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Chlorinated Biphenyl Quinones and Phenyl-2,5-benzoquinone Differentially Modify the Catalytic Activity of Human Hydroxysteroid Sulfotransferase hSULT2A1

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Abstract

Human hydroxysteroid sulfotransferase (hSULT2A1) catalyzes the sulfation of a broad range of environmental chemicals, drugs, and other xenobiotics in addition to endogenous compounds that include hydroxysteroids and bile acids. Polychlorinated biphenyls (PCBs) are persistent environmental contaminants, and oxidized metabolites of PCBs may play significant roles in the etiology of their adverse health effects. Quinones derived from oxidative metabolism of PCBs (PCB-quinones) react with nucleophilic sites in proteins and also undergo redox cycling to generate reactive oxygen species. This, along with the sensitivity of hSULT2A1 to oxidative modification at cysteine residues led us to hypothesize that electrophilic PCB-quinones react with hSULT2A1 to alter its catalytic function. Thus, we examined the effects of four phenylbenzoquinones on the ability of hSULT2A1 to catalyze the sulfation of the endogenous substrate, dehydroepiandrosterone (DHEA). The quinones studied were 2'-chlorophenyl-2,5-benzoquinone (2'-Cl-BQ), 4'-chlorophenyl-2,5-benzoquinone (4'-Cl-BQ), 4'-chlorophenyl-3,6-dichloro-2,5-benzoquinone (3,6,4'-triCl-BQ), and phenyl-2,5-benzoquinone (PBQ). At all concentrations examined, pretreatment of hSULT2A1 with the PCB-quinones decreased catalytic activity of hSULT2A1. Pretreatment with low concentrations of PBQ, however, increased the catalytic activity of the enzyme, while higher concentrations inhibited catalysis. A decrease in substrate inhibition with DHEA was seen following preincubation of hSULT2A1 with all of the quinones. Proteolytic digestion of the enzyme followed by LC/MS analysis indicated PCB-quinone- and PBQ-adducts at Cys55 and Cys199, as well as oxidation products at methionines in the protein. Equilibrium binding experiments and molecular modeling suggested that changes due to these modifications may affect the nucleotide binding site and the entrance to the sulfuryl acceptor binding site of hSULT2A1.

Keywords

Sulfotransferase; SULT2A1; Electrophiles; PCB; Quinones; Adducts; Oxidation

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Introduction

Polychlorinated biphenyls (PCBs) constitute a family of industrial chemicals that were widely employed in transformers, hydraulic fluids, lubricating oils, coolants, plasticizers, building materials and other uses during the mid-twentieth century.¹ Although production of these chemicals was banned in the late 1970s, their presence in the environment^{2–5} and their adverse health effects⁶ persist. In addition to those PCBs that are derived from previous uses (sometimes referred to as “legacy” PCBs), it is increasingly apparent that new sources of PCBs (*i.e.*, “nonlegacy” PCBs) are unintended byproducts of manufacturing processes.^{3,7}

While many of the PCB congeners that contain higher numbers of chlorine atoms are relatively resistant to biodegradation, PCBs with lower numbers of chlorine atoms (*e.g.*, 5 or less) are more readily metabolized. Such metabolites can be involved in a variety of toxic responses including carcinogenesis, endocrine disruption, and others.^{8–15} Mammalian metabolism of the lower chlorinated PCBs is often initiated by oxidative biotransformations catalyzed by cytochrome P450 enzymes, and these reactions can ultimately yield phenol and quinone metabolites. Phenolic metabolites of PCBs, known as hydroxylated PCBs (OHPCBs), are subject either to further oxidative reactions or to conjugation reactions such as those catalyzed by cytosolic sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs).⁸ Metabolites of PCBs bearing a quinone functionality (PCB-quinones) have been of particular interest due to their reactivity with cellular macromolecules^{16–20} as well as their ability to generate reactive oxygen species.²¹ Such events have been linked to the initiation of carcinogenesis seen with some of the lower chlorinated PCBs.¹¹ Although the reactivity of PCB-quinones has often limited their detection *in vivo*, protein adducts derived from quinone metabolites of 2,2',5,5' tetrachlorobiphenyl have been identified in the livers and brains of rats treated with this lower chlorinated PCB.¹⁸

Mammalian sulfation of xenobiotics, such as OHPCBs, as well as endogenous chemicals like hormones, neurotransmitters, and bile acids, is catalyzed by enzymes belonging to a superfamily of cytosolic sulfotransferases (SULTs).^{22–26} While sulfation reactions are often considered a detoxication process, they can also lead to the formation of electrophilic cations that are capable of forming irreversible adducts with nucleophilic sites on DNA or proteins.^{27–31} Additionally, it has recently been shown that sulfation of some lower chlorinated OHPCBs leads to products that reversibly bind with high affinity to the thyroid hormone transport protein, transthyretin.¹⁵

Among the SULTs, human hydroxysteroid sulfotransferase (hSULT2A1) is responsible for catalyzing the sulfation of many biological hydroxysteroids, like dehydroepiandrosterone (DHEA), androgens, pregnenolone, and bile acids.^{32–34} This enzyme also catalyzes the sulfation of many varied xenobiotics,^{23,35–38} of which several OHPCBs have been previously noted.^{39,40} Since the catalytic activity of hSULT2A1 is affected by formation of disulfide bonds at key cysteine residues,⁴¹ we have hypothesized that PCB-quinones may alter catalytic function of the enzyme by reaction with these cysteines. Such changes in catalytic efficiency of the enzyme might then affect both its physiological functions in metabolism of steroid hormones and bile acids as well as its role in xenobiotic sulfation.

Experimental Procedures

Chemicals

Adenosine 3',5'-diphosphate sodium salt (PAP), methylene blue, dehydroepiandrosterone (DHEA), 2-mercaptoethanol, and 4-vinylpyridine were purchased from Sigma-Aldrich (St. Louis, MO). Adenosine 3'-phosphate, 5'-phosphosulfate (PAPS) was purchased from Sigma-Aldrich and further purified according to a previously described procedure⁴² to

obtain at least 98% purity (determined by HPLC analysis). Modified trypsin and Glu-C (both sequencing grade) were from Promega (Madison, WI). SDS-PAGE was carried out with 12% precast gels purchased from Bio-Rad Labs (Hercules, CA). [³H]-Dehydroepiandrosterone (94.5 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). 8-Anilidonaphthalene-1-sulfonic acid ammonium salt (ANS) was purchased from Fluka (Steinheim, Germany). 2'-Chlorophenyl-2,5-benzoquinone (2'-Cl-BQ), 4'-chlorophenyl-2,5-benzoquinone (4'-Cl-BQ), and 4'-chlorobiphenyl-3,6-dichloro-2,5-benzoquinone (3,6,4'-triCl-BQ) were synthesized and characterized as previously described.^{43,44} Phenyl-2,5-benzoquinone (PBQ) was purchased from MP biomedical (Solon, OH) and recrystallized from ethanol prior to use. The chemical structures and abbreviated names of all quinones used are seen in Figure 1. An additional note on nomenclature is that two of the compounds studied are quinones derived from monochlorinated biphenyls. Since the monochlorinated biphenyls corresponding to these quinones are systematically named as PCB congeners (i.e., PCB 1 and PCB 3),^{45,46} we refer to them as PCB-quinones. All other chemicals used were of the highest purity commercially available.

Expression and purification of recombinant hSULT2A1

Human SULT2A1 was expressed in *Escherichia coli* BL21 (DE3) cells,⁴⁷ and recombinant hSULT2A1 was obtained from the lysed *E. coli* cells through extraction and purification as previously described.⁴⁸ Homogeneity of the purified protein was determined by SDS-PAGE with Coomassie brilliant blue staining. A single protein band with a 34 kDa relative molecular mass was observed, and this was consistent with the previously reported subunit mass of hSULT2A1.⁴⁹ Protein content was determined using the modified Lowry procedure⁵⁰ with bovine serum albumin as standard.

Pretreatment of hSULT2A1 with PCB-quinones

Prior to incubation of hSULT2A1 with various quinones, dithiothreitol (DTT) that remained from the purification of hSULT2A1 was removed by chromatography using a PD-10 column (1.45 × 5.0 cm; GE Healthcare, Pittsburgh, PA) as described previously.⁴¹ Removal of DTT to a concentration less than 0.01 mM was then verified by a standard assay for determination of thiols.⁵¹ Following the removal of DTT, hSULT2A1 was incubated for 1 hour at 25 °C with the indicated concentrations of PCB-quinones or PBQ in the same buffer solution that had been used for chromatography on the PD-10 column (i.e., 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 10 % (v/v) glycerol, and 0.05 % (v/v) Tween 20). Prior to addition to this incubation mixture, PCB quinones or PBQ were dissolved in absolute ethanol. In order to reduce oxygen concentration, argon was added to the buffer solutions before these incubations, and reactions of hSULT2A1 with the quinones were conducted in sealed tubes with an argon atmosphere above the solution. Aliquots of hSULT2A1 that had undergone reaction with a specific quinone in this procedure were then utilized in the kinetic studies and ligand-binding experiments described below. Concentrations of enzyme, quinone, and appropriate substrates or products are provided within the descriptions of the individual experiments.

Kinetic studies on the sulfation of DHEA catalyzed by hSULT2A1

Assays to determine the rate of sulfation of DHEA catalyzed by hSULT2A1 following pretreatment of the enzyme with PCB-quinones (0.45 μM of hSULT2A1 in the preincubation step) were carried out in a total volume of 0.2 mL of 0.25 M potassium phosphate at pH 7.4 containing 0.2 mM PAPS and the indicated concentration of a mixture of [³H]-DHEA and unlabeled DHEA. This solution was incubated at 37 °C for 2 min prior to addition of 30 ng (in a 2 μL volume) of either control or quinone-treated hSULT2A1 that resulted from the pretreatment at 1 h and 25 °C as described above. The complete assay

mixture was then incubated at 37 °C for either 3 or 4 min, with the reaction time adjusted to maintain initial velocity conditions. The enzymatic reaction was terminated by the addition of 0.8 mL of 50 mM potassium hydroxide, and quantitation of the DHEA-sulfate formed in the reaction was carried out as previously described.⁵² Three replicates were determined at each concentration of DHEA, and the kinetic constants were determined by nonlinear curve-fitting using SigmaPlot 11.0 (Systat Software, Chicago, IL). These kinetic constants, $\pm$ standard error of the fit, were determined with either the Michaelis-Menten equation or the substrate inhibition equation seen below, where v is the initial velocity of the reaction, $V_{\max}$ is the maximal velocity, K_m is the Michaelis constant, $[S]$ is the substrate (DHEA) concentration, and K_i is the substrate inhibition constant.

$$v = V_{\max} / (1 + (K_m/[S]) + ([S]/K_i))$$

Inhibition of hSULT2A1 activity by PCB-quinones in the absence of pre-incubation

Assays to determine whether the inhibition of hSULT2A1 was dependent upon pre-incubation of the PCB-quinone with the enzyme were carried out using homogeneous hSULT2A1 that had been prepared as above for removal of exogenous thiols. Individual PCB-quinones (final concentration of 50 nM) were added to a standard 0.2 mL assay mixture for sulfation of 0.5 μ M DHEA (described above), and the reaction was initiated by addition of 30 ng (in a 2 μ L volume) of hSULT2A1. The mean $\pm$ standard deviation of three replicates was determined for each assay.

Studies on the binding of DHEA to hSULT2A1

The binding of DHEA to either the unmodified hSULT2A1 or the enzyme modified by pretreatment with PCB-quinones was determined using the fluorescent probe ANS. These studies were conducted in 0.25 M potassium phosphate buffer, pH 7.4, with a 200 μ M final concentration of ANS and 2 μ g of either PCB-quinone pretreated or untreated hSULT2A1 (*i.e.*, an enzyme concentration of 12 nM based on the M_r of dimeric hSULT2A1) in 1.0 mL total volume. The solution was placed in a quartz cuvette with 1.0 cm excitation path length and 0.4 cm emission path length. The mixture was then incubated at 37 °C in a PerkinElmer LS55 luminescence spectrometer (PerkinElmer, Inc., Waltham, MA) for 2 min prior to titration. After each addition of DHEA, the solution was mixed well, and a 10-s incubation time was allowed before taking the reading. Fluorescence emission was measured with an excitation wavelength of 410 nm and an emission wavelength of 480 nm. The entrance and the exit slits were both set at 5 nm, and the shutters of the fluorimeter were closed between measurements. All solutions were filtered with a Millex-GS 0.22 μ m filter (Millipore, Billerica, MA) prior to use. Dilution factors were applied for each addition of DHEA, and each determination was carried out in duplicate. The means and standard errors of K_d values were calculated by fitting the data to a two-site binding equation corrected for non-specific binding (SigmaPlot 11.0, Systat Software, Chicago, IL).

Studies on the binding of PAP to hSULT2A1

The effects of quinone-treatment on the binding of PAP to hSULT2A1 were determined by monitoring the change in intrinsic fluorescence of the protein at 37 °C in 0.25 M potassium phosphate buffer, pH 7.4, with an excitation wavelength of 290 nm and an emission wavelength of 347 nm. The entrance and the exit slits were set at 5 nm and 7 nm, respectively, and the shutters of the fluorimeter were closed between measurements. Either pretreated or untreated hSULT2A1 was added (15 nM final concentration based on the M_r of the dimeric protein) together with DHEA (0 μ M, 0.5 μ M, or 50 μ M final concentration) into the potassium phosphate buffer in a quartz cuvette 20 min prior to the titration.

Titration was carried out by addition of aliquots of a solution of PAP to each the mixture to reach final concentrations of PAP ranging from 0.5 μ M to 300 μ M. After each addition of PAP, the solution was mixed and incubated in the chamber for 10 s before determining the fluorescence. A dilution factor for each addition of PAP solution was applied to calculate the change in fluorescence, and each determination was carried out in duplicate. All solutions used in this assay were filtered with a Millex-GS 0.22 μ m filter prior to the experiment. The means and standard errors of K_d values were calculated by fitting the data to a two-site binding equation (SigmaPlot 11.0, Systat Software, Chicago, IL).

Locations of structural modification in hSULT2A1 upon reaction with PCB-quinones and PBQ

The structural modification of hSULT2A1 by quinones was detected and characterized by LC/MS. Prior to these experiments, residual DTT in the purified hSULT2A1 was removed via a PD-10 size exclusion chromatography column eluted with 50 mM Tris-HCl buffer, pH 8.0. Aliquots of hSULT2A1, each containing 15 μ g of enzyme, were incubated with various PCB-quinones or PBQ in a 25 μ L volume at 25 $^{\circ}$ C for 1 hour followed by addition of 4-vinylpyridine into the solution (45 mM final concentration of 4-vinylpyridine). Following incubation of this mixture for an additional 1 hour, proteolytic digestion was carried out by adding 0.6 μ g of sequencing grade trypsin in 50 mM Tris-HCl buffer (pH 8.0) and acetonitrile (final 10% v/v) in a total volume of 0.1 mL, and incubating at 37 $^{\circ}$ C for 16 h. In those cases where a secondary digestion was performed, 1 μ g of sequencing grade Glu-C was added immediately after the 16 h tryptic digestion, and the mixture was incubated at 25 $^{\circ}$ C for another 16 hours. All of the digestions were ended by adding 2 μ L of glacial acetic acid. All samples were subsequently analyzed with a Thermo LCQ Deca quadrupole ion trap mass spectrometer (ThermoElectron, San Jose, CA) interfaced with a Dionex LC (Dionex, Sunnyvale, CA). Chromatographic separation was carried out with a Supelco Discovery Bio Wide Pore C18 (2.1 $\times$ 150 mm) column (Sigma-Aldrich, St. Louis, MO) using a 10 μ L injection volume. The LC mobile phase was 0.1% (v/v) formic acid in water (A) and acetonitrile with 0.1% (v/v) formic acid (B). The initial gradient conditions were 5% B held for 2 min, and then the solvent composition was increased linearly to 40% B over 80 minutes at a flow rate of 200 μ L/min. MS data were collected using positive electrospray ionization over the mass range of 300–2000.

Visualization of locations of structural modification in hSULT2A1

Examination of the relationships between the modifications detected by LC/MS and the structure of hSULT2A1 was accomplished using Sybyl X (Tripos; St. Louis, MO). The X-ray structure of hSULT2A1 with PAP bound⁵³ was obtained from the Protein Data Bank (PDB file: 1EFH). The structure of hSULT2A1 with DHEA bound⁵⁴ was obtained from PDB file 1J99. Locations of the modified residues are depicted using PyMol (v. 1.5.0.4; Schrödinger, LLC, New York, NY).

Statistical Analysis

Assays of the rates of sulfation catalyzed by hSULT2A1, either with untreated enzyme or with enzyme that had been pretreated with a quinone, were conducted in triplicate, and data are reported as the mean $\pm$ standard deviation. The results were analyzed by a paired two-tailed t-test, and a value of $p < 0.05$ defined statistical significance. Kinetic constants were determined by nonlinear regression fit either to the Michaelis-Menten equation or to the substrate inhibition equation described above. Equilibrium dissociation constants for ligands (K_d values) were obtained by fitting duplicate determinations of binding at varied ligand concentrations to a two-site binding equation (SigmaPlot 11.0, Systat Software, Chicago, IL). Kinetic constants and K_d values are reported $\pm$ the standard error of the fit, and

statistical significance at $p < 0.05$ was determined by unpaired t-test of the best-fit values and standard errors.

Results

Catalytic regulation of hSULT2A1 by PCB-quinones and PBQ

In order to determine the effects of these quinones on the catalytic function of hSULT2A1, increasing concentrations of PCB-quinones or PBQ were incubated with hSULT2A1 before an aliquot was subjected to determination of its catalytic activity. Following 100-fold dilution of the enzyme into a standard assay, the catalytic activities of either pretreated or untreated hSULT2A1 were determined using $0.5 \mu\text{M}$ ^3H -DHEA as substrate. A concentration-dependent reduction in the catalytic activity of hSULT2A1 was observed in all cases where the enzyme was pretreated with PCB-quinones (Figure 2). Among the three PCB-quinones examined in this study, 3,6,4'-triCl-BQ was the most potent in decreasing the catalytic activity of hSULT2A1.

Based on previous observations of the inhibitory effects of hydroxylated PCBs on hSULT2A1,³⁹ we examined the possibility that the diluted PCB-quinones were direct, reversible inhibitors of the enzyme, without a need for preincubation to allow irreversible modification. Since a 100-fold dilution of the highest concentration of PCB-quinone utilized in the preincubations would yield a 50 nM concentration of the quinone in the final assay, 50 nM of each PCB-quinone was added to a standard assay for sulfation of $0.5 \mu\text{M}$ DHEA catalyzed by untreated hSULT2A1. The reaction was initiated by addition of the enzyme, and the result showed that, in the presence of either 2'-Cl-BQ or 4'-Cl-BQ at 50 nM, there was no significant decrease in the rate of sulfation catalyzed by hSULT2A1. For the assay with 50 nM 3,6,4'-triCl-BQ, there was a small decrease in the catalytic activity of hSULT2A1. Nonetheless, this observed decrease only accounted for a small part of the total loss of enzyme activity that occurred with hSULT2A1 that had undergone reaction with 3,6,4'-triCl-BQ prior to dilution (Figure 3).

PBQ was utilized to explore the extent to which chlorination of a biphenyl quinone determines effects on the catalytic function of hSULT2A1. Indeed, the effects of PBQ were markedly different from those observed with the three PCB-quinones. Preincubation of hSULT2A1 with concentrations of PBQ of either $0.5 \mu\text{M}$ or $1.0 \mu\text{M}$ caused an increase in the catalytic activity of hSULT2A1 upon dilution into a standard assay for sulfation of DHEA, while reaction of the enzyme with $5.0 \mu\text{M}$ or higher concentrations of PBQ yielded decreases in catalytic activity (Figure 4).

The kinetic characteristics of DHEA-sulfation catalyzed by hSULT2A1 revealed fundamental differences in catalysis depending upon whether or not the enzyme had been modified by reaction with PBQ or one of the PCB-quinones. The substrate inhibition of unmodified hSULT2A1 that is seen with increasing concentrations of DHEA is well known.^{49,52} After pretreatment with either PBQ or each of the three PCB-quinones, the modified enzyme displayed changes in substrate inhibition characteristics in all cases except 4'-Cl-BQ. Indeed, no substrate inhibition was observed for the sulfation of DHEA catalyzed by hSULT2A1 that had been modified by reaction with 2'-Cl-BQ, 3,6,4'-triCl-BQ, or PBQ (Table 1).

Effects of PCB-quinones and PBQ on the binding of DHEA and PAP to hSULT2A1

The affinities for the binding of substrates and products with hSULT2A1 play important roles in the regulation of the catalytic activity as well as in the substrate inhibition characteristics of the enzyme. Thus, in addition to determining changes in the substrate inhibition characteristics of hSULT2A1 upon reaction with PCB-quinones and PBQ, we also

examined the potential for reaction with these quinones to alter binding of the substrate DHEA as well as the product PAP to the enzyme.

Equilibrium dissociation constants were determined for the interaction of DHEA with hSULT2A1 that had been modified by reaction with PCB-quinones or PBQ. This was determined by displacement of the fluorescent probe ANS by titration with increasing concentrations of DHEA. The percentage change in fluorescence intensity throughout the titration with DHEA was fit to a two-site binding model, and the resulting equilibrium dissociation constants are summarized in Table 2. With the exception of a decrease in K_{d1} for 3,6,4'-triCl-BQ, there were no significant changes in the binding of DHEA to hSULT2A1 that had been pretreated with PCB-quinones or PBQ.

Previous studies have revealed the involvement of PAP and DHEA in the formation of ternary dead-end complexes that are important in substrate inhibition seen with sulfotransferases.^{52,55–58} The binding of PAP to the unmodified hSULT2A1 as well as the enzyme that had been modified by reaction with each quinone was determined by monitoring the intrinsic fluorescence of the protein in response to titration with increasing concentrations of PAP. Significant changes in the binding of PAP were observed for the enzyme preparations that were pretreated with PCB-quinones (Table 3). Following pretreatment with either 2'-Cl-BQ or 4'-Cl-BQ, there was no detectable binding of PAP to the enzyme in the absence of DHEA. 3,6,4'-triCl-BQ-pretreatment led to a significant decrease in both K_{d1} and K_{d2} for PAP-binding. For hSULT2A1 pretreated with 10 μ M PBQ, there was no significant change in the binding of PAP to the enzyme in the absence of DHEA. When the enzyme was allowed to react with 1.0 μ M PBQ, however, there was a significant change in the dissociation constant K_{d1} for PAP determined in the absence of DHEA (Table 4).

Since previous studies had indicated that the presence of DHEA can affect the binding of PAP to untreated hSULT2A1,⁵² we examined the binding of PAP following PCB-quinone reaction of the enzyme carried out in the presence of either 0.5 μ M or 50 μ M DHEA. With the exception of 3,6,4'-triCl-BQ-pretreated hSULT2A1, there were significant differences in the effect of reaction of the enzyme with these PCB-quinones in the presence or absence of DHEA (Table 3).

Structural modifications of hSULT2A1 identified by LC/MS

While changes in the binding of DHEA and PAP to hSULT2A1, as well as altered kinetic characteristics, were seen following reaction of the enzyme with PCB-quinones and PBQ, these results did not provide specific information on the potential structural changes in the protein that governed those effects. In order to investigate the specific alterations in amino acids resulting from reaction with PBQ and the three PCB-quinones, 15 μ M hSULT2A1 was incubated with 250 μ M quinone (either PBQ or each of the three PCB-quinones) for 1 h in 50 mM Tris-HCl buffer, pH 8.0, at 25 °C. In order to facilitate the proteolytic digestion and LC/MS analysis, a higher concentration of hSULT2A1 was used, however, the molar ratio of quinone to protein in these pre-incubations (i.e., 17:1) was within the range of molar ratios used in the pre-incubation of the enzyme with quinones that was employed for the studies on enzyme kinetics. Following reaction with quinone, the hSULT2A1 was subjected to proteolytic digestion and analysis by LC/MS.

As seen in Table 5, protein adducts were observed at Cys55 and Cys199 with 2'-Cl-BQ, 4'-Cl-BQ and PBQ. These adducts retained the original chlorine atom, thus suggesting that Michael-type additions to form substituted hydroquinones had occurred. Additionally, oxidation products of the adducted hydroquinones were formed producing the quinone adducts with 2'-Cl-BQ- and 4'-Cl-BQ-pretreatment. For the 3,6,4'-triCl-BQ-pretreated

hSULT2A1, a dichlorinated adduct was found in the digested peptides containing Cys55 and Cys199, indicating a chlorine-displacement reaction. In addition, singly and doubly charged ions with m/z corresponding to the quinone adduct – 2 were also observed with all pretreatments, which indicated a possible covalent bond formation between the adducted quinone ring and the terminal lysine residue on the peptide. PCB-quinone adducts at Cys154 were not detected in these studies (Table 5).

In addition to the cysteine adducts, several methionines (*i.e.*, Met223, Met 228 and Met 137) were found to be oxidized to methionine sulfoxides following treatment with these quinones (Table 5). The positions of the modified cysteine and methionine residues in relation to the crystal structures of hSULT2A1 with either PAP or DHEA bound are illustrated in Figure 5.

Discussion

Quinones are well known as a class of biologically active electrophiles that can undergo redox cycling to cause oxidative stress and also react with nucleophilic sites on DNA, RNA and proteins to elicit toxic responses.⁵⁹ Reduced cysteine residues in proteins, as well as non-protein thiols, are significant cellular targets for many quinones,^{60,61} although reactivity at lysine, histidine, and other residues in proteins also occurs. Indeed, lysine-rich motifs have been identified in the formation of quinone-adducts with some proteins.^{62,63} Quinone metabolites of PCBs have been shown to form protein adducts, and this process has been linked to toxic responses.^{16–18,20} For our initial studies on the reaction of lower chlorinated PCB-quinones with hSULT2A1, we chose two mono-chlorinated congeners, each with a chlorine atom in the non-benzoquinone ring, a tri-chlorinated congener with two chlorine atoms in the benzoquinone ring, and the non-chlorinated PBQ for comparison. Moreover, the parent PCBs from which the three chlorinated biphenyl quinones are derived have been previously identified in urban air samples.^{4,64}

Previous studies on cytosolic sulfotransferases have clearly indicated that modification of key cysteine residues by formation of disulfide bonds can modulate the catalytic function of these enzymes.^{41,55,57,65–68} While most of these SULTs have been members of family 1, we have recently reported that the catalytic activity of human hydroxysteroid sulfotransferase hSULT2A1 is also sensitive to formation of disulfide bonds at key cysteine residues.⁴¹ Alteration of the structure and function of hSULT2A1 through oxidative post-translational changes at cysteine residues led us to hypothesize that changes in catalytic function may also result from reaction of the enzyme with electrophiles that form adducts at those cysteine residues. Such adduct formation would likely alter sulfation with concomitant effects on the metabolism of xenobiotics as well as endogenous molecules. Our interest in the role of sulfation in the metabolism of PCBs with lower numbers of chlorine atoms led us to investigate the potential for quinone metabolites of these PCBs to alter sulfation reactions catalyzed by hSULT2A1. Such sulfation reactions could range from key metabolic reactions in hydroxysteroid and bile acid metabolism to the detoxication or, in some cases, bioactivation of xenobiotics containing alcohol, phenol, or amine functional groups.

Investigations of the effects of oxidative modification of cysteine residues in hSULT2A1 have shown decreases in catalytic activity.⁴¹ Indeed, our current results on the modification of hSULT2A1 with three PCB-quinones also showed consistent decreases in catalytic activity. There was, however, an increase in the catalytic activity at some concentrations of the non-chlorinated biphenyl quinone, PBQ. While increases in catalytic function have been previously observed in rSULT1A1,^{55,57,66,68} a major family 1 SULT in the rat, our current results with low concentrations of PBQ are the first indication that such an increase in catalytic activity may occur following modification of cysteines in a human family 2 SULT.

Thus, reaction of hSULT2A1 with electrophilic quinones can either increase or decrease the catalytic activity of the enzyme depending on the structure and concentration of the quinone.

Previous investigations of the mechanism for regulation of rSULT1A1 by formation of disulfide bonds identified alterations near the PAPS/PAP binding site of the enzyme as critical for increases and decreases in the catalytic function.^{55,57,68} In particular, Cys 66 was identified as a key residue in oxidative regulation of rSULT1A1.⁶⁸ Mutation of this cysteine to serine resulted in an enzyme that retained catalytic activity, but lost the ability to be regulated by disulfide bond formation.⁶⁸ Molecular modeling studies of rSULT1A1 have shown that cysteine 66 is near, but not within, the PAPS/PAP binding site of the enzyme, and formation of a protein-glutathione disulfide bond at Cys 66 results in subtle structural changes that alter binding of PAP.⁵⁵ More recently, this oxidative regulation of rSULT1A1 has also been observed within intact hepatic tissue slices.⁶⁵

As a probe for changes in the nucleotide-binding site in hSULT2A1 following reaction with quinones, we examined alterations in the ability of hSULT2A1 to bind PAP. With PBQ, pretreatment of the enzyme at a concentration of 1 μ M caused a decreased affinity for binding of PAP (*i.e.*, an increase in K_d value), while a higher concentration of 10 μ M yielded no significant difference in binding of PAP when compared to the untreated control. hSULT2A1 can form dead-end inhibitory complexes of the enzyme, acceptor substrate, and PAP,⁵² and these may be viewed as analogous to the ternary complexes formed in rSULT1A1. A decrease in binding affinity for PAP might therefore weaken this dead-end complex and, up to a point, accelerate the rate of reaction. Additional changes in protein structure occurring at higher concentrations of quinone, however, would decrease the overall rate of reaction. While the data in Table 4 are consistent with this hypothesis, more extensive experimentation will be required to fully understand the structural and functional changes in hSULT2A1 that are occurring to yield this biphasic response to PBQ.

Another manifestation of alterations in the structure of the hSULT2A1 by reaction with quinones lies in changes in substrate inhibition that are seen when compared with the sulfation of DHEA by the unmodified enzyme. Substrate inhibition was no longer observed for DHEA sulfation following pretreatment of the enzyme with 2'-Cl-BQ, 3,6,4'-triCl-BQ, or PBQ, and the K_i value was significantly increased (*i.e.*, less substrate inhibition) following reaction with 4'-Cl-BQ. Moreover, there are structural changes occurring subsequent to the binding of nucleotide that affect the binding of DHEA and other substrates.^{69,70} Our results indicate that the reaction of hSULT2A1 with quinones also caused complex changes in the binding of nucleotide in the presence or absence of DHEA. For example, reaction with either of the two mono-chlorinated quinones (*i.e.*, 2'-Cl-BQ and 4'-Cl-BQ) caused an inability of the enzyme to bind PAP in the absence of DHEA, however, the conformational change resulting from binding of DHEA to the modified enzyme allowed binding of the nucleotide.

In contrast to the other quinones, pretreatment of hSULT2A1 with 3,6,4'-triCl-BQ resulted in an enzyme with lower K_d values for PAP whether in the presence or absence of DHEA. This indicated that there are different mechanisms operating for different congeners of PCB-quinones. Indeed, when the kinetic assays for sulfation of DHEA are considered, reaction with 3,6,4'-triCl-BQ caused a decrease in both the V_{max} as well as K_m values, and this was in contrast to results obtained following pretreatment of hSULT2A1 with either 2'-Cl-BQ or 4'-Cl-BQ. As noted below, these differences between the mono-chlorinated and the tri-chlorinated quinones in nucleotide-binding and reaction kinetics may be related to structural differences arising from differences in the chemical reactivities of the individual quinones. Additionally, there may be steric considerations that differentially alter the extent of conformational changes in the protein that affect the nucleotide-binding site. Such

conformational changes are likely to have subtle but important effects on the stability of inhibitory dead-end complexes that affect the overall rate of catalysis. This interpretation is supported by the fact that the value of k_{cat}/K_m for DHEA as substrate for the enzyme (a measure of the catalytic efficiency of the sulfuryl transfer to DHEA in a catalytically productive complex) is not appreciably changed by reaction of hSULT2A1 with any of the quinones (Table 1).

LC/MS analysis of the adducts formed upon reaction of hSULT2A1 with PBQ and the three PCB-quinones provided additional insight into the mechanisms for alteration of the catalytic function of this enzyme. Adducts were detected at two of the three cysteines in hSULT2A1, Cys55 and Cys199, and these were both in the vicinity of the nucleotide binding site. One of the peptides containing Cys199, ICQFLGK, yielded an adduct with a mass two units lower than that of the expected quinone adduct. This may have been due to the quinone adduct undergoing an intramolecular Michael-type reaction with the terminal amine of the lysine in this heptapeptide. Oxidation of the resulting hydroquinone would provide a quinone adduct of the appropriate mass. Furthermore, since examination of larger peptides containing this amino acid sequence failed to identify similar intramolecular adducts at this site, we propose that this particular peptide-adduct is a result of an intramolecular cyclization reaction by the cysteine adduct that occurred after proteolytic digestion. No other reaction with lysine residues in hSULT2A1 was observed. This, however, was not surprising due to the fact that the sequence of this enzyme does not contain any of the lysine motifs previously identified in other proteins as being reactive with quinones.^{62,63}

Since the cysteines in hSULT2A1 that were found to be reactive with quinones in this study, Cys 55 and Cys 199, are highly conserved within the human SULT2 enzymes,⁷¹ we would expect that other members of the SULT2 family may also exhibit analogous effects upon reaction with PCB-quinones. This will require further examination, as will the possibility that PCB-quinones may react with cysteines in human SULT1 enzymes to alter catalysis. Our current results on the dependence of these catalytic changes on the individual structures of PCB-quinone congeners and, presumably, the nature of specific protein conformational changes that result, indicate that any simple extrapolations to assume completely identical effects in multiple SULTs may be difficult.

Our results also indicate the importance of quinone redox cycling in the modification of hSULT2A1. For both PBQ and 4'-Cl-BQ, peptide adducts included both the hydroquinone and quinone adducts. A Michael-type addition to the quinone would initially yield a hydroquinone adduct; that hydroquinone might then be involved in redox cycling after formation of a semiquinone radical by reaction either with a quinone or other one-electron oxidant. Previous model studies have indicated the formation of semi-quinone radicals when glutathione was reacted with PCB-quinones, with subsequent formation of reactive oxygen species like superoxide radicals ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\text{OH}^{\bullet}$).⁷² Thus, we suspect that reactions of these quinones with cysteine(s) in the enzyme might also result in the formation of reactive oxygen species. Indeed, we observed three oxidized methionines in all cases when the enzyme was treated with quinones. The selectivity of oxidation (*i.e.*, oxidation of other methionines was not observed), suggested that the oxidation was not an artifact of sample preparation either during or after proteolysis. Also, the oxidation of methionine residues was dependent upon the presence of quinones, as earlier experiments on modification of cysteines by disulfide bond formation did not yield methionine sulfoxides.⁴¹ It is interesting to note that two of the three methionines that were oxidized following incubation with these quinones (*i.e.*, Met223 and Met228) are adjacent to and within a region (residues 224–253) that has been recently proposed^{69,70} to form a dynamic active site cap that is important in determining specificity of hSULT2A1 for sulfuryl acceptor substrates. These results from molecular dynamics simulations further

implicate a region (residues 230–238) at the entrance to the DHEA-binding site as important in these conformational changes.⁷⁰ While the methionines modified in our reactions with quinone are near this site, the extent to which they might influence conformational changes that are important for substrate binding remains to be determined. Additionally, it will be necessary to address the potential for complex interactions between structural changes at the cysteine residues due to adduct formation with the quinone and structural changes due to methionine oxidation to be responsible for alterations in catalytic rate for a single substrate.

In summary, quinones derived from oxidative metabolism of PCBs have effects on the catalytic function of hSULT2A1 that are dependent upon the structure of the quinone. The decrease in activity of hSULT2A1 in the sulfation of DHEA seen with all concentrations of these PCB-quinones is in contrast to an increase in catalytic activity of the enzyme seen with low concentrations of the non-chlorinated PBQ. Our results on structural modifications of the enzyme upon reaction with the PCB-quinones and PBQ indicate that future extensive studies on the kinetics and specificity of modification of the enzyme by these and other quinones are warranted. Indeed, our findings on the locations of modification of the protein by these quinones will guide mutagenesis studies to determine the effect of individual amino acid modification on catalytic activity and mechanism. Finally, this is, to our knowledge, the first report that reaction of an electrophilic quinone with a human sulfotransferase can increase catalytic activity of the enzyme. This suggests that, depending upon the structure of the reactive species, electrophilic modification of human sulfotransferases may either increase or decrease metabolic sulfation. Alterations in the sulfation of endogenous substrates such as hydroxysteroids may constitute previously unconsidered toxic consequences of PCB-quinones and other electrophilic quinones. On the other hand, the toxicities of xenobiotics that depend upon sulfation for either detoxication or metabolic activation will also be affected by such modifications of the structure and function of SULTs.

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Abbreviations

ANS	8-anilinonaphthalene-1-sulfonic acid
2'-Cl-BQ	2'-chlorophenyl-2,5-benzoquinone
4'-Cl-BQ	4'-chlorophenyl-2,5-benzoquinone
3,6,4'-triCl-BQ	4'-chlorophenyl-3,6-dichloro-2,5-benzoquinone
DHEA	dehydroepiandrosterone
hSULT2A1	human cytosolic sulfotransferase 2A1
OHPCB	hydroxylated polychlorinated biphenyl
PAP	adenosine 3',5'-diphosphate
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PBQ	phenyl-2,5-benzoquinone
PCB	polychlorinated biphenyl

PCB-quinone	polychlorinated biphenyl bearing a quinone functionality
rSULT1A1	rat cytosolic sulfotransferase 1A1
SULT	cytosolic sulfotransferase
Tris-HCl	[tris(hydroxymethyl)aminomethane] hydrochloride

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Figure 1.
Chemical structures and abbreviated nomenclature of the quinones used.

Figure 2.

Catalytic activity of hSULT2A1 following a 1 hour pretreatment of the enzyme with different concentrations of PCB-quinones and 100-fold dilution into an assay for sulfation of 0.5 μM DHEA. The statistical significance ($p < 0.05$) of changes in the catalytic activity of the PCB-quinone-pretreated group compared to the untreated enzyme is indicated by an *. Values are the means $\pm$ S.D. of 3 determinations.

Figure 3.

Examination of the potential for direct, reversible inhibition of hSULT2A1 by the PCB-quinones. Assays for the sulfation of DHEA were conducted without any pre-treatment of the enzyme with quinone. Each quinone was added at a final concentration of 50 nM, and the assay was initiated by the addition of the enzyme. The statistical significance ($p < 0.05$) of differences in the catalytic activity of the hSULT2A1 in the presence of each PCB-quinone compared to the activity of the enzyme without PCB-quinone is indicated by an *. Values are the means $\pm$ S.D. of 3 determinations.

Figure 4.

The effect of pretreatment of hSULT2A1 with the indicated concentrations of PBQ followed by 100 fold dilution into an assay for sulfation of 0.5 μM DHEA. Statistically significant ($p < 0.05$) differences from the untreated enzyme are noted with an *. Values are the means $\pm$ S.D. of 3 determinations.

Figure 5. Locations of amino acids modified by reaction with PBQ and the three PCB quinones examined in this study. A) PDB structure 1EFH with PAP bound is shown with the positions of the two cysteines where adducts were observed (Cys 55 and Cys 199) and the locations of the three methionines where the formation of sulfoxides was observed. B) PDB structure 1J99 with DHEA bound (two possible orientations of DHEA in the structure) is provided to indicate location of the DHEA-binding site in hSULT2A1 in relation to those amino acids modified by reaction with PBQ and the three PCB quinones.

Summary of kinetic constants for hSULT2A1-catalyzed sulfation of DHEA following pretreatment of the enzyme with PCB-quinones or PBQ^a

Table 1

	hSULT2A1 only	Pretreatment			
		2'-Cl-BQ	4'-Cl-BQ	3,6,4'-triCl-BQ	PBQ
V_{max} (nmol/min/mg)	178±35	349±15 ^b	372±46 ^b	115±2 ^b	239±9.5 ^b
K_m (μM)	1.3±0.4	3.3±0.3 ^b	4.1±0.7 ^b	0.9±0.1	1.8±0.2
K_i (μM)	6.2±2.4	N/A ^b	17.2±5.6	N/A ^b	N/A ^b
K_{cat}/K_m (min ⁻¹ μM ⁻¹)	4.6	3.6	3.1	4.3	4.5

^aThe hSULT2A1 was pretreated, either with or without the indicated quinone (10 μM), at 25 °C for 1 hour. An aliquot of the resulting enzyme was diluted 100-fold by addition to a standard assay mixture to determine the rate of sulfation using 200 μM PAPS and concentrations of DHEA ranging from 0.25 μM to 10 μM. An entry of N/A in the table indicates that no significant substrate inhibition occurred. Values for V_{max} , K_m , and K_i are presented as ± the standard error determined by nonlinear regression fit of kinetic data to either the Michaelis-Menten equation or to the equation for substrate inhibition described in "Experimental Procedures." The values for k_{cat} were calculated from the mean of the corresponding V_{max} using a subunit relative molecular mass of 33,765 for hSULT2A1.

^b K_m , V_{max} or K_i was significantly different ($p<0.05$) from the corresponding kinetic constants for untreated hSULT2A1.

Table 2

Equilibrium dissociation constants for DHEA-binding to hSULT2A1 with and without pretreatment of the enzyme with 10 μ M PCB-quinone or PBQ^a

DHEA binding	hSULT2A1 only	Pretreatment		
		2'-Cl-BQ	4'-Cl-BQ	3,6,4'-triCl-BQ PBQ
K_{d1} (μ M)	0.7 $\pm$ 0.1	0.6 $\pm$ 0.1	0.6 $\pm$ 0.1	0.3 $\pm$ 0.03 ^b 0.5 $\pm$ 0.08
K_{d2} (μ M)	12 $\pm$ 5 μ M	23 $\pm$ 50	20 $\pm$ 10	15 $\pm$ 0.6 23 $\pm$ 14

^a K_d values were obtained by fitting the data to a two-site binding model.

^b K_d value was significantly different ($p < 0.05$) from the corresponding dissociation constant for untreated hSULT2A1.

Table 3

Equilibrium dissociation constants for PAP-binding to either untreated or quinone-pretreated hSULT2A1 in the absence or presence of either 0.5 μ M or 50 μ M DHEA^a

PAP binding	hSULT2A1 only	Pretreatment		
		2'-Cl-BQ	4'-Cl-BQ	3,6,4'-triCl-BQ PBQ
<u>no DHEA</u>				
K_{d1} (μ M)	1.9 $\pm$ 0.2	N/A ^{ab}	N/A	0.9 $\pm$ 0.3 ^b 0.7 $\pm$ 0.9
K_{d2} (μ M)	540 $\pm$ 100	N/A	N/A	105 $\pm$ 12 ^b 452 $\pm$ 206
<u>0.5 μM DHEA</u>				
K_{d1} (μ M)	0.9 $\pm$ 0.2	3.3 $\pm$ 0.6 ^b	4.8 $\pm$ 2.3	1.8 $\pm$ 0.4 ^b 1.3 $\pm$ 0.4
K_{d2} (μ M)	248 $\pm$ 92	407 $\pm$ 81	270 $\pm$ 45	180 $\pm$ 39 268 $\pm$ 72
<u>50 μM DHEA</u>				
K_{d1} (μ M)	1.1 $\pm$ 0.3	1.2 $\pm$ 0.5	2.1 $\pm$ 0.7	1.3 $\pm$ 0.3 5.8 $\pm$ 2.0 ^b
K_{d2} (μ M)	264 $\pm$ 36	N/A	136 $\pm$ 29 ^b	218 $\pm$ 52 553 $\pm$ 237

^a K_{d1} values were obtained by fitting the data to a two-site binding model. N/A indicates that no significant binding of PAP was observed. Pretreatment of the enzyme was carried out at 10 μ M quinone.

^b K_{d1} value was significantly different ($p<0.05$) from the corresponding dissociation constant for untreated hSULT2A1.

Table 4

Equilibrium dissociation constants for PAP-binding to untreated hSULT2A1, 1 μ M PBQ-pretreated hSULT2A1, and 10 μ M PBQ-pretreated hSULT2A1. All determinations of PAP-binding to pretreated hSULT2A1 were conducted in the presence of 0.5 μ M DHEA.

PAP binding	hSULT2A1 only	Pretreatment	
		1 μ M PBQ	10 μ M PBQ
$K_{d1}(\mu\text{M})$	0.9 $\pm$ 0.2	5.7 $\pm$ 1.3 ^a	1.3 $\pm$ 0.4
$K_{d2}(\mu\text{M})$	248 $\pm$ 92	455 $\pm$ 138	269 $\pm$ 73

^a K_d value was significantly different ($p<0.05$) from the corresponding dissociation constant for untreated hSULT2A1.

Table 5

Modification of cysteines and methionines following reaction of hSULT2A1 with PBQ and PCB-quinones.

Modified Cysteine	Peptide Structure	Predicted Mass (M+H) ⁺	Mass found
3,6,4'-triCl-BQ			
Cys55-Di-Cl-BQ	DEDVIILTPKSGTNWLAIEILCLMHKS	3710.7	1237.7 (M+3H) ³⁺
Cys199-Di-Cl-BQ	RTIEKICQFLGKLEPEELNLILK	2924.0	1462.0 (M+2H) ²⁺
Cys199-Di-Cl-BQ	ICQFLGKLEPEELNLILK	2451.2	1225.9 (M+2H) ²⁺ 817.2 (M+3H) ³⁺
Cys199-Di-Cl-BQ ^d	ICQFLG	1056.4	1056.2 (M+H) ⁺ 528.4 (M+2H) ²⁺
2'-Cl-BQ			
Cys55-2'-Cl-BQ	SGTNWLAIEILCLMHKS	2018.9	1010.3 (M+2H) ²⁺ 673.9 (M+3H) ³⁺
Cys199-2'-Cl-BQ ^b	ICQFLGK	1024.4	1024.3 (M+H) ⁺ 512.6 (M+2H) ²⁺
Cys199-2'-Cl-BQ ^{a,b}	ICQFLG	1022.4	1022.3 (M+H) ⁺
4'-Cl-BQ			
Cys55-4'-Cl-HQ	SGTNWLAIEILCLMHKS	2020.9	1011.3 (M+2H) ²⁺ 674.5 (M+3H) ³⁺

Modified Cysteine	Peptide Structure	Predicted Mass (M+H) ⁺	Mass found
Cys199-4'-Cl-BQ ^b		1024.4	1024.3 (M+H) ⁺ 512.6 (M+2H) ²⁺
Cys199-4'-Cl-HQ		2419.2	1210.4 (M+2H) ²⁺ 807.0 (M+3H) ³⁺
Cys199-4'-Cl-BQ ^{a,b}		1022.4	1022.3 (M+H) ⁺ 511.7 (M+2H) ²⁺
PBQ			
Cys55-HQ		1986.9	993.4 (M+2H) ²⁺ 663.0 (M+3H) ³⁺
Cys199-BQ ^{a,b}		988.6	988.4 (M+H) ⁺
All treatments			
Met137 sulfoxide	O-(DVLVSGYFFWKNMK)	1749.9	875.2 (M+2H) ²⁺
Met223 sulfoxide	O-(NSSFQSMK)	944.4	944.4 (M+H) ⁺
Met228 sulfoxide	O-(MSNYLLSVDYVVDK)	1748.8	1748.6 (M+H) ⁺

^aIndicates a mass of the quinone adduct -2.

^bIndicates a peptide resulting from the digestion procedure using both trypsin and Glu-C.