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

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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-tet-octylphenol is 2.8 µM). Additionally, after treatment of TE 671 cells with 0.005–0.5 µM 4-tet-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 6 June 2007]

Endocrine disruptors (EDs) have been studied quite extensively in environmental biology (Topperti 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 (Tancre 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 inappropriate 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 (Vallette et al. 2001), probably because these neurosteroids modulate acetylcholine release and y-aminojuglyric 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 inde- pendently 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 hydroxy- steroids, including pregnenolone, but can also sulfate various xenosterogens and drugs. SULT 2A1 is unusual in that, although it has a K_I 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 physiological 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.”

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We thank members of the Queen Elizabeth Hospital Clinical Transplant Unit and The Liver Research Laboratories for assistance with collection of liver tissue.

This study was supported by the European Commission’s Quality of Life Programme, Key Action Environment and Health, contract QLK4-CT-2002-02637.

The authors declare they have no competing financial interests.

Received 22 May 2006; accepted 23 October 2006.
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 Haén 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 (E2), and diethylstilbestrol (DES) from Sigma (Poole, UK).

All cell culture reagents were purchased from PAA Laboratories Ltd. (Yevoyl, UK). TE 671 cells were obtained from the European Collection of Cell Cultures (Porton Down, UK). RNA-Be 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 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 transection 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 sodium edetate (0.1 mM) at pH 7.0 by a method developed for determining E2 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 Hemoctometer (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 E2 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-Be, 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 E2, or DES (in quadruplicate for each dose). RNA pellets were resuspended in 25 µL of 1× RNA Secure 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 ΔΔCt 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

| Primer | Sequence (5' → 3') | No. of base pairs | cDNA size (bp) | Oligo annealing temperature (°C) |
|--------|-------------------|-------------------|----------------|---------------------------------|
| HPRT1 Forward | CCTGCTTACATTAACAAAGCAGT | 25 | 282 | 56.1 |
| HPRT1 Reverse | CCTGCTTACCATTCITCACCAGC | 24 | 63.5 |
| CDO1 Forward | CAGGAAGGGCCAGATGCAAGCAG | 21 | 360 | 59 |
| CDO1 Reverse | AGGGACCCGAAATTGACATT | 21 | 59.7 |
| SUOX Forward | AGCCACACATCCTCCCTCACCCC | 21 | 494 | 58.2 |
| SUOX Reverse | TCCTGAGCAGCTCCACACCA | 21 | 59.2 |
| PAPSS1 Forward | GAATGTTAGAGGGCGCCCGCTGT | 21 | 667 | 60.5 |
| PAPSS1 Reverse | AGGGGCGATTGCTGACAGCATT | 21 | 59.4 |
one-way analysis of variance (ANOVA) followed by Dunnet multiple comparisons test, using GraphPad Instar 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) 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-µ-octylphenol (Figure 1) showed significant dose-dependent decreases in SUOX ($p < 0.01$) and CDO1 ($p < 0.01$) 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-µ-tetraoctylphenol—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 $K_{i}$ of the enzyme for this substrate, the Michaelis-Menten equation ($v = V_{\text{max}} [S]/K_{\text{m}} + [S]$) simplifies to $v = V_{\text{max}} K_{i}/K_{\text{m}}$. In the presence of a competitive inhibitor, the rate is given by $v = V_{\text{max}} (1 + i/K_{i})$, 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-µ-tetraoctylphenol, 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-µ-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-µ-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...
retardants has also been linked to adverse effects on both memory and learning (Fonnun et al. 2006). Although the genetic mechanisms were not analyzed 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 steroid function.

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