAPSS1 mRNA levels ($p < 0.01$).

Bisphenol A dimethacrylate treatment did not affect CDO1, however, SUOX and PAPSS1 ($p < 0.0001$) mRNA expression levels both decreased in a dose-dependent manner. Diisodecyl phthalate treatment (Figure 2) showed extremely significant dose-dependent decreases in CDO1 ($p < 0.01$) and SUOX ($p < 0.001$) mRNA levels, and a clear decreasing trend was also observed for PAPSS1. Treatment with bis(2-ethylhexyl)phthalate (Figure 3) showed dose-dependent decreases in *CDO1, SUOX* and *PAPSS1* ($p < 0.004$) expression levels. Treatment with 2,4-dichlorophenol and 4-chloro-3-methylphenol did not show significant decreases in CDO1, SUOX and PAPSS1 mRNA levels.

Treatment with the other compounds had no effect on CDO1, SUOX and PAPSS1 mRNA expression levels.

Discussion

Chemicals used as plasticizers are released into the environment in large quantities. They are hydrophobic and are able to both bioaccumulate (Van der Oost et al. 2003) and cross the blood–brain barrier (Waterhouse 2003). The results from the present study clearly show that some plasticizers can inhibit SULT 2A1 at micromolar concentrations. Concentrations of the alkylphenols in polluted waters can reach 10–70 nM, and these lipophilic compounds have been found to bioaccumulate 400-fold in estuarine fish (see Kirk et al. 2003). Nevertheless, even in the case of 4-*tert*-octylphenol—the most potent of the inhibitors in this study and one of the most commonly used plasticizing agents—it is unlikely that it reaches micromolar concentrations in the human CNS. However, because the physiologic concentration of DHEA is very much lower than the K_m of the enzyme for this substrate, the Michaelis-Menten equation ($v = VS/[K_m + S]$, where v is the rate of reaction, V is the maximum rate of reaction, S is the substrate concentration, and K_m is the Michaelis constant) simplifies to $v = VS/K_m$. In the presence of a competitive inhibitor, the rate is given by $v = VS/K_m(1 + i/K_i)$, where i is the inhibitor concentration. Hence, even relatively low concentrations of inhibitor will interfere with DHEA sulfation. Long-term accumulation of plasticizers in brain tissue may therefore reduce the efficiency of memory processes as pregnenolone sulfate and DHEAS are required for acetyl choline release and receptor modulation.

From these results, it appears that the presence of a single benzyl or phenyl group and another compact hydrophobic side-chain are both important for tight binding to the active site. Benzyl butyl phthalate is three times more potent than the aliphatic dibutyl phthalate, which is many times more potent than its longer-chained counterparts. Similarly, 4-*tert*-octylphenol, which has a compact, hydrophobic side-chain, binds nearly five times more tightly than its straight-chain isomer. However, although a single aromatic group appears to aid binding, bisphenol A, which has two such moieties, is a less potent inhibitor.

The real-time RT-PCR results also showed significant dose-dependent decreases in CDO1, SUOX, and PAPSS1 mRNA expression levels in TE 671 cells treated for 24 hr with 0.005–0.5 µM 4-*n*-octylphenol, diisodecyl phthalate, or bis(2-ethylhexyl)phthalate. Endocrine-disrupting effects of some plasticizers may therefore also be a consequence of modulation of expression of enzymes supplying PAPS for hormone sulfation. Our results indicate that exposure to 4-*n*-octylphenol, diisodecyl phthalate, or bis(2-ethylhexyl)phthalate may negatively affect the sulfate supply pathway. This could result in reducing PAPS production, giving increased levels of free hormones and decreased capacity for detoxification via sulfate conjugation

Although *in vitro* studies with tissue homogenates and human cell lines cannot necessarily be correlated with the experiences *in vivo*, it is increasingly evident from recent work (Chen et al. 2006) that DHEAS plays an important role in memory function. In addition. animal experiments have shown that chemicals of the type used in the present study inhibit cognitive function (MacLusky et al. 2005), alter synaptic plasticity (Kawato 2004), and affect the acquisition of memory (Carr et al. 2003). Similarly, exposure to polychlorinated biphenyls and brominated fire

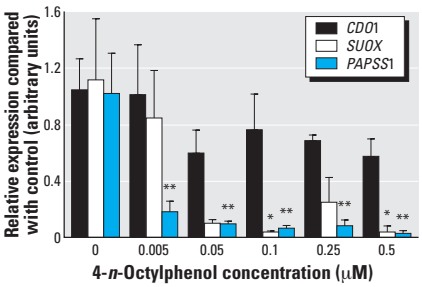

Figure 1. Real-time RT-PCR results for TE 671 cells treated for 24 hr with 4-*n*-octylphenol. Data are means ± SEs ($n = 4$).
*Significantly different from 0 µM control at $p < 0.05$. **Very significant at $p < 0.01$ (ANOVA with Dunnet multiple comparisons test).

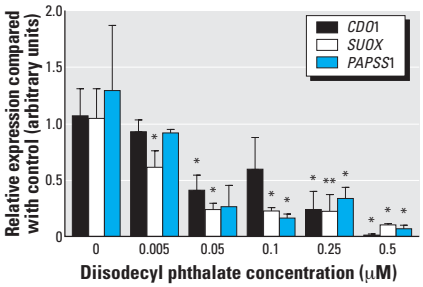

Figure 2. Real-time RT-PCR results for TE 671 cells treated for 24 hr with diisodecyl phthalate. Data are means ± SEs ($n = 4$).
*Significantly different from 0 µM control at $p < 0.05$. **Very significant at $p < 0.01$ (ANOVA with Dunnet multiple comparisons test).

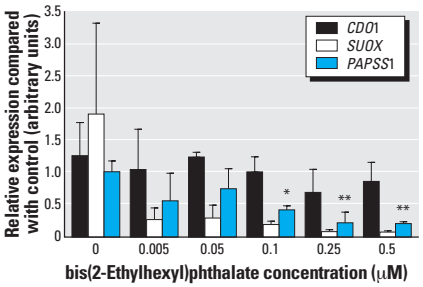

Figure 3. Real-time RT-PCR results for TE 671 cells treated for 24 hr with bis(2-ethylhexyl)phthalate. Data are means ± SEs ($n = 4$).
*Significantly different from 0 µM control at $p < 0.05$. **Very significant at $p < 0.01$ (ANOVA with Dunnet multiple comparisons test).

retardants has also been linked to adverse effects on both memory and learning (Fonnum et al. 2006). Although the genetic mechanisms were not explored in detail, the authors of this article have analyzed the first 4,000 bases of the upstream flanking region of the *PAPSS1* gene using the MatInspector program (Genomatix Software GmbH, Munich, Germany). This revealed two potential estrogen response elements 483–501 and 2916–2934 bases upstream of the transcription start site, and two aryl hydrocarbon receptor response elements 39–61 and 346–368 bases upstream of the transcription start site. In addition, there were other potential nuclear hormone receptor response elements and numerous cAMP response elements. Endocrine disruptors, including these plasticizers, often have weak estrogenic properties, interact with the aryl hydrocarbon receptor (Bonefeld-Jørgensen et al. 2007), and influence a range of cell signaling pathways. The reduction in *PAPSS1* expression could occur via any of these routes.

In conclusion, the results from these experiments suggest that plasticizers may have deleterious effects on physiologic function including neuronal pathways and that, where possible, alternatives should be sought that do not interact with critical metabolic pathways. The information provided by the present study creates novel avenues for hazard identification and risk assessments and has shown that environmental contaminants may interact with nonreproductive steroidal function.

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