HETEROPTROPIC MODULATION OF SULFOTRANSFERASE 2A1 ACTIVITY BY CELECOXIB: PRODUCT RATIO SWITCHING OF ETHYNYLESTRADIOL SULFATION

Donghui Cui, Catherine L. Booth-Genthe, Edward Carlini, Brian Carr, and Michael L. Schrag

Drug Metabolism Department, Merck Research Laboratories, West Point, Pennsylvania

Received February 17, 2004; accepted July 30, 2004

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

The major sulfated product of 17α-ethynylestradiol (EE) after incubations with 3′-phosphoadenosine-5′-phosphosulfate and recombinant human sulfotransferase 2A1 (SULT2A1), or liver cytosol, is the 3-O-sulfate of EE. However, when celecoxib is also present in the incubation, sulfation is switched (in a concentration-dependent manner) from the 3-O-position to the 17β-O-position of ethynylestradiol. In incubations with recombinant SULT2A1, increasing concentrations of celecoxib decreased the $V_{\text{max}}$ of 3-O-sulfate product formation by 3- to 4-fold, with no major change in the $K_m$ value. For 17β-O-sulfate formation, increasing concentrations of celecoxib resulted in an 8-fold decrease in the $K_m$ and a 7-fold increase in $V_{\text{max}}$. Celecoxib not only modulated the regioselectivity of the enzyme, but also activated the enzyme such that total sulfated product exceeded product formation by the native enzyme, 3- to 4-fold (at 250 μM celecoxib). Finally, $IC_{50}$ values obtained by varying celecoxib concentrations (0–250 μM) at fixed concentrations of EE showed that 3-O-sulfation was inhibited by celecoxib to the same extent, independent of the concentration of EE. In addition, the apparent kinetic constant for celecoxib (as measured by EE 17β-O-sulfation) decreased 2-fold in the presence of high concentrations of EE, consistent with the potential for celecoxib to bind to either the enzyme-EE complex or to free enzyme. Taken as a whole, these data suggest that celecoxib is acting as a heterotropic modulator of SULT2A1 activity, most likely involving a separate noncompetitive binding site.

Materials and Methods

Reagents. [3H]17α-Ethynylestradiol (49.1 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Insect cell (Sf9) cytosol containing cDNA-expressed human sulfotransferase 2A1, as well as control cytosol (devoid of human SULTs), was purchased from PanVera Corp (Madison, WI), or was derived from cloned and expressed in-house material (described below). Pooled (10 organ donors) preparations of liver cytosol were obtained from Tissue Transformation Technologies (Edison, NJ), EE, and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Celecoxib was obtained via extraction from a 100-ng commercial capsule formulation (described below). Capsules were obtained from a commercial pharmacy (Myoderm Medical Supply, Norristown, PA). EE 3-O-sulfate standard was obtained from Steraloids (Newport, RI). All other reagents were purchased from vendors at the best obtainable grade.

Cloning and Expression of SULT2A1. SULT2A1 was amplified from human cDNA (BD Biosciences Clontech, Palo Alto, CA) by PCR using laboratory screened a library of commercially available compounds in an effort find isof orm-selective inhibitors for sulfotransferases that could aid in reaction phenotyping of EE. This screen was performed with either EE or 1-hydroxyxyprene as the reporter substrate for enzymatic activity (Ma et al., 2003). One unusual finding was that celecoxib modulated the kinetic and regioselective specificity of EE sulfation by SULT2A1. The following report describes initial studies of this phenomenon.

ABBREVIATIONS: SULT, sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; EE, 17α-ethynylestradiol; PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; OH-PCB, hydroxylated polychlorinated biphenyl; $K_{\text{m(app)}}$, apparent $K_m$. 

1260
forward and reverse primers (5'→3') AGGTCTACCATCCTGCTGGAG-
GATTC and GAGTTTGGACGTTATCTCCATGGGAA, respectively. The
gel-purified PCR product was ligated into pCR3.1 (Invitrogen, Carlsbad,
CA). Restriction enzymes PstI and XbaI were added to the 5' and 3' ends of
SULT2A1 by using forward and reverse primers 5'→3' GAGGAGACTG-
CAGTCTACATGCTGCGACGATT and TCTTCACCTATAGTGATTCATCAG-
GGAAACG, respectively. The PstI-XbaI-digested PCR product was ligated
into pBlueBac4.5 (Invitrogen) and further subcloned into the pFASTBAC1
vector (Invitrogen) between the PstII-HindIII multicloning site. The plasmid
was analyzed by sequence analysis using an Applied Biosystems 310 auto-
mated sequencer (Applied Biosystems, Foster City, CA).

Generation of recombinant human SULT2A1 baculovirus was generated using the Bac-
to-Bac expression system (Invitrogen). Briefly, DH10BAC cells were trans-
formed with pFASTBAC1-SULT2A1 plasmid to generate SULT2A1 bacmid. Generation of recombinant bacmid was confirmed by PCR amplification. Sf9
cells were transfected with SULT2A1 recombinant bacmid, and the resulting recombinant virus was amplified after two consecutive rounds of infection. Cytosol isolated from Sf9 cells infected with recombinant SULT2A1 was evaluated for expression by immunoblot detection using human SULT2A1 polyclonal antibody (PanVera).

Extraction of Celecoxib. Four 100-mg capsules of commercially available celecoxib were disassembled and the contents were mixed as a suspension in 100 ml of ethyl acetate. The mixture was washed three times in a separatory funnel with brine, a saturated solution of sodium carbonate, and a 1% solution of acetic acid. The organic layer was then dried over magnesium sulfate and evaporated under vacuum to yield approximately 290 mg of white solid. Mass spectrometry (m/z 380) and NMR analysis (1H NMR [CD3CN] δ 2.37 [s, 3H], 6.41 [s, 2H], 6.95 [s, 1H], 7.21 [d, J = 8.2 Hz, 1H], 7.24 [d, J = 8.2 Hz, 1H], 7.5 [d, J = 8.7 Hz, 1H], 7.91 [d, J = 8.7 Hz, 1H]) was consistent with the structure of celecoxib. Mass spectrometry was performed by direct infusion of a methanol solution on a Quantum Mass Spectrometer (Thermo Finnigan, San Jose, CA), and NMR spectra were collected on a Varian Innova 500 MHz NMR (Varian Inc., Palo Alto, CA) equipped with a cold probe. Analysis of the extracted celecoxib by HPLC-UV, LC-MS, and NMR did not show the presence of any significant detectable impurities (i.e., ≤2%).

Incubations. Stock solutions of [3H]EE, potassium phosphate buffer (50 mM, pH 7.4), MgCl2 (1 mM), and enzyme were mixed to yield a reaction
volume of 0.45 ml. The amount of expressed SULT2A1 added to each reaction was adjusted so that no more than 10% substrate depletion occurred over the course of a typical incubation (~3 μg). Solvent effects were minimized by adding control cytosol to each reaction mixture (before incubation) such that the total protein concentration of the reaction was adjusted so that no more than 10% substrate depletion occurred over the course of a typical incubation (~3 μg). Solvent effects were minimized by adding control cytosol to each reaction mixture (before incubation) such that the total protein concentration of the reaction was 15 μg/ml (Ma et al., 2003). Incubations performed with human liver cytosol contained 10 to 12 μg/ml in each reaction. Reaction mixtures were preincubated for 5 min in a shaking water bath at 37°C. Catalysis was initiated with the addition of PAPS (50 μl of 200 μM solution) and continued for 10 min, under linear conditions. Celecoxib stock solutions were made with ethanol, and the final volume of solvent in the reaction mixture did not exceed 0.5% (v/v).

All incubations were terminated by the addition of acetonitrile (100 μl), followed by vortexing and centrifugation. The resultant supernatant was transferred into new test tubes and analyzed by HPLC without further workup.

HPLC Analysis. HPLC analyses were conducted on a Hewlett-Packard 1100 gradient system. Separation and quantification of [3H]EE 3-O-sulfate and [3H]EE 17-O-sulfate were achieved on a reverse phase C18 column (4.6 × 150, 5 μm) using a mobile phase consisting of: 25 mM ammonium formate (A; pH 3) and 0.1% formic acid in acetonitrile (B; constant flow rate of 1.0 ml/min). The following gradient was used: 0 min, 70% A; 20 min, 45% A; 22 min, 20% A; 24 min, 20% A; 25 min, 70% A. The column was equilibrated at 70% A for at least 5 min before injection of the next sample.

Radioactivity was quantitated postcolumn using a Radiomatic Flo-One detector (Radiomatic Instruments, Tampa, FL). Flowscint II scintillation cocktail (PerkinElmer Life and Analytical Sciences) was utilized at a flow rate of 3.0 ml/min. The 3-O-sulfate of ethynylestradiol was identified by comparing radioactivity with the retention time of an authentic standard. The identity of EE 17-O-sulfate was verified by comparing online radioactive traces with LC-MS data (HPLC conditions as described above) collected with a Finnigan TSQ7000 (Thermo Finnigan). MS analysis was carried out with electrospray ionization in the negative ion mode. The capillary temperature was 230°C and the ionization energy was maintained at 5.0 kV.

Data Analysis. Analysis of the data and curve fitting (to obtain Kmax, Vmax, and IC50 values) was accomplished using the method of nonlinear least-
squares, as performed by algorithms contained in the program Sigma Plot (SPSS Inc., Chicago, IL). In the current studies, substrate inhibition was not significant over the EE concentration range used. Therefore, substrate-velocity data were fit mathematically using a simple Michaelis-Menten model (eq. 1). Values for Ks (eq. 2) were determined by fitting a curve to percentage remaining activity (relative to solvent control) using eq. 2. The term Imax in eq. 2 is equal to the observed percentage of maximal, saturable inhibition in IC50 experiments (in this case, ~92%). The term s in eq. 2 is a modulator of curve shape.

\[
\text{velocity} = \frac{V_{\text{max}} \cdot S}{K_{\text{m}} + S}
\]

\[
\text{IC}_{50} = \frac{100 - I_{\text{max}}}{1 + \left(\frac{x}{I_{\text{max}}}\right)} - I_{\text{max}}
\]

Results and Discussion

In the presence of increasing concentrations of celecoxib (0–250 μM) and a fixed concentration of EE (2.5 μM), PAPS (20 μM), and recombinant SULT2A1 (~15 μg/ml total protein), product formation was switched from the 3-O-sulfate to the 17β-O-sulfate of EE in a concentration-dependent manner (Figs. 1 and 2). Figure 3 shows that the ratio of the 17β-O- to 3-O-sulfated product saturates with increasing celecoxib concentration, for both expressed SULT2A1 and liver cytosol. This observation confirms that product switching is not simply an artifact of an expressed system.

In addition, when the concentration of PAPS was varied from 0.05 μM to 50 μM, the ratio of 17β-O-sulfate to the 3-O-sulfate did not

![Fig. 1. The chemical structures of 17α-ethynylestradiol and celecoxib.](image)

![Fig. 2. HPLC radioactivity traces of SULT2A1-mediated EE (2.5 μM) sulfation in the presence of 0, 5, and 250 μM celecoxib.](image)
change and was ~1. The experiment used a fixed concentration of 5 μM celecoxib, 2.5 μM EE, and recombinant SULT2A1 (~15 μg/ml total protein, data not shown). The measured $K_m$ of PAPS for EE sulfation was 0.9 μM (recombinant SULT2A1, data not shown), which is similar to a previously published value (Falany et al., 1989).

As a consequence, the PAPS range tested above included concentrations less than and substantially greater than the measured PAPS $K_m$. Thus, different saturation states of the PAPS binding site had no effect on the EE sulfate product ratio as determined by 5 μM celecoxib and 2.5 μM EE. It was therefore concluded that a competitive interaction between celecoxib and PAPS was not involved in product ratio switching.

Assuming a simple Michaelis-Menten model (eq. 1 for substrate-velocity profiles of 3-O- and 17β-O-sulfate product formation), kinetic parameters were determined at different fixed concentrations of celecoxib (Tables 1 and 2). In Table 1, increasing concentrations of celecoxib decreased the $V_{max}$ of 3-O-sulfate product formation by 3- to 4-fold, with no obvious change in the $K_m$ value ($K_m$ standard errors overlap). In Table 2, increasing concentrations of celecoxib resulted in an 8-fold decrease in the $K_m$ of 17β-O-sulfation and a 7-fold increase in $V_{max}$. A comparison of the $V_{max}$ data in Table 1 with data in Table 2 indicates that celecoxib not only modulates the regioselectivity of the enzyme sulfation but also activates the enzyme such that total sulfated product exceeds product formation of the native enzyme by 3- to 4-fold. Finally, Table 3 shows kinetic parameters obtained by varying the celecoxib concentration at fixed concentrations of EE. Increasing concentrations of celecoxib inhibited EE 3-O-sulfate formation (see IC50 values) to the same extent independent of the concentration of EE. In addition, the kinetic constant for celecoxib ($K_{sof(app)}$ as measured by EE 17β-O-sulfate formation) decreased 2-fold in the presence of high concentrations of EE, indicating that celecoxib may bind tighter to the enzyme-EE complex than to free enzyme.

The data in Tables 1 and 3 yield insight into the binding of celecoxib. In Table 1, celecoxib decreased the $V_{max}$ of 3-O-sulfate formation, with no major effect on $K_m$ (values are within S.E.). These data alone would suggest that celecoxib binds to a separate effector site that is distinct in location to the binding site of EE (Segel, 1975). Moreover, the data presented in Table 3 demonstrate that increasing concentrations of celecoxib inhibited EE 3-O-sulfate formation with a similar IC50 independent of EE concentration, a characteristic of noncompetitive inhibition (Segel, 1975). Finally, in Table 3, increasing concentrations of EE decreased the apparent kinetic constant for celecoxib (as measured by EE 17β-O-sulfation). As noted above, this suggests that formation of the enzyme-EE complex does not inhibit

### TABLE 1

**Kinetic constants describing the 3-O-sulfation of EE in the presence of cDNA-expressed human SULT2A1 at different fixed concentrations of celecoxib**

<table>
<thead>
<tr>
<th>Celecoxib (μM)</th>
<th>3-O-Sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (μM)</td>
</tr>
<tr>
<td>0</td>
<td>0.191 ± 0.05</td>
</tr>
<tr>
<td>1.25</td>
<td>0.177 ± 0.03</td>
</tr>
<tr>
<td>2.5</td>
<td>0.186 ± 0.02</td>
</tr>
<tr>
<td>5</td>
<td>0.185 ± 0.06</td>
</tr>
<tr>
<td>10</td>
<td>0.235 ± 0.07</td>
</tr>
<tr>
<td>50</td>
<td>0.216 ± 0.06</td>
</tr>
<tr>
<td>125</td>
<td>0.182 ± 0.04</td>
</tr>
<tr>
<td>250</td>
<td>0.250 ± 0.04</td>
</tr>
</tbody>
</table>

### TABLE 2

**Kinetic constants describing the 17β-O-sulfation of EE in the presence of cDNA-expressed human SULT2A1 at different fixed concentrations of celecoxib**

<table>
<thead>
<tr>
<th>Celecoxib (μM)</th>
<th>17-O-Sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (μM)</td>
</tr>
<tr>
<td>0</td>
<td>0.628 ± 0.09</td>
</tr>
<tr>
<td>1.25</td>
<td>0.311 ± 0.02</td>
</tr>
<tr>
<td>2.5</td>
<td>0.265 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>0.23 ± 0.07</td>
</tr>
<tr>
<td>10</td>
<td>0.128 ± 0.02</td>
</tr>
<tr>
<td>50</td>
<td>0.092 ± 0.03</td>
</tr>
<tr>
<td>125</td>
<td>0.075 ± 0.06</td>
</tr>
<tr>
<td>250</td>
<td></td>
</tr>
</tbody>
</table>
binding of celecoxib, which also is consistent with separate binding sites for EE and celecoxib.

Additional work will be required to define the location of the celecoxib binding site and the mechanism by which the product ratio switch of EE sulfation occurs. Presently, there are many reports in the sulfotransferase literature that implicate multisite binding. For example, it has been previously shown by Zhang et al. (1998) that SULT1E1 is capable of binding two molecules of estradiol per subunit, and a kinetic model was proposed that incorporated multiple binding. When it was assumed that the second binding site was inhibitory to catalysis, then substrate inhibition (observed for estradiol sulfation) could adequately be described by the model. These investigators suggested that inhibition occurred through the binding of estradiol to a separate allosteric site.

Substrate inhibition is common to sulfotransferase substrate-velocity profiles and has been observed notably with SULT1A1 and SULT1A3. In a recent paper by Gamage et al. (2003), two molecules of 4-nitrophenol were found in the active site of crystallized human SULT1A1. These investigators proposed that substrate inhibition could be explained by reduced catalytic efficiency of an enzyme active site containing two molecules of 4-nitrophenol, as opposed to one. Moreover, the structure of SULT2A1 complexed with dehydroepiandrosterone has revealed alternate substrate binding orientations, one in a catalytic mode and another in an orientation postulated to give rise to substrate inhibition (Rehse et al., 2002).

The phenomenon of multiple binding sites is observed not only for substrates, but also for inhibitors. Hydroxylated polychlorinated biphenyls (OH-PCBs) have been shown to be potent noncompetitive inhibitors of estradiol sulfation by human SULT1E1 (Kester et al., 2000). When human SULT1E1 was crystallized with bound OH-PCBs, it was discovered that the inhibitor was located within the substrate binding site (Shevtsov et al., 2003). The authors noted that this would be expected to result in competitive inhibition of estradiol, in contrast to the noncompetitive inhibition reported by Kester et al. (2000). Noncompetitive inhibition has also been observed for salicylic acid (Vietri et al., 2000), and mixed noncompetitive inhibition has been reported for quercetin (Eaton et al., 1996).

Based upon the results reported herein, it appears that the product switching induced by celecoxib involves separate binding sites for both the substrate and the effector. This raises two interesting possibilities for celecoxib. First, celecoxib could bind to a separate allosteric site, eliciting a protein conformational change, which in turn may alter the orientation of EE in the binding site. The new EE orientation would be postulated to produce the 17β-O-sulfate. This hypothesis is similar to that proposed by Zhang et al. (1998). Alternatively, celecoxib may bind to a subset of the active site (by analogy with cytochrome P450 3A4; see below), and the resulting co-occupancy of the active site by EE and celecoxib could alter the orientation of EE to achieve 17β-O-sulfation.

As an example of the last possibility, in a seminal paper by Shou et al. (1994), it was noted that phenanthrene dramatically decreased the $V_{\text{max}}$ of cytochrome P450 3A4 (CYP3A4)-mediated oxidation of 7,8-benzoflavone but had little to no effect on the $K_{\text{cat}}$. The results reported by Shou et al. (1994) are similar to those found here (Table 1). Moreover, phenanthrene was also shown to be a substrate for CYP3A4, which strongly suggested that both phenanthrene and 7,8-benzoflavone had access to the active site and were probably bound in it simultaneously. In the present case, however, it was not possible to demonstrate that celecoxib was a substrate for SULT2A1 because the molecule lacks a free hydroxyl group that may be sulfated (Fig. 1). With the CYP3A4 analogy in mind, it is questionable whether the active site of SULT2A1 is large enough to accommodate a steroid nucleus (i.e., EE) and celecoxib simultaneously, and still display kinetic behavior consistent with noncompetitive inhibition, although it should be noted that this type of contradiction was also observed for crystallized SULT1E1 bound with OH-PCBs (vide supra).

To our knowledge, this is the first account of a compound that produces product switching for a substrate of sulfotransferase. However, it is possible that this phenomenon actually may be more common than its absence in the literature would suggest. This is due to the fact that many investigators in the sulfotransferase field have adopted the barium precipitation method as a means of assessing enzymatic activity (Foldes and Meek, 1973). The precipitation method measures total radioactivity of sulfated product by scintillation counting and does not provide information regarding the site of sulfation. Thus, product switching would not be observed in the many studies in which this technique has been used.

**Acknowledgments.** We acknowledge Dr. Raju Subramanian and Paul Krolikowski for technical assistance (NMR), and Cliff Fisher (Barker Institute, Groton, CT) for insightful comments during the preparation of the manuscript.

**References**


Address correspondence to: Dr. Michael Schrag, Amgen, One Amgen Center Drive, 1-1-A, Thousand Oaks, CA 91320-1799. E-mail: mschrag@amgen.com