Involvement of Human Cytochrome P450 2D6 in the Bioactivation of Acetaminophen

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ABSTRACT:

Acetaminophen (APAP), a widely used analgesic and antipyretic agent, can cause acute hepatic necrosis in both humans and experimental animals when consumed in large doses. It is generally accepted that N-acetyl-p-benzoquinone imine (NAPQI) is the toxic, reactive intermediate whose formation from APAP is mediated by cytochrome P450. Several forms of P450 in humans, including 2E1, 1A2, 2A6, 3A4, have been shown to catalyze the oxidation of APAP to NAPQI. We now present evidence which demonstrated that human cytochrome P450 450D6 (CYP2D6) is also involved in the bioactivation of APAP. The formation of NAPQI from APAP by cDNA-expressed CYP2D6 was examined. \(K_m\) and \(V_{\text{max}}\) values were 1.76 mM and 3.02 nmol/min/nmol of P450, respectively, such that the efficiency of CYP2D6 in the conversion of APAP to NAPQI is approximately one-third of that of CYP2E1. The contribution of CYP2D6 to the total formation of NAPQI from APAP (1 mM) in human liver was investigated using quinidine (1 \(\mu\)M) as a CYP2D6-specific inhibitor, and varied from 4.5 to 22.4% among 10 livers, with an average at 12.6%. The correlation between the contribution of CYP2D6 to NAPQI formation in human liver microsomes and the CYP2D6 activity probed by the \(O\)-demethylation of dextromethorphan was studied, and found to be strong \((r^2 = 0.85)\), and significant \((P < .0001)\). Our findings indicate that CYP2D6, one of the major P450 isoforms in humans and also one of the pharmacogenetically important isoforms, may contribute significantly to the formation of the cytotoxic metabolite NAPQI, especially in CYP2D6 ultra-rapid and extensive metabolizers and at toxic doses of APAP when plasma APAP concentrations reach 2 mM or more.

Cytochrome P450 2E1 (CYP2E1) has been considered to be a major isoform responsible for the bioactivation of APAP in humans (Raucy et al., 1989; Patten et al., 1993). Recent studies with transgenic CYP2E1 knockout mice have demonstrated that this isoform is a major enzyme involved in the metabolism and toxicity of APAP in mice (Lee et al., 1996). The apparent kinetic parameters, \(K_m\) and \(V_{\text{max}}\), for the metabolism of APAP to NAPQI by purified human CYP2E1 were recently determined by our laboratory to be 1.29 mM and 6.87 nmol/min/nmol of P450, respectively (Chen et al., 1998). A few other human isoforms including 1A2 (Raucy et al., 1989), 2A6 (Chen et al., 1998), and 3A4 (Thummel et al., 1993) have also been shown to catalyze the oxidation of APAP to NAPQI, although some of these isoforms may not contribute significantly to hepatotoxicity caused by APAP in mice (Tonge et al., 1998; Zaher et al., 1998) or humans (Sarich et al., 1997).

In human liver, the five isoforms that are primarily responsible for the metabolism of most drugs include CYP3A4, CYP2D6, CYP2C9, CYP2C19, and CYP1A2 (Guengerich, 1985). CYP3A4 and CYP2D6 are involved in the metabolism of about 50% and 30% of marketed drugs, respectively. It is important to evaluate the interactions between known drugs or new chemical entities and CYP2D6 to understand or predict potential drug-drug interactions. In fact, CYP2D6 and CYP3A4 are the two isoforms that are used routinely to evaluate new compounds for potential drug interaction problems in the early stages of drug discovery and development in the pharmaceutical industry. CYP2D6 is also one of the pharmacogenetically important isoforms. The metabolism of over a hundred drugs has been linked to the CYP2D6 polymorphism. Therefore, a knowledge of the involvement of CYP2D6 in the metabolism of a drug can provide information...
about the mechanism of the interindividual variability in its pharmacological activity and toxicity.

In the present paper, we present evidence which demonstrates that CYP2D6 is also involved in the bioactivation of APAP. We have examined the metabolism of APAP by cDNA-expressed and purified CYP2D6 and determined the kinetic parameters for the oxidation reaction. The contribution of CYP2D6 to the total formation of NAPQI in human liver microsomes was also investigated using quinidine as a CYP2D6-specific inhibitor, and by comparing the relative contribution of CYP2D6 to NAPQI formation and the \( O \)-demethylation of dextromethorphan as a probe substrate.

### Materials and Methods

**Chemicals.** APAP, DLPC, GSH, NADPH, quinidine, and 5-sulfosalicylic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Dextromethorphan and dextrorphan were from Research Biochemicals International (Natick, MA). GS-APAP and 3-OH-APAP were synthesized as described (Forte et al., 1984; Harvison et al., 1988).

**Preparation of Human Liver Microsomes.** Microsomes were prepared as described (Thummel et al., 1993). Microsomal protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

**Expression and Purification of Human CYP2D6.** CYP2D6 cDNA was obtained from Dr. U. Zanger (Evert et al., 1997) in which the artifactual base mutation at position 1120 had been reverted to CYP2D6*1. To create recombinant CYP2D6 baculovirus, the CYP2D6*1 clone was placed in the multiple cloning site of pFastBac (Life Technologies Bac-to-BacTM system). This transfer vector takes advantage of a bacterial intermediate, which allows for easy selection of a clonal population of recombinant viral DNA by using lacZ(a) color selection (Lucow et al., 1993). Following transfection and virus production, the expression of CYP2D6 in Tricoplusia ni insect cells suspension culture from recombinant baculovirus proceeded essentially as described for human CYP2C9 variants (Haining et al., 1996). Purification of active P450 was carried out using octyl-Sepharose chromatography, followed by DEAE-cellulose and hydroxyapatite column chromatography steps, also as described in detail elsewhere (Chen et al., 1996; Haining et al., 1996; Koenigs et al., 1999).

**Expression and Purification of Recombinant Rat NADPH-P450 Oxidoreductase.** Recombinant rat NADPH-P450 oxidoreductase was expressed and purified from bacterial cultures as previously described (Shen et al., 1991). Recombined CYP2D6 Incubation. Human CYP2D6 was reconstituted with recombinant rat NADPH-cytochrome P450 reductase and DLPC at a molar ratio of 1:2:600. The incubation mixture containing 0.15 \( \mu \)M P450, selected concentrations of APAP, 5 mM GSH, and 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 100 \( \mu \)l was preincubated for 3 min at 37°C. The reaction was initiated by addition of NADPH, whose final concentration was 0.5 mM. The assay was conducted at 37°C for 15 min and terminated by addition of 10 \( \mu \)l of 33% (w/v) sulfosalicylic acid.

**Human Liver Microsomal Incubations.** The microsomal incubation mixture containing 1 mg of microsomal protein, quinidine (0 or 1 \( \mu \)M), and 50 mM potassium phosphate buffer (pH 7.4) was preincubated at 37°C for 5 min. NADPH was added, and the reaction continued for 15 min. APAP oxidation was initiated by addition of APAP, GSH, and NADPH. The final concentrations of APAP, GSH, and NADPH, in a final volume of 250 \( \mu \)l were 1, 5, and 1.5 mM, respectively. The reaction was conducted at 37°C for 15 min and terminated by the addition of 25 \( \mu \)l of 33% (w/v) sulfosalicylic acid.

**HPLC Analysis of the Formation of GS-APAP.** GS-APAP formed from both incubations was quantified on a Hewlett-Packard 1090 II/L system with an authentic synthetic standard. A Hewlett-Packard 1049A electrochemical detector was connected to the UV detector in tandem and was set at a constant voltage of 0.6 V. Separations were performed on a 5-\( \mu \)m Alltech Econosil C18 column (4.6 mm \( \times \) 25 cm). The mobile phase was 25 mM ammonium phosphate buffer (pH 5.3) containing 10% methanol with a solvent flow rate of 1.0 ml/min.

**CYP2D6 Activity Assays of Human Liver Microsomes.** The incubation mixture containing 1 mg of microsomal protein and 0.1 mM dextromethorphan was preincubated at 37°C for 5 min. The reaction was initiated by the addition of NADPH whose final concentration was 1.5 mM. The final volume was 250 \( \mu \)l. The assay was conducted at 37°C for 15 min, and terminated by the addition of 25 \( \mu \)l of 70% perchloric acid. The mixture was centrifuged for 10 min at 16,000 g, and the supernatant was analyzed for the formation of dextrophan. The product was quantified on a Hewlett-Packard 1090 II/L system with an authentic standard by measuring the UV absorbance at 280 nm. The separation was performed on a 5-\( \mu \)m Alltech Econosil C18 column (4.6 mm \( \times \) 25 cm), with a mobile phase of 0.1 M HClO\(_2\)-NaClO\(_2\) buffer (pH 2.2) and acetoniitrile whose concentration (v/v) was from 10 to 80% over a 25-min linear gradient at a flow rate of 1 ml/min.

**Data Analysis.** For kinetic analysis, the following APAP concentrations were used: 0.3, 0.6, 2.4, 4.8, 9.6, and 19.2 mM. The kinetic parameters for the formation of NAPQI from APAP by purified recombinant CYP2D6 were determined by nonlinear regression analysis of the rate data based on Michaelis-Menten equation using the statistical program EnzymeKinetics V1.4. The correlation study was conducted with the statistical program StatView 4.5. All analysis was performed on the mean values of duplicate experiments. In all cases, duplicate values did not vary by more than 10%.

**Results and Discussions.** To characterize the involvement of human CYP2D6 in the bioactivation of APAP, the metabolism of APAP by baculovirus-expressed and purified human CYP2D6 was first examined. The formation of

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**Fig. 1. APAP oxidation pathways.**

**Fig. 2. NAPQI formation by baculovirus-expressed and purified human CYP2D6.**

The mean of duplicate incubations is plotted, and the line represents the predicted data from the nonlinear regression fit.
NAPQI from APAP was assayed as its glutathione conjugate, GS-APAP. The major isoform responsible for the bioactivation of APAP, human CYP2D6, appeared to be a relatively low-affinity, high-turnover enzyme for the catalysis of the oxidation of APAP. The ratio of \( V_{\text{max}} / K_m \), which indicates the efficiency of CYP2D6 in the conversion of APAP to its toxic metabolite, NAPQI, is approximately one-third of that of human CYP2E1. It has been demonstrated that cytochrome \( b_5 \) shows a stimulatory effect on \( P450 \) 2E1-mediated APAP oxidation. Therefore, we examined the catalytic activities of CYP2D6 on the formation of GS-APAP from APAP in a reconstituted system containing NADPH-P450 reductase and DLPC in the presence and absence of cytochrome \( b_5 \). Cytochrome \( b_5 \) did not show a significant effect on the formation rate of GS-APAP from APAP (data not shown).

The contribution of CYP2D6 to the total formation of NAPQI in human liver microsomes was next investigated using quinidine. The mean values of duplicate experiments are reported, and the duplicate values did not vary by more than 10%.

Table 1: Inhibition of NAPQI Formation by Quinidine in Human Liver Microsomes

<table>
<thead>
<tr>
<th>Liver ID</th>
<th>Velocity</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{pmol/mg protein/min} )</td>
<td>%</td>
</tr>
<tr>
<td>-Quinidine</td>
<td>+Quinidine</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>26.1</td>
<td>22.5</td>
</tr>
<tr>
<td>2</td>
<td>91.4</td>
<td>80.5</td>
</tr>
<tr>
<td>3</td>
<td>86.6</td>
<td>76.1</td>
</tr>
<tr>
<td>4</td>
<td>79.4</td>
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<tr>
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<td>56.5</td>
</tr>
<tr>
<td>6</td>
<td>28.1</td>
<td>21.8</td>
</tr>
<tr>
<td>7</td>
<td>140.9</td>
<td>117.8</td>
</tr>
<tr>
<td>8</td>
<td>35.2</td>
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<tr>
<td>9</td>
<td>57.5</td>
<td>54.9</td>
</tr>
<tr>
<td>10</td>
<td>24.1</td>
<td>20.2</td>
</tr>
</tbody>
</table>

Inhibition

\begin{align*}
\text{Inhibition} &= \frac{100 - C_{\text{APAP}}}{C_{\text{APAP}}} \times 100 \\
&= \frac{100 - 0.325}{0.325} \times 100 \\
&= 67.5 \%
\end{align*}

The formation of the nontoxic catechol metabolite, 3-OH-APAP, was not measurable, and related kinetic parameters were not determined.

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References


Evert B, Eichbein ML, Haubruck H and Zanger UM (1997) Functional properties of CYP2D6 1 (wild type) and CYP2D6 7 (His324Pro) expressed by recombinant baculovirus in insect cells. Nuern-Schmiedeberg's Arch Pharm 335:289–318.


