Cytochrome P450 2E1 is the Principal Catalyst of Human Oxidative Halothane Metabolism in Vitro

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Accepted for publication December 6, 1996

ABSTRACT

The volatile anesthetic halothane undergoes substantial biotransformation generating metabolites that mediate hepatotoxicity. Aerobically, halothane undergoes cytochrome P450-catalyzed oxidation to trifluoroacetic acid (TFA), bromide and a reactive intermediate that can acetylate liver proteins. These protein neo-antigens stimulate an immune reaction that mediates severe hepatic necrosis ("halothane hepatitis"). This investigation identified the human P450 isoform(s) that catalyze oxidative halothane metabolism. Halothane oxidation by human liver microsomes was assessed by TFA and bromide formation. Eadie-Hofstee plots of TFA and bromide formation were both nonlinear, suggesting the participation of multiple P450s. Microsomal TFA and bromide formation were inhibited 45 to 66% and 21 to 26%, respectively, by the P450 2A6 inhibitors 8-methoxypсорalen and coumarin, 84 to 90% by the P450 2E1 inhibitor 4-methylpyrazole and 55% by diethyldithiocarbamate, an inhibitor of both P450 2A6 and 2E1. Selective inhibitors of P450s 1A, 2B6, 2C9/10, 2D6 and 3A4 did not affect halothane oxidation. At saturating halothane concentrations (2.4 vol%), P450s 1A, 2B6, 2C9/10, 2D6 and 3A4 did not affect halothane oxidation. At saturating halothane concentrations, respectively. Further kinetic analysis using cDNA-expressed P450 and liver microsomes clearly demonstrated that P450 2E1 is the high affinity/low capacity isoform (Km = 0.030-0.053 vol%) and P450 2A6 is the low affinity/high capacity isoform (Km = 0.77-1.2 vol%). Evidence was also obtained for substrate inhibition of P450 2E1. The in vitro clearance estimates (Vmax/Km) for microsomal P450 2E1 (4.3-5.7 ml/min/g) were substantially greater than those for microsomal P450 2A6 (0.12-0.21). These clearances, as well as rates of apparent halothane oxidation predicted from kinetic parameters in conjunction with plasma halothane concentrations measured during clinical anesthesia in humans, demonstrated that both P450 2E1 and P450 2A6 contribute to human halothane metabolism, and that P450 2E1 is the predominant catalytic isoform.

Halothane is one of the more widely used volatile anesthetics in the world. Halothane undergoes extensive biotransformation with approximately 50% of an administered dose metabolized by reductive and oxidative pathways, both evident during routine anesthesia (Carpenter et al., 1986). Numerous epidemiological, clinical and laboratory investigations have established that halothane metabolism mediates both a mild and severe form of hepatic toxicity (for recent reviews see Cousins et al., 1989; Ray and Drummond, 1991; Gut et al., 1995). Mild hepatic reactions occur in up to 20% of halothane anesthetics (Ray and Drummond, 1991) and are evidenced clinically by elevated postoperative liver enzymes (AST, ALT, GST) (de Groot and Noll, 1983; Akita et al., 1989; Sato et al., 1990). Current theories suggest that this mild, subclinical hepatotoxicity is attributable to anaerobic reductive halothane metabolism that results in free radical generation and lipid peroxidation (de Groot and Noll, 1983; Sato et al., 1990; Awad et al., 1996). The severe form of hepatic toxicity is a rare but often fatal fulminant hepatic necrosis, commonly known as “halothane hepatitis” (Ray and Drum-
Clinically, halothane hepatitis occurs in approximately 1:6,000 to 35,000 halothane anesthetics (Ray and Drummond, 1991) but is fatal in 75% of these cases (Cousins et al., 1989). It is manifested by fever, jaundice and grossly elevated serum transaminase concentrations. Pathologically, it is characterized by massive centrilobular necrosis. The cytotoxicity associated with halothane hepatitis is consistent with an immunological reaction to trifluoroacetylated (TFA) liver protein neo-antigens. These TFA-antigens derive from acylation of native liver proteins that, in susceptible individuals, serve as neo-antigens that stimulate the formation of anti-TFA-protein antibodies. On reexposure to halothane or certain other volatile anesthetics, these antibodies initiate an immunological cascade that ultimately results in “halothane hepatitis.” TFA-protein formation results from oxidative halothane metabolism. Investigations suggest that the amount of antigen formation, and thus, the rate and extent of halothane metabolism may be a critical regulatory factor in the onset of halothane hepatitis (Christ et al., 1988a, 1988b; Pohl et al., 1989; Kenna et al., 1990). For example, in immunoblot analysis, the sera from six patients with halothane hepatitis cross-reacted with neo-antigens of halothane-and enflurane-treated rats but not with isoflurane-treated animals (Christ et al., 1988a). The relative amount of cross-reaction (halothane-enflurane-isoflurane) correlates with the extent of metabolism of the three anesthetics. Therefore, the seminal event in immune-based halothane hepatitis is P450-catalyzed oxidative halothane metabolism (Kenna et al., 1987).

The anaerobic reductive pathway of halothane metabolism has been well-described (Ray and Drummond, 1991; Spracklin et al., 1996). The oxidative pathway is shown in figure 1. Under sufficient oxygen tensions, halothane undergoes P450-catalyzed oxidation to trifluoroacetyl chloride, with concomitant loss of bromine. This unstable intermediate undergoes further reactions, including: 1) hydrolysis to yield the nontoxic metabolite TFA; 2) binding to phospholipids (Muller and Srier, 1982) and 3) acetylation of tissue proteins to form the TFA-protein adducts (Ray and Drummond, 1991). To date, a number of the TFA-modified proteins have been identified. These include protein disulfide isomerase, microsomal carboxylesterase, calreticulin, stress protein ERP72 and ERp99/endoplasm/GRP 94 in microsomes (Gut et al., 1995), and glutathione-S-transferase in cytosol (Brown and Gandolfi, 1994). However, the precise antigens that cause halothane hepatitis are unknown.

The mechanism and consequences of oxidative halothane metabolism have been well studied; nonetheless, clinicians are still unable to identify which patients will develop anti-TFA proteins, or which patients will develop halothane hepatitis. However, it is clear that P450-catalyzed oxidative halothane metabolism is the critical initiating event, and inhibition of P450-catalyzed halothane oxidation is a promising potential clinical strategy to prevent halothane hepatitis (Kharasch et al., 1996). Nevertheless, the exact identity of the P450 isoform(s) that catalyze oxidative metabolism of halothane in humans is unknown. Previous investigations have suggested a role for P450 2E1 with possible involvement of P450 2A6 (Brown et al., 1995; Kharasch et al., 1996). Disulfiram, an effective P450 2E1 inhibitor, significantly diminished halothane oxidation in humans (Kharasch et al., 1996), however disulfiram may inhibit P450 2A6 as well as P450 2E1. Therefore, the purpose of this investigation was to clarify the human liver P450 isoform(s) responsible for oxidative halothane metabolism.

**Materials and Methods**

Halothane was purchased from Halocarbon Laboratories (N. Augusta, SC). Furafylline and sulfaphenazole were generous gifts from Drs. Kent Kunze and William F. Trager, respectively (University of Washington, Seattle, WA). Sodium trifluoracetate, sodium bromide and chlorodifluoroacetic acid were purchased from Fluka Chemical Co. (Ronkonkoma, NY) and were of the highest purity available. Microsomes containing individual cDNA-expressed cytochrome P450 isoforms were purchased from Gentest (Woburn, MA). Unless specified, all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available. All buffers and reagents were prepared with high-purity (≥18.2 MΩ cm) water (Milli-Q, Millipore, Bedford, MA).

Microsomes were prepared from human livers as described previously (Kharasch and Thummel, 1993). Microsomal protein concentrations were determined by the method of Lowry et al. (1951). Total microsomal cytochrome P450 content was determined from the reduced minus oxidized carbon monoxide difference spectrum (Estabrook et al., 1972).

Halothane metabolism was determined in scintillation vials (24.4 ml) containing human liver microsomes (2 mg/ml), halothane and NADPH (2 mM) in a final volume of 1.0 ml potassium phosphate buffer (50 mM, pH 7.4). The reaction was initiated by the addition of halothane that was added either undiluted (2 μl, producing a headspace concentration of 2.4 vol% at 37°C) for experiments at saturating substrate concentrations, or diluted in methanol (final aqueous methanol concentration 0.2%) for experiments at subsaturating sub-

![Fig. 1. Mechanism of TFA and bromide formation from P450-catalyzed halothane oxidation.](image)
strate concentrations. Preliminary experiments showed that this methanol concentration did not affect the rate of halothane oxidation. Incubations were routinely carried out for 60 min at 37°C unless indicated otherwise. Reactions were quenched by quantitatively transferring the reaction mixture to a 2-ml polypropylene vial containing trichloroacetic acid (10 μl of a 6 N aqueous solution; 60 μmol) and mixing thoroughly for 10 sec. The internal standard chlorodifluoroacetic acid (25 μl of a 1 mM aqueous solution; 25 nmol) was then added and the solution vortexed for another 10 sec. The vials were placed on ice for 15 min and then centrifuged at 16,000 × g for 30 min. The resulting supernatant was filtered through a 0.2 μm DIMEX syringe filter (Millipore) directly into an autosampler vial. Experiments using cDNA-expressed protein were carried out similarly using typical protein concentrations of 1 mg/ml and incubation times of 60 or 90 min.

Metabolites and internal standards were analyzed by ion HPLC with conductivity detection. Analytes were performed using a DX-300 HPLC-IC system (Dionex Corp. Sunnyvale, CA), consisting of an AGP gradient pump, LCM-3 chromatography module, CDM-3 conductivity detector, ASM-3 autosampler, IonPac AS11 analytical column and AG11 guard column and an ASRS-1 anion self-regenerating suppressor operating in the autosuppression recycle mode. Dionex AI-450 software was used to control the hardware, detector signal acquisition and chromatographic peak integration. Injections of 50 μl were made via an autosampler utilizing 0.5-ml polypropylene vials equipped with 20-μm filter caps (PolYVial, Dionex Corp.). The sodium hydroxide concentration was initially 0.75 mM for 5 min, linearly decreased to 0.5 mM at 0.1 mM/min, linearly increased to 3.0 mM over 10.5 min, increased to 80 mM at 25 mM/min and held at 80 mM for 5 min. The concentration was then linearly decreased to 0.75 mM at 14.5 mM/min, and the column allowed to reequilibrate at this concentration for 7 min. The eluant flow rate was 2.0 ml/min and the detector sampling rate was 0.20 sec.

Under these conditions, the retention times for trifluoroacetate, bromide and chlorodifluoroacetic acid were 6.1, 7.8 and 11.2 min, respectively. Trichloroacetic acid did not interfere with analyte quantitation. Standards were prepared by adding aqueous TFA (0.25-40 nmol), sodium bromide (0.25-40 nmol) and the internal standard to microsomal mixtures prepared similarly to the incubation mixtures except for the omission of halothane. Standard curves for TFA and bromide were constructed from peak area ratios of metabolite to internal standard. Standard curves were linear over the concentration range 0 to 40 μM (r² = 0.99). The lower limit of quantitation (0.1 μM) was defined as a signal to noise ratio of 3:1. Quantitation of metabolite TFA and bromide was accomplished by comparing sample peak area ratios to those of the standard curve.

For kinetic experiments measuring TFA and bromide formation as a function of substrate concentration, headspace halothane concentrations were measured as previously described (Spracklin et al., 1996). Briefly, incubation mixtures were prepared identically to those used to assess TFA and bromide formation except that NADPH was omitted and the vial was sealed with a rubber septum instead of a screw cap. After 10 min at 37°C, an aliquot of the headspace gas from the reaction vial was transferred to another sealed vial. Headspace GC/MS analysis was used to quantitate halothane. Microsomal halothane concentrations were measured by a previously reported method used to measure whole blood halothane concentrations (Kharasch et al., 1996). After 10 min at 37°C, an aliquot of the reaction mixture was added to heptane. After centrifugation, an aliquot of the heptane was analyzed by gas chromatography to quantitate halothane.

Experiments with isoform-selective P450 inhibitors were conducted at the following final concentrations: 7.8-benzoflavone (P450 1A, 16 μM), furafylline (P450 1A2, 20 μM), 8-methoxypsoralen (P450 2A6, 28 μM), coumarin (P450 2A6, 36 μM), orphenadrine (P450 2B6, 5 μM), sulfaphenazole (P450 2C9/10, 3.6 μM), (S)-mephentoin (P450 2C19, 100 μM), quinidine (P450 2D6, 45 nM), 4-methylpyrazole (P450 2E1, 540 μM), diethylidithiocarbamate (P450 2E1, 100 μM), troleandomycin (P450 3A4, 100 μM), ketoconazole (P450 3A4, 90 nM), n-octylamine (P450, 3 mM). All inhibitors were added in potassium phosphate buffer except 7.8-benzoflavone, 8-methoxypsoralen, (S)-mephentoin, troleandomycin, ketoconazole and n-octylamine which were diluted in methanol (final methanol concentration 0.2%). Substrate and inhibitor concentrations were chosen to theoretically suppress more than 80% of isoform activity based on published Kₗ values. In experiments using the competitive inhibitors 7.8-benzoflavone, coumarin, 8-methoxypsoralen, sulfaphenazole, (S)-mephentoin, quinidine, 4-methylpyrazole and ketoconazole, the inhibitor was added followed by a solution of halothane (2 μl of 10% (v/v) halothane/methanol, equivalent to 0.2 μl halothane or 0.24 vol%, final methanol concentration 0.2%), and the reaction was initiated by the addition of the NADPH. Reactions were carried out at 37°C for 60 min and then quenched with trichloroacetic acid as described above. Incubations containing the mechanism-based inhibitors furafylline, orphenadrine, diethylidithiocarbamate, troleandomycin and n-octylamine were first preincubated at 37°C for 15 min with NADPH under aerobic conditions, after which time halothane was added (2 μl; 2.4 vol%). Reactions were carried out at 37°C for 60 min and then quenched as described previously. Kinetic and inhibitor experiments were performed in a single liver that was competent in all the major drug metabolizing P450 isozymes.
absence (fig. 2B) of NADPH illustrate that no TFA or bromide formation was observed in the absence of NADPH. TFA and bromide formation increased linearly with time for 60 min (fig. 2C). Formation of both metabolites was also linear with protein concentration up to 5 mg/ml (fig. 2D). Adequate sensitivity was obtained at 2.0 mg/ml which was used for subsequent incubations.

Anesthetic doses delivered during surgery are measured by the concentration (in volumes percent) in inspired gas. Tissue anesthetic concentrations are most closely reflected by those in expired alveolar gas (end-tidal concentration), but actual hepatic concentrations corresponding to those in respiratory gas are unknown. Therefore halothane was added to microsomes in sealed vials and the concentration of halothane in both the headspace gas and the microsomal suspensions was measured (fig. 3). There was a linear relationship between halothane concentrations (vol%) in the headspace gas and the amount of halothane added up to 3 μl. Similarly, there was a linear relationship between halothane concentrations (μM) in the microsomal suspension and the amount of halothane added, for halothane additions up to 1 μl. Aqueous solubility was limited at higher halothane concentrations. The relationship between headspace and solution concentrations is shown in the inset to figure 3. For example, a headspace concentration of 0.6 vol% corresponded to a suspension concentration of 400 μM, resulting from 0.5 μl of halothane. Calculation of a microsome:gas partition coefficient (λ) from the measured microsomal halothane concentrations yielded a value of 2.2. This is in good agreement with the blood:gas partition coefficient (A = 2.3) reported for halothane (Eger, 1985). Thus the blood:gas partition coefficient appears to be a reasonable approximation for the microsome: gas partition coefficient.

The substrate concentration dependence of halothane oxidation in human liver microsomes was examined over the range of 0 to 3 μl added, producing a headspace concentration of 0 to 3.6 vol% (fig. 4). Eadie-Hofstee plots for both TFA and bromide formation were nonlinear, suggesting the participation of multiple enzymes in halothane oxidation. Experimental data were fit to a two-enzyme Michaelis-Menten model by nonlinear regression analysis. The parameters obtained are summarized in table 1. For TFA formation, the apparent parameters obtained were V_{max(1)} = 130 pmol/min/mg protein, K_{m(1)} = 0.045 vol% (30 μM); V_{max(2)} = 94 pmol/min/mg protein, K_{m(2)} = 1.2 vol% (800 μM). For bromide, the apparent parameters were V_{max(1)} = 170 pmol/min/mg protein, K_{m(1)} = 0.045 vol% (30 μM); V_{max(2)} = 130 pmol/min/mg protein, K_{m(2)} = 0.94 vol% (630 μM).

To identify the P450 isoforms responsible for oxidative halothane metabolism, the effect of isoform-selective P450 inhibitors on rates of TFA and bromide formation were determined (fig. 5). The P450 2A6-selective inhibitor 8-methoxypsoralen (Maenpaa et al., 1994), used as a competitive inhibitor, decreased the rate of TFA and bromide formation by 45 and 66%, respectively. The P450 2A6 substrate com-

Fig. 2. NADPH-, time- and protein-dependence of TFA and bromide formation from P450-catalyzed halothane oxidation. Ion HPLC chromatograms showing TFA and bromide formation in a 60 min incubation of halothane with human liver microsomes as described in “Methods.” Incubations were carried out in a single liver that was competent in all the major drug metabolizing P450 isoforms. Incubations were performed in the (A) presence or (B) absence of NADPH. Also shown are rates of TFA and bromide formation as a function of (C) time (2 mg/ml protein) and (D) protein concentration (incubation time was 60 min).

Fig. 3. Relationship between halothane addition and measured halothane concentrations. Halothane concentrations were measured in the headspace (triangles) and in the microsomal suspension (circles) after addition of halothane to sealed vials containing human liver microsomes (2 mg/ml) and equilibrated at 37°C for 10 min. The inset shows the relationship between halothane concentrations measured in the headspace and the microsomal suspension (r = 0.99; P < .001).
Kinetic Parameters for TFA and bromide formation from P450-catalyzed halothane oxidation by HLM and cDNA-expressed P450

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>TFA</th>
<th>Br</th>
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<tr>
<td></td>
<td>V_max</td>
<td>K_m</td>
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<tr>
<td>HLM^a</td>
<td></td>
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<tr>
<td>High affinity</td>
<td>130</td>
<td>0.045</td>
</tr>
<tr>
<td>cDNA^c</td>
<td>2E1</td>
<td>0.30</td>
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<tr>
<td>HLM</td>
<td>Low affinity</td>
<td>94</td>
</tr>
<tr>
<td>cDNA</td>
<td>2A6</td>
<td>0.59</td>
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^a Rates of halothane oxidation by human liver microsomes expressed as pmol/min/mg protein; Rates of halothane oxidation by cDNA-expressed P450 expressed as pmol/min/pmol P450.

^b Human liver microsomes.

^c cDNA-expressed P450.

To identify further the isoforms responsible for oxidative halothane metabolism, the rates of TFA and bromide formation catalyzed by human liver microsomes and cDNA-expressed P450 were examined (fig. 6). At saturating halothane concentrations (2 µl = 2.4 vol%), P450 2A6 (0.45 pmol/min/pmol P450) and P450 2B6 (0.47 pmol/min/pmol P450) catalyzed significant and comparable rates of TFA formation. P450 2A12 (0.050 pmol/min/pmol P450), P450 2C9 (<0.001 pmol/min/pmol P450), P450 2D6 (0.036 pmol/min/pmol P450), P450 2E1 (0.032 pmol/min/pmol P450), and P450 3A4 (0.010 pmol/min/pmol P450) catalyzed much less TFA formation. However, at lower substrate concentrations (0.25 µl = 0.30 vol%), these lower substrate concentrations (0.25 µl = 0.30 vol%) halothane concentrations (0.25 µl = 0.30 vol%), these lower substrate concentrations (0.25 µl = 0.30 vol%), these lower substrate concentrations (0.25 µl = 0.30 vol%), these lower substrate concentrations (0.25 µl = 0.30 vol%), these lower substrate concentrations (0.25 µl = 0.30 vol %) and low (0.10 µl = 0.12 vol%) halothane concentrations. At saturating halothane concentrations (2.4 vol%), among a panel of 20 livers, there was a highly significant linear correlation between coumarin hydroxylase (P450 2A6) activity and both TFA (r = 0.90; P < .001) (fig. 7A) and bromide formation (r = 0.85; P < .001) (fig. 7D). Similarly, there was a significant linear correlation between P450 2A6 protein content and both TFA (r = 0.71; P < .001) and bromide (r = 0.63; P < .003) formation (fig. 7, B and E). Conversely, at these halothane concentrations, the correlation between chlorozoxazone hydroxylase (P450 2E1) activity and both TFA or bromide formation was less significant (P < .11 and .05, respectively) and exhibited considerably greater scatter (fig. 7, C and F). As a further refinement of the analysis, multiple linear re-
phenadrine, 5 μM; furafylline, 20 μM; octylamine, 3 mM.

(P450 2E1) activity and both TFA (r = 0.93) and bromide (r = 0.91) formation catalyzed by cDNA-expressed P450 2A6 and 2E1 at saturating halothane concentrations (2 μl = 2.4 vol%) and cDNA-expressed P450 as described in “Methods.” The inset shows rates of TFA and bromide formation catalyzed by cDNA-expressed P450s 2A6 and 2E1 at sub-saturating halothane concentrations (0.25 μl = 0.30 vol%).

To define further the roles of P450 2A6 and 2E1 in oxidative halothane metabolism, the substrate dependence of halothane oxidation was examined using cDNA-expressed P450s 2A6 and 2E1 (figs. 9 and 10). Eadie-Hofstee plots for TFA and bromide formation by cDNA-expressed P450 2A6 were linear, and the data were fit to a one enzyme Michaelis-Menten model using nonlinear regression analysis (fig. 9). Kinetic parameters are summarized in table 1. For TFA formation, Vₘₐₓ was 0.59 pmol/min/pmol P450 and Kᵢ was 1.2 vol% (800 μM). For bromide formation, Vₘₐₓ was 0.87 pmol/min/pmol P450 and Kᵢ was 0.77 vol% (510 μM). By comparison, the oxidation of halothane by P450 2E1 was more complex (fig. 10). At very low halothane concentrations (0.012-0.25 vol%), the oxidation of halothane was examined (fig. 11). The results are consistent with the known mechanism of halothane oxidation in which TFA

gression analysis for P450 2A6 and 2E1 activity yielded slightly improved correlation coefficients compared with linear regression analysis for P450 2A6 alone. The correlation coefficients (r) obtained using multiple linear regression were 0.93 (P < .001) for TFA formation and 0.91 (P < .001) for bromide formation. There was no significant correlation between either TFA or bromide formation and P450 1A2, 2C9 or 2D6 activities. There was a significant correlation between TFA and bromide formation and P450 2A6 and 3A4 activity. However, this was due to coexpression of P450s 2A6 and 3A4 activities (r = 0.59; P < .006). The relationship between metabolite formation and P450 content was also examined at subsaturating halothane concentrations (fig. 8). For reactions at this lower halothane concentration (0.12 vol%), the relationship between metabolite formation and P450 2A6 and 2E1 activities was opposite to that observed at saturating halothane concentrations. Among a panel of 15 livers, there was a positive correlation between chlorzoxazone hydroxylase (P450 2E1) activity and both TFA (r = 0.48; P < .08) and bromide (r = 0.63; P < .02) formation (fig. 8, B and D). Conversely, there was no significant correlation between coumarin hydroxylase (P450 2A6) activity and either TFA or bromide formation (fig. 8, A and C). There was not a significant correlation between TFA or bromide formation and P450 1A2, 2C9, 2D6 or 3A4 activities. These results further supported the predominant catalytic participation of P450 2E1 at low, subsaturating halothane concentrations, and P450 2A6 at higher halothane concentrations.
and bromide are formed in equimolar amounts. At both saturating (2 μl = 2.4 vol%) and subsaturating (0.10 μl = 0.12 vol%) halothane concentrations, there was a highly significant linear correlation between TFA and bromide formation rates among the liver microsomes examined.

**Discussion**

The results of this investigation demonstrate that human liver microsomal oxidative halothane metabolism is catalyzed by multiple P450 isoforms. Near complete inhibition of TFA and bromide formation by the nonselective P450 inhibitor n-octylamine suggests that oxidative halothane metabolism is catalyzed exclusively by P450. Eadie-Hofstee plots of human liver microsomal TFA and bromide formation were both nonlinear, consistent with a reaction catalyzed by two or more P450 isoforms. TFA and bromide formation were each decreased by inhibitors of two different isoforms. Experiments using cDNA-expressed P450 proteins showed that multiple P450 isoforms catalyzed oxidative halothane metabolism. Finally, in a panel of human livers, there was a correlation of TFA and bromide formation with one isoform at saturating halothane concentrations and a correlation with a different isoform at subsaturating halothane concentrations.

Several lines of investigation were used to determine that P450s 2A6 and 2E1 are the isoforms which catalyze oxidative halothane metabolism in human liver microsomes. These included: 1) Effects of isoform-selective inhibitors and competitive substrates on rates of TFA and bromide formation, 2) rates of TFA and bromide formation by cDNA-expressed P450 isoforms and 3) correlation of TFA and bromide formation with isoform activity in a panel of human liver microsomes. Moreover, a similar experimental strategy using different halothane concentrations suggested that P450 2E1 is the predominant catalyst at subsaturating halothane concentrations and 2A6 is the predominant catalyst at saturating halothane concentrations. Inhibitors of P450 2A6 (8-methoxypsoralen and coumarin) and P450 2E1 (4-methylpyrazole and DDC) significantly decreased the rates of both TFA and bromide formation. At saturating halothane concentrations, high rates of TFA and bromide formation were catalyzed by cDNA-expressed P450 2A6. However, at subsaturating halothane concentrations, the rate of metabolite formation was significantly decreased by inhibitors of P450 2E1.
formation by P450 2E1 exceeded that of 2A6. At saturating halothane concentrations, cDNA-expressed P450 2B6 catalyzed rates of metabolite formation comparable to that of 2A6. However, the P450 2B6-selective inhibitor orphena- drine had no effect on halothane oxidation, and P450 2B6 is minimally expressed in human livers (Yamano et al., 1989; Mimura et al., 1993; Shimada et al., 1994). The cumulative evidence suggests that P450 2B6 does not play a significant role in oxidative halothane metabolism in human liver microsomes. Finally, in a panel of human liver microsomes, for reactions at saturating halothane concentrations, there was a significant linear correlation between both TFA and bromide formation and P450 2A6 activity and content. The correlation was improved by a multiple linear regression analysis using both P450 2A6 and 2E1 activities. At subsaturating halothane concentrations, there was a significant linear correlation between both TFA and bromide formation and P450 2E1 activity. A previous investigation suggested that P450 2E1 is not the principal catalyst of oxidative metabolism of halothane by human liver microsomes (Brown et al., 1995). The present data do not support this conclusion and clearly demonstrate that both P450s 2A6 and 2E1 are the isoforms that catalyze oxidative halothane metabolism in human liver microsomes. These results suggest further that at low halothane concentrations, P450 2E1 is the predominant catalyst and at high concentrations, P450 2A6 is the predominant catalyst of halothane oxidation.

Kinetic analysis of halothane oxidation by human liver
P450 2E1 is the high affinity, low capacity enzyme (table 1) that catalyzes halothane oxidation (0.045 vol%) is in super agreement (1.2 vol% for both). There was also excellent agreement between the high affinity $K_m$ values derived from both enzyme sources are in super agreement (1.2 vol% for both). There was also excellent agreement between the high affinity $K_m$ values for bromide formation obtained for both microsomes and cDNA-expressed P450 2E1 (0.045 and 0.053 vol%, respectively). Similarly, the low affinity $K_m$ values derived from both enzyme sources are in excellent agreement (0.94 and 0.77 vol%, respectively). The $K_m$ values obtained for TFA and bromide formation were comparable in both microsomes and expressed P450 in every case, consistent with the known mechanism of halothane oxidation whereby TFA and bromide are formed in equimolar amounts. Most impressively, the $K_m$ value for the high affinity isoform catalyzing microsomal halothane oxidation (0.045 vol%) is in remarkable agreement with that reported ($K_m = 0.029\%$) for human halothane metabolism in vivo, in which halothane was administered over the alveolar concentration range 0.0007 to 0.13% (Cahalan et al., 1982). These investigations explicitly demonstrated that P450 2E1 is the high affinity, low capacity catalyst and P450 2A6 is the low affinity, high capacity catalyst of halothane oxidation.

Compelling evidence was obtained to suggest that human liver microsomal and cDNA-expressed P450 2E1 are subject to substrate inhibition at high concentrations of halothane. Furthermore, this substrate inhibition occurs at halothane concentrations that occur during anesthesia. Simple Michaelis-Menten kinetics did not adequately model the kinetic data for cDNA-expressed P450 2E1. Rather, the experimental data for cDNA-expressed P450 2E1-catalyzed halothane oxidation to both TFA and bromide were best modeled by incorporating a term for substrate inhibition. Evidence for substrate inhibition of P450 2E1 in human liver microsomes was also obtained. At halothane concentrations exceeding 4 vol%, the rates of microsomal TFA and bromide formation decreased (data not shown). Furthermore, the two enzyme Michaelis-Menten model of the microsomal data predicted that unlike the data for cDNA-expressed enzymes, the high affinity isofrom was the high capacity isofrom. Although the apparent parameters correctly modeled the data, the absolute values for $V_{max}$ were inaccurate because the simple model did not include a term for substrate inhibition. Attempts to model microsomal halothane oxidation including a component for substrate inhibition were unsuccessful because there was not a unique solution to the equation. Substrate inhibition has also been observed previously for the P450-catalyzed metabolism of 2,2-dichloro-1,1,1-trifluoroethane (HCFC-123), a halothane congener (Vinegar et al., 1994). HCFC-123 metabolism could only be accurately described when the physiological based pharmacokinetic model included a term for substrate inhibition (Andersen et al., 1987).

These results provide a biochemical rationale for observations in patients where halothane metabolism decreased as administered halothane concentrations increased (Cascorbi et al., 1970; Cahalan et al., 1981; Carpenter et al., 1986). Patients receiving 0.11% halothane metabolized a greater proportion (55%) of halothane than did those receiving 0.44% halothane (41%) (Cahalan et al., 1981). Also, in a valiant study by Cascorbi and co-workers (1970), the authors were injected with radioactive halothane, both with and without concomitant halothane anesthesia. Halothane metabolism was greater when the subjects were not anesthetized. Two investigations in animals (Eckes and Buch, 1985; Lind and Gandolfi, 1993) have also demonstrated that at high halothane concentrations, halothane metabolism was inhibited; however, as halothane concentrations decreased after cessation of anesthesia, metabolism increased. The present results
provide a biochemical explanation for all these observations. In vivo halothane metabolism decreases as halothane concentrations increase because the P450 isoform that catalyzes the majority of halothane oxidation, P450 2E1, is subject to substrate inhibition.

The ultimate objective of human microsomal investiga-

Fig. 12. Predicted halothane oxidation catalyzed by P450s 2E1 and 2A6. A, Blood halothane concentrations for patients undergoing a 3-hr halothane anesthetic were measured up to 9 hr postanesthesia and extrapolated to 4 days (inset). B, Rates of predicted TFA formation from halothane oxidation catalyzed by microsomal P450s 2E1 and 2A6. The rates were calculated using blood halothane concentrations in (A) and kinetic parameters obtained from nonlinear regression analysis of microsomal TFA formation. C, Rates of predicted TFA formation from halothane oxidation catalyzed by cDNA-expressed P450s 2E1 and 2A6. For various relative contents of P450s 2E1 and 2A6, the rates of halothane oxidation were calculated using blood halothane concentrations in (A) and kinetic parameters obtained from nonlinear regression analysis of TFA formation catalyzed by cDNA-expressed P450s 2E1 and 2A6.
tions in vitro is the understanding and prediction of human drug metabolism in vivo. The kinetic analysis of halothane oxidation demonstrates that at clinically relevant concentrations, both P450 2E1 and 2A6 participate in halothane oxidation. Typical inspired halothane concentrations during anesthesia are 0.4 to 3 vol%, which exceeds the apparent \( K_m \) values for both P450 2E1 and 2A6 predicted from both microsomes (0.045 and 1.2 vol%, respectively) and from cDNA-expressed P450 2A6 (0.053 and 1.2 vol%, respectively). Furthermore, the following analysis predicts that P450 2E1 catalyzes the majority of halothane oxidation during and after anesthesia, while P450 2A6 catalyzes meaningful amounts of halothane oxidation only at the high concentrations present during halothane administration. The blood halothane concentrations for patients undergoing a 3-hr halothane anesthetic were measured during anesthesia and for 9 hr postanesthesia, and were extrapolated to 4 days (fig. 12A). Based on the metabolism calculated using the apparent kinetic parameters derived from microsomal data, P450 2E1 is predicted to catalyze the majority of oxidative halothane metabolism (fig. 12B). Additionally, the kinetic parameters obtained from analysis of halothane oxidation by cDNA-expressed P450 also predict that P450 2E1 is the major catalyst of halothane oxidation (fig. 12C). The metabolism calculated for various relative P450 2E1 and 2A6 contents indicated that P450 2E1 is the predominant catalytic isoform, and that a 2E1:2A6 ratio of > 1 most closely reflected the observed microsomal data. In good agreement, the liver that was used for the kinetic experiments ranked second in P450 2E1 content and sixth in P450 2A6 content (by relative IOD) among the 20 human livers examined, thus suggesting a 2E1:2A6 ratio of > 1 in this liver. In further support for the predominant role of P450 2E1 in vivo was the observation that the in vivo \( K_m \) is identical to that for P450 2E1. Finally, the in vitro clearance estimates (\( V_{\text{max}}/K_m \)) for microsomal P450 2E1 (4.3-5.7 ml/min/g) were substantially greater than those for microsomal P450 2A6 (0.12-0.21). Notably, these in vitro predictions are concordant with in vivo results. The present in vitro models predict a predominant role for P450 2E1 in halothane oxidation and in vivo results also suggested that P450 2E1 was a predominant catalyst of halothane oxidation (Kharasch et al., 1996). In vivo, disulfiram, an inhibitor of P450 2E1 (Kharasch et al., 1993), and possibly P450 2A6, significantly decreased urinary TFA and bromide excretion after halothane anesthesia. Thus, in vitro studies using human liver microsomes appear to be a useful model for understanding in vivo human halothane oxidation.

One final point deserving mention is the relevance of animal models. P450 2E1 is highly conserved among various animal species which suggests that animal results concerning P450 2E1-catalyzed metabolism may be extrapolated to humans. In animals, numerous investigations have established the role of P450 2E1 in halothane oxidation both in vitro (Loesch et al., 1987; Gruenke et al., 1988) and in vivo (Eckes and Buch, 1985; Rice et al., 1987; Lind et al., 1990). In rats, the role of P450 2E1 in TFA-neoantigen formation (Kenna et al., 1990) has also been suggested. These animal studies are consistent with the present results describing P450 2E1 as the major catalyst of human halothane oxidation in vitro, and with those showing a predominant role for P450 2E1 in human halothane oxidation in vivo (Kharasch et al., 1996). Therefore, animal models describing the role of P450 2E1 in halothane hepatotoxicity may be relevant to humans. Animal models have also demonstrated a role for phenobarbital-induced P450 isoforms in halothane oxidation in vitro (Gruenke et al., 1988) and in vivo (Jenner et al., 1990), suggesting the participation of non-2E1 isoforms. P450 2B enzymes are the major phenobarbital-induced isoforms in rats. Experiments with cDNA-expressed P450 2B6, as well as previous experiments (Gruenke et al., 1988; Jenner et al., 1990), demonstrated the activity of P450 2B toward halothane oxidation. Indeed, other P450 2E1 substrates can also be metabolized by P450 2B at high substrate concentrations (Nakajima et al., 1990; Nakajima et al., 1992). However, P450 2B6 is not routinely expressed in human liver, and thus, does not appear to be a significant catalyst of human halothane oxidation. Therefore, animal models describing the role of phenobarbital-induced P450s in halothane oxidation must be interpreted with caution.

The mild and severe forms of hepatotoxicity mediated by halothane metabolism are believed to arise from different routes of biotransformation, with different clinical sequela. The present investigation, coupled with recent findings (Spracklin et al., 1996), provide biochemical evidence that the mild and severe halothane hepatotoxicities indeed arise from different routes of biotransformation. Furthermore, these disparate pathways of metabolism are catalyzed by different P450 isoforms. P450 2A6 and P450 3A4 catalyze reductive halothane metabolism while P450 2E1 and 2A6 catalyze oxidative halothane metabolism. Halothane is a unique substrate in that it readily undergoes both oxidative and reductive metabolism. The underlying basis for this apparent oxygen-dependence of isoform specificity is currently unknown.

The identification of the enzymes that catalyze halothane oxidation is an important step in understanding halothane hepatitis. These investigations have identified P450 2E1 as the major catalyst of oxidative halothane metabolism using an in vitro model. This model has been used to rationalize in vivo results in humans, where P450 2E1 was identified as the predominant catalyst of oxidative halothane metabolism. Thus, the microsomal in vitro model is an accurate predictor of in vivo human halothane oxidation.

Acknowledgments

The authors thank Dr. Douglas S. Mautz for the measurement of blood halothane concentrations.

References


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