Human Halothane Reduction In Vitro by Cytochrome P450 2A6 and 3A4: Identification of Low and High $K_M$ Isoforms

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The volatile anesthetic halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is extensively metabolized in humans, with approximately 50% of an absorbed dose undergoing hepatic biotransformation (Carpenter et al., 1986). Halothane is a unique substrate, undergoing both oxidative and reductive P450-catalyzed dehalogenation during clinical anesthesia, with each metabolic pathway subtending a different form of hepatic toxicity (Cousins et al., 1989; Gut et al., 1995; Jenner et al., 1990; Ray and Drummond, 1991). Oxidative hepatic metabolism mediates a rare, often fatal, immune-based fulminant hepatic necrosis (“halothane hepatitis”) (Kenna et al., 1988; Ray and Drummond, 1991), whereas reductive metabolism mediates a common, mild, and subclinical hepatotoxicity (de Groot and Noll, 1983; Sato et al., 1990).

The mechanism of halothane oxidation and hepatic necrosis has been well described (Bourdi, 1996; Gut et al., 1993, 1995; Ray and Drummond, 1991). Under sufficient oxygen tension, halothane undergoes P450-catalyzed oxidation to a reactive acyl chloride intermediate, which may trifluoroacetylate tissue proteins. In susceptible individuals, these act as neoantigens to stimulate formation of anti-trifluoroacetylated antibodies that, upon re-exposure to halothane or other trifluoroacetylated volatile anesthetics (enflurane, isoflurane, or desflurane), mediate an immune response culminating in fulminant hepatic necrosis. The rate and extent of oxidative halothane metabolism is considered crucial factors in determining susceptibility to halothane hepatitis (Christ et al., 1988a, 1988b; Kenna et al., 1990; Pohl et al., 1989).

The mechanism of halothane reduction and mild hepatotoxicity has been similarly well described (Ray and Drummond, 1991). Under anaerobic conditions, halothane undergoes P450-catalyzed reduction to an unstable radical intermediate (Ahr et al., 1982), which may abstract a hydrogen atom to form the volatile metabolite 2-chloro-1,1,1-trifluoroethane (CTE). 2) undergo a second P450-catalyzed reduction and loss of fluoride to give the volatile metabolite 2-chloro-1,1,1-trifluoroethene (CDE). 3) bind covalently to microsomal phospholipids (Muller and Srier, 1982) or proteins such as P450 causing suicide inactivation (Baker et al., 1991; Manno et al., 1992), or 4) initiate microsomal lipid peroxidation (Akita et al., 1989; Awad et al., 1996; de Groot and Noll, 1983; Sato et al., 1990). These sequelae of halothane reduction are the putative causes of mild halothane hepatotoxicity, which is manifested by mildly elevated postoperative liver enzymes (Akita et al., 1989; de Groot and Noll, 1983; Sato et al., 1990). Of greater clinical significance is the impaired mixed function oxidase activity (Cousins et al., 1987), which ensues from P450-halothane metabolite complex formation (Baker et al., 1991; Manno et al., 1992) and/or lipid peroxidation (Awad et al., 1996; de Groot and Noll, 1983). Halothane reduction accounts for approximately 1–6% of total metabolism (Wark et al., 1990), and mild hepatotoxicity occurs in up to 25% of patients undergoing halothane anesthesia (Ray and Drummond, 1991).

Recent investigations identifying the P450 isoforms catalyzing halothane oxidation and reduction have revealed a curious isoform specificity. Human hepatic oxidative halothane metabolism is catalyzed predominantly by P450 2E1 in vitro and in vivo, and to a lesser extent by P450 2A6 (Kharasch et al., 1996; Madan and Parkinson et al., 1996; Spracklin et al., 1997). In contrast, human hepatic reductive halothane metabolism is catalyzed principally by P450s 2A6 and 3A4 (Spracklin et al., 1996). More specifically, P450s 2E1 and 2A6 were identified as the low and high $K_M$ isoforms, respectively, catalyzing halothane oxidation (Spracklin et al., 1997). In contrast, the identity of the low and high $K_M$ isoforms catalyzing halothane reduction remains unknown. The purpose of this investigation was to provide this identification.

Materials and Methods

Assays of halothane metabolism were conducted essentially as described previously (Spracklin et al., 1996). Briefly, reaction mixtures contained human liver microsomes (1 mg/ml), halothane, and an NADPH-generating system in

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1 Abbreviations used are: CTE, 2-chloro-1,1,1-trifluoroethane; CDE, 2-chloro-1,1,1-difluoroethene.

Fig. 1. Halothane reduction to CDE and CTE by cDNA-expressed P450.

Symbols denote observed metabolite formation. Lines represent rates predicted using Michaelis-Menten kinetic parameters derived from nonlinear regression analysis of the experimental data.
0.1 M potassium phosphate buffer (pH 7.4). Incubations (10 min, 37°C) were performed in sealed vials (11.8 ml), purged with prepurified nitrogen to ensure anaerobic conditions, and quenched with 20% perchloric acid. Metabolites (CTE and CDE) were analyzed by gas chromatography/mass spectrometry with selected-ion monitoring and headspace sampling, without further sample preparation. A Hewlett-Packard (Wilmington, DE) 5890 series II gas chromatograph-5971 mass selective detector and 7694 headspace sampler, with a DB-VRX fused silica capillary column (30 m × 0.32 mm × 1.8-µm film thickness) (J&W Scientific, Folsom, CA). Incubations using cDNA-expressed P450 (Genent, Woburn, MA) were carried out similarly, using protein concentrations of 1 mg/ml and incubation times of 20 min. Halothane concentrations were determined by gas chromatography/mass spectrometry as described previously (Spracklin et al., 1997).

Experiments with isoenzyme-selective inhibitors of P450 2A6 (8-methoxypsoralen) and 3A4 (troleandomycin) were conducted at headspace halothane concentrations of 0.02 and 0.2 vol%, produced by adding 0.01 and 0.12 ml of halothane (in acetonitrile), respectively. Concentrations of 8-methoxypsoralen and troleandomycin (28 and 100 µM, respectively) were chosen to theoretically suppress >80% of isoform activity, based on published K<sub>M</sub> values (Maenpaa et al., 1994; Newton et al., 1995). Michaelis-Menten kinetic parameters were determined by nonlinear regression analysis (SigmaPlot 5.01; Jandel Scientific, San Rafael, CA). Results are expressed as the mean ± SD of three experiments.

Results and Discussion
cDNA-expressed P450s 2A6 and 3A4 both catalyze halothane reduction under saturating conditions (Spracklin et al., 1996). To specifically define the kinetic role of these expressed human P450 isoforms in halothane reduction, the concentration dependence of CDE and CTE formation was examined (fig. 1). For CDE and CTE formation by P450 2A6, saturation kinetics were observed, Eadie-Hofstee plots were linear, and the rate data were fit to a one-enzyme Michaelis-Menten model by nonlinear regression analysis. For CDE formation, V<sub>max</sub> was 0.049 pmol/min/pmol P450, and K<sub>M</sub> was 0.026 pmol vol%; for CTE formation, V<sub>max</sub> was 0.18 pmol/min/pmol P450, and K<sub>M</sub> was 0.027 pmol vol% (table 1). Similarly, CDE and CTE formation by cDNA-expressed P450 3A4 exhibited saturation kinetics, Eadie-Hofstee plots were linear, and data were also fit to a one-enzyme Michaelis-Menten model using nonlinear regression analysis. For CDE formation, V<sub>max</sub> was 0.027 pmol/min/pmol P450, and K<sub>M</sub> was 0.20 pmol vol%; for CTE formation, V<sub>max</sub> was 0.21 pmol/min/pmol P450, and K<sub>M</sub> was 0.52 pmol vol% (table 1). These results indicated P450 2A6 as the high-affinity and P450 3A4 as the low-affinity catalyst of halothane reduction.

To substantiate these findings, in human liver microsomes, the effects of the P450 2A6-selective inhibitor 8-methoxypsoralen and the P450 3A4-selective inhibitor troleandomycin were examined (table 2). Halothane concentrations were chosen to reflect the K<sub>M</sub> values obtained for both cDNA-expressed P450 and human liver microsomes. At halothane concentrations corresponding to the predicted low K<sub>M</sub> (0.02 pmol vol%), 8-methoxypsoralen inhibited both CDE and CTE formation by 60–70%, whereas troleandomycin inhibited CDE and CTE formation by only 22–24%. Conversely, at halothane concentrations corresponding to the predicted high K<sub>M</sub> (0.2 pmol vol%), 8-methoxypsoralen inhibited CDE and CTE formation by 26–43%, whereas troleandomycin inhibited CDE formation by 35% but CTE formation by only 5%. At saturating halothane concentrations (3.2 pmol vol%), troleandomycin inhibited CDE and CTE formation by 31–46%. Greater inhibition by 8-methoxypsoralen occurred at halothane concentrations corresponding to the low K<sub>M</sub> values, whereas more inhibition by troleandomycin occurred at halothane concentrations at or above the high K<sub>M</sub> values. These results, in conjunction with previous observations (Spracklin et al., 1996), are consistent with the assignment of P450s 2A6 and 3A4 as the high- and low-affinity catalysts, respectively, of human hepatic microsomal halothane reduction. Furthermore, there was good agreement between the K<sub>M</sub> values obtained with cDNA-expressed P450 and those from human liver microsomes (table 1).

Typical pulmonary halothane concentrations during surgical anesthesia are 0.4–1 vol%, which exceed the apparent K<sub>M</sub> values of P450s 2A6 and 3A4 (0.02 and 0.2 pmol vol%), suggesting that both high and low K<sub>M</sub> P450 isoforms will participate in human halothane reduction in vivo during surgical anesthesia. This is consistent with the observation that the P450 3A4 inducers phenytoin and phenobarbital (Wrighton et al., 1986) substantially enhanced reductive halothane metabolism and significantly increased the incidence of mild halothane hepatotoxicity, respectively, in patients (Jenner and Stevens, 1992) substantially enhanced reductive halothane metabolism and significantly increased the incidence of mild halothane hepatotoxicity, respectively, in patients (Jenner et al., 1990; Nomura et al., 1986). Halothane is relatively fat-soluble and slowly eliminated following administration. For example, blood halothane concentrations during anesthesia (at a pulmonary concentration of 1%) were 40%–70% K<sub>M</sub> during the first hour of anesthesia, and 30%–50% K<sub>M</sub> during the second hour of anesthesia.

### Table 1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>CDE</th>
<th>CTE</th>
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<tbody>
<tr>
<td>HLM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>High affinity</td>
<td>2.4</td>
</tr>
<tr>
<td>cDNA&lt;sup&gt;b&lt;/sup&gt; 2A6</td>
<td>0.049</td>
<td>0.026</td>
</tr>
<tr>
<td>HLM</td>
<td>Low affinity</td>
<td>18</td>
</tr>
<tr>
<td>cDNA 3A4</td>
<td>0.027</td>
<td>0.20</td>
</tr>
</tbody>
</table>

<sup>a</sup> Human liver microsomes [from (Spracklin et al., 1996)].
<sup>b</sup> cDNA-expressed P450.

### Table 2

<table>
<thead>
<tr>
<th>Halothane (vol%)</th>
<th>8-Methoxypsoralen</th>
<th>Troleandomycin</th>
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<tr>
<td></td>
<td>CDE</td>
<td>CTE</td>
</tr>
<tr>
<td>0.02</td>
<td>40 ± 9</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>0.2</td>
<td>74 ± 5</td>
<td>57 ± 5</td>
</tr>
<tr>
<td>3.2</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
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Halothane reduction is expressed as % of control (uninhibited) activity. *Not determined.*
and high $K_M$ isoforms for oxidative (27–36-fold) compared with reductive (15–20-fold) halothane metabolism. The present results highlight additional novel aspects of human halothane metabolism. Oxidation is catalyzed by P450s 2E1 and 2A6, as the low and high $K_M$ isoforms, with no apparent role for P450 3A4. Anaerobic reduction is catalyzed by P450s 2A6 and 3A4, as the low and high $K_M$ isoforms, with no apparent role for P450 2E1. Furthermore, not only does P450 2A6 switch from the high $K_M$ to the low $K_M$ isoform in the presence and absence of oxygen, respectively, but the difference in aerobic and anaerobic $K_M$ values (0.8–1.5% vs. 0.02%; 500–800 μM vs. 14 μM) is substantial. The mechanistic basis for these oxygen-dependent differences is presently unknown.

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**References**


