

# Halothane-dependent Lipid Peroxidation in Human Liver Microsomes Is Catalyzed by Cytochrome P4502A6 (CYP2A6)

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**Background:** Halothane is extensively (approximately 50%) metabolized in humans and undergoes both oxidative and reductive cytochrome P450-catalyzed hepatic biotransformation. Halothane is reduced under low oxygen tensions by CYP2A6 and CYP3A4 in human liver microsome to an unstable free radical, and then to the volatile metabolites chlorodifluoroethene (CDE) and chlorotrifluoroethane (CTE). The free radical is also thought to initiate lipid peroxidation. Halothane-dependent lipid peroxidation has been shown in animals *in vitro* and *in vivo* but has not been evaluated in humans. This investigation tested the hypothesis that halothane causes lipid peroxidation in human liver microsomes, identified P450 isoforms responsible for halothane-dependent lipid peroxidation, and tested the hypothesis that lipid peroxidation is prevented by inhibiting halothane reduction.

**Methods:** Halothane metabolism was determined using human liver microsomes or cDNA-expressed P450. Lipid peroxidation was quantified by malondialdehyde (MDA) formation using high-pressure liquid chromatography–ultraviolet analysis of the thiobarbituric acid–MDA adduct. CTE and CDE were determined by gas chromatography–mass spectrometry.

**Results:** Halothane caused MDA formation in human liver microsomes at rates much lower than in rat liver microsomes. Human liver microsomal MDA production exhibited biphasic enzyme kinetics, similar to CDE and CTE production. MDA production was inhibited by the CYP2A6 inhibitor methoxsalen but not by the CYP3A4 inhibitor troleandomycin. Halothane-dependent MDA production was catalyzed by cDNA-expressed CYP2A6 but not CYP3A4 or P450 reductase alone. CYP2A6-catalyzed MDA production was inhibited by methoxsalen or anti-CYP2A6 antibody.

**Conclusions:** Halothane causes lipid peroxidation in human liver microsomes, which is catalyzed by CYP2A6, and inhibition of halothane reduction prevents halothane-dependent lipid peroxidation *in vitro*.

HALOTHANE undergoes both oxidative and reductive metabolism in humans *in vivo*.<sup>1</sup> Even with patients breathing 100% oxygen, there are portions of the liver with sufficiently low oxygen tension to permit reductive metabolism, owing in part to halothane-dependent reductions in hepatic blood flow. Halothane is reduced by

cytochrome P450 (CYP) to the 2-chloro-1,1,1-trifluoroethyl radical, which has been demonstrated in rodent livers and bile *in vitro* and *in vivo*.<sup>2-6</sup> This radical may abstract hydrogen to form the stable metabolite chlorotrifluoroethane (CTE) or undergo further P450-catalyzed reduction to yield chlorodifluoroethene (CDE) and inorganic fluoride. Recently, human liver microsomal halothane reduction to CTE and CDE *in vitro* was shown to be catalyzed principally by CYP2A6 and CYP3A4.<sup>7</sup> These were identified as the low- and high-K<sub>m</sub> isoforms, respectively.<sup>8</sup>

Halothane radicals may also react with tissue macromolecules, such as cytochrome P450, to form a catalytically inactive enzyme-metabolite complex<sup>9,10</sup> and with lipids to form a number of products.<sup>11,12</sup> It is generally accepted that halothane radicals initiate lipid peroxidation, at least in animals.<sup>12,13</sup> Considerable evidence for halothane-dependent lipid peroxidation in rodents has been presented. Halothane-dependent lipid peroxidation was demonstrated *in vitro* using microsomes and hepatocytes from uninduced and phenobarbital-induced rats, rabbits, and guinea pigs,<sup>11,12,14-17</sup> in livers excised from rats and guinea pigs treated with halothane *in vivo* even under normoxic conditions,<sup>18,19</sup> and noninvasively in phenobarbital-induced hypoxic rats *in vivo*.<sup>20</sup> Awad *et al.*<sup>19</sup> recently demonstrated halothane-dependent lipid peroxidation in rats *in vivo*, using quantification of F<sub>2</sub>-isoprostanes in plasma and excised livers, which was increased under hypoxic conditions. Enhancement of lipid peroxidation was specific to halothane and not observed with enflurane, isoflurane, or desflurane in microsomes or *in vivo*.<sup>18,19</sup> Lipid peroxidation in guinea pigs is greater than in rats and has been attributed to greater rates of reductive halothane metabolism.<sup>21</sup> Inhibition of halothane reduction in guinea pig liver microsomes decreased lipid peroxidation.<sup>16</sup> Halothane reduction and consequent lipid peroxidation are considered by some to mediate mild halothane hepatotoxicity.<sup>1,18,20</sup>

In contrast to rodents, little is known about halothane effects on lipid peroxidation in humans. Halothane-dependent lipid peroxidation has never been evaluated in human liver microsomes. The purpose of this investigation was to test the hypothesis that anaerobic reduction of halothane causes lipid peroxidation in human liver microsomes and to identify P450 isoforms responsible for halothane-dependent lipid peroxidation. In general, when anesthetic toxicity is related to anesthetic metabolism, modulation of anesthetic metabolism may have the more important effect of modifying metabolism-

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based toxicity. For example, inhibition of CYP2E1-dependent halothane oxidation decreased trifluoroacetylated neoantigen formation.<sup>22</sup> Although it has been shown that halothane reduction *in vitro* is prevented by inhibiting CYP2A6 and CYP3A4 activity,<sup>7</sup> it is not known whether the consequence of halothane reduction, namely lipid peroxidation, is prevented by inhibiting P450 activity. Therefore, the second purpose of this investigation was to determine whether inhibition of halothane reduction prevents lipid peroxidation in human liver microsomes.

## Materials and Methods

Halothane was obtained from Ayerst Laboratories, Inc. (New York, NY). CDE and CTE were purchased from PCR, Inc. (Gainesville, FL). Microsomes containing individual cDNA-expressed P450 isoforms, P450 reductase, or both were purchased from Gentest, Inc. (Woburn, MA). Monoclonal anti-CYP2A6 antibody was prepared and characterized as monospecific as previously described<sup>23</sup> and was the generous gift of Yang Sai, Ph.D. (University of Washington, Seattle, WA). Other reagents were purchased from Sigma Chemical Co. (St Louis, MO). A thiobarbituric acid (TBA) solution was prepared adding 6 ml NaOH, 1 M, to 1% aqueous TBA. After heating with stirring until dissolved, 0.1 ml concentrated HCl was added. The solution was decolorized with Norit activated charcoal, filtered, and adjusted to pH 2.5 with concentrated HCl. All buffers and reagents were prepared with high-purity water. Microsomes were prepared from human livers as described previously.<sup>24</sup> Experiments were done with several livers; data presented were from HL141. Animal experiments were approved by the University of Washington Animal Care and Use Committee in accordance with American Association for Accreditation of Laboratory Animal Care guidelines. Microsomes were prepared from livers of uninduced and phenobarbital-, pregnenolone-, and isoniazid-induced rats as described previously.<sup>25</sup>

Halothane metabolism was determined in reaction mixtures (0.5 ml) containing human liver microsomes (2 mg/ml) or cDNA-expressed P450 (1.6 mg/ml), halothane, and 2 mM NADPH in 50 mM Tris-HCl-150 mM KCl buffer (pH 7.4) as described previously.<sup>7</sup> Incubation vials were sealed with septa and flushed with prepurified nitrogen (99.995%) for 3 min. Halothane was then added, either 2  $\mu$ l or diluted in acetonitrile (final aqueous acetonitrile concentration 0.2%) for experiments at subsaturating substrate concentrations. After 3 min preincubation, the reactions were initiated by injecting NADPH through the septum. Incubations were routinely performed at 37°C for 60 min, except for the time course experiment. Reactions were quenched by addition of 10% trichloroacetic acid (75  $\mu$ l) into the sealed

vials, followed by 2% butylated hydroxy toluene in methanol (10  $\mu$ l). Gas samples were removed using a gas-tight syringe, and CDE and CTE were analyzed by headspace gas chromatography-mass spectrometry using a Hewlett-Packard (Wilmington, DE) 5890 gas chromatograph interfaced to an HP5971 mass-selective detector and an HP7694 headspace sampler, with a DB-VRX capillary column (30 m  $\times$  0.32 mm  $\times$  1.8  $\mu$ m film thickness; J&W Scientific, Folsom, CA).<sup>7</sup> Halothane concentrations were also analyzed by this assay for the concentration experiment. After removal of the gas sample, the microsomal mixture was centrifuged at 14,000 rpm for 10 min. Malondialdehyde (MDA) was quantified as the TBA adducts by high-pressure liquid chromatography.<sup>26,27</sup> Microsomal supernatant (0.3 ml) was combined with 0.3 ml TBA solution and heated in a boiling water bath for 15 min. The subsequent TBA-derivatized products were cooled and extracted by vortexing with 0.75 ml *n*-butanol. After centrifuging, the organic layer was evaporated to dryness, reconstituted in mobile phase, and analyzed by high-pressure liquid chromatography using a Hewlett-Packard 1050 system and a Microsorb MV C18 column (4.6 mm  $\times$  250 mm  $\times$  5  $\mu$ m; Rainin, Walnut Creek, CA). The mobile phase was acetonitrile: 5 mM phosphate buffer (pH 7.0) (15:85) at a flow rate of 1.0 ml/min, and the MDA-TBA adduct was detected at 515 nm. An MDA standard curve (0, 0.2, 0.4, 1, and 2  $\mu$ M) was prepared daily by adding 1,1,3,3-tetramethoxypropane to the buffer and microsomes and analyzing as described for the unknown. MDA concentrations in zero time blanks or incubations without halothane were subtracted from those incubations containing halothane, as appropriate.

Experiments with isoform-selective P450 inhibitors used 5  $\mu$ M methoxsalen (CYP2A6) and 100  $\mu$ M troleandomycin (CYP3A4). Inhibitors were added in acetonitrile (final concentration 0.2%) to incubations that were then preincubated with NADPH at 37°C for 10 (methoxsalen) or 15 (troleandomycin) min under aerobic conditions to permit isoform-selective mechanism-based inhibition. The vials were then placed on ice, sealed, and purged with nitrogen, and then 2  $\mu$ l halothane was added to initiate the reductive halothane reaction. Reactions were performed at 37°C for 60 min and then quenched as described previously. Experiments using anti-CYP2A6 antibody were preincubated at 37°C for 5 min before adding NADPH.

Regression and statistical analyses were performed using SigmaPlot and SigmaStat (SPSS, Chicago, IL). Experiments were typically performed in triplicate, and results are expressed as mean  $\pm$  SD.

## Results

Initial experiments characterized the time dependence of MDA production (fig. 1). Human liver microsomes

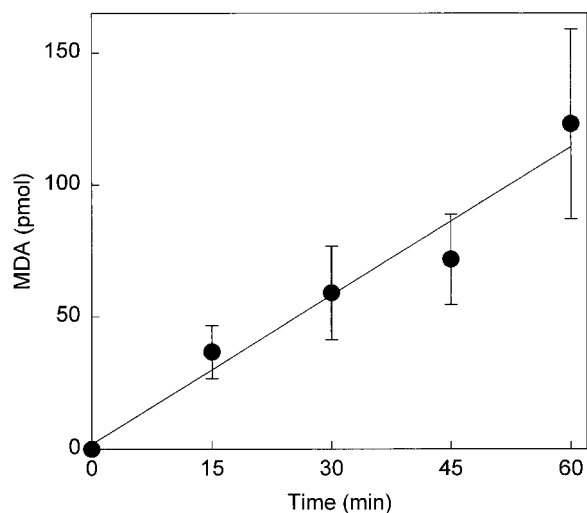


Fig. 1. Time dependence of malondialdehyde (MDA) production. Human liver microsomes were incubated with NADPH and halothane at saturating concentrations under anaerobic conditions. Each data point is the mean of triplicate incubations.

(HL141) were incubated with halothane and NADPH under anaerobic conditions. MDA production increased linearly for 60 min. CTE and CDE formation paralleled that of MDA (not shown), as described previously.<sup>7</sup> MDA formation was also supported by NADH, although at lower rates (58% compared with NADPH), and was negligible in the absence of either cofactor (not shown). MDA formation was also markedly reduced by 76% when incubations were conducted using room air. Halothane-dependent lipid peroxidation in microsomes from other human livers was less than in HL141; therefore, this liver was used for subsequent kinetic and inhibition experiments.

Halothane-dependent MDA production by human liver microsomes was compared with that in rat liver microsomes (fig. 2). Microsomes from rats pretreated with isoniazid (CYP2E1 induction), phenobarbital (CYP2B, 2C, and 3A induction), pregnenolone (CYP3A induc-

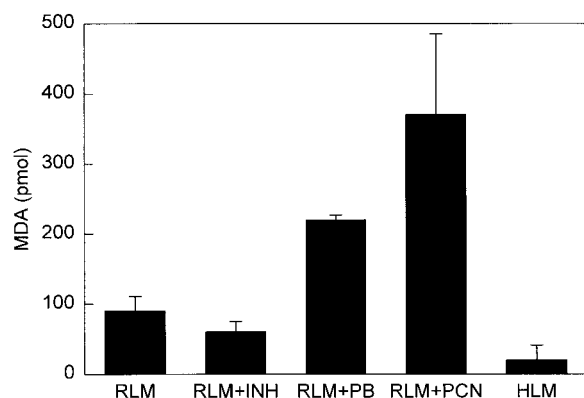


Fig. 2. Halothane-dependent malondialdehyde (MDA) formation by human (HLM) and rat (RLM) liver microsomes. Rats were induced with isoniazid (INH), phenobarbital (PB), pregnenolone (PCN), or nothing. The halothane concentration was 1%. Each data point is the mean of triplicate incubations.

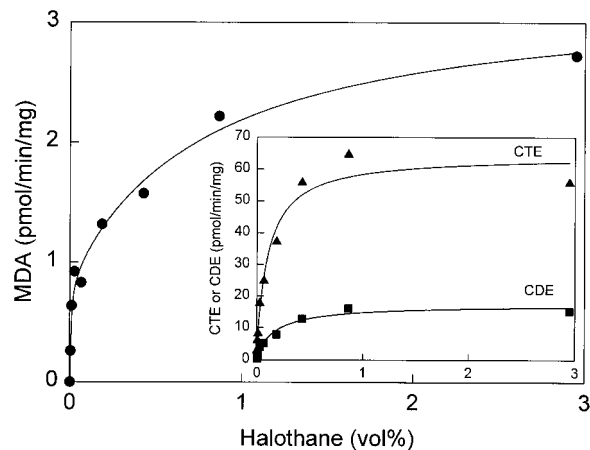


Fig. 3. Dependence of human liver microsomal malondialdehyde (MDA) production on halothane concentration. Reactions were performed as described in Materials and Methods, with varying halothane concentration (0–2  $\mu$ l, producing a concentration of 0–3.5% (v/v)). Data points represent observed values. Lines represent values predicted from parameters determined by nonlinear regression analysis using a two-enzyme model. CTE = chlorotrifluoroethane; CDE = chlorodifluoroethane.

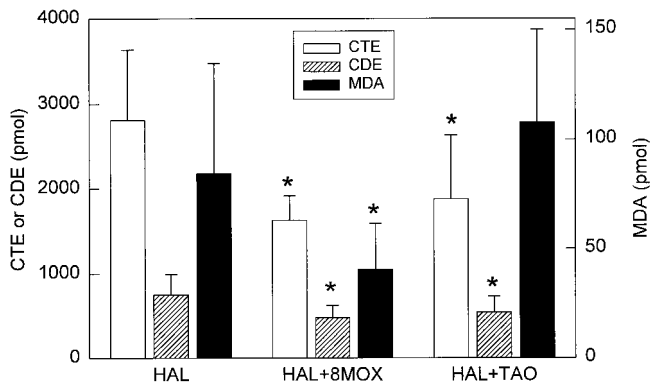
tion), or nothing were evaluated. MDA formation with all rat liver microsomes, including uninduced rats, was higher than with human liver microsomes. Among induced rats, microsomes from phenobarbital- and pregnenolone-pretreated rats produced the greatest amounts of MDA.

The halothane concentration dependence of MDA production was studied using microsomes from one human liver (fig. 3). MDA production was concentration-dependent and saturable, and exhibited biphasic kinetics with respect to substrate concentration. MDA production was fit to a two-enzyme system by nonlinear regression analysis. The parameters ( $\pm$  standard error of the parameter estimate) obtained were  $V_{\max 1} = 0.86 \pm 0.21$  pmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg<sup>-1</sup>,  $K_{m1} = 0.0062 \pm 0.0047\%$ ,  $V_{\max 2} = 2.41 \pm 0.28$  pmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg<sup>-1</sup>, and  $K_{m2} = 0.81 \pm 0.39\%$ . CTE and CDE formation were also measured and similarly exhibited biphasic kinetics.

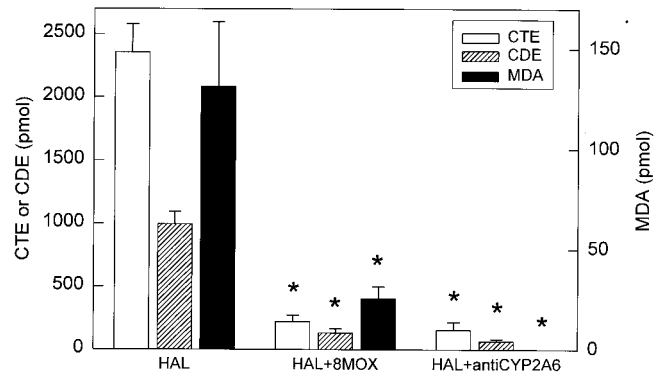
To identify the P450 isoforms responsible for the halothane-dependent lipid peroxidation in human liver microsomes, the effect of isoform-selective P450 inhibitors was determined (fig. 4). Halothane-reductive metabolism in human liver microsomes was previously shown to be catalyzed by CYP2A6 and CYP3A4<sup>7</sup>; therefore, the effect of the CYP2A6 and CYP3A4 inhibitors, methoxsalen and troleanomycin, were evaluated. MDA production was inhibited 52% by methoxsalen but was not decreased by troleanomycin.

To further identify the isoforms responsible for halothane-dependent lipid peroxidation, rates of MDA production catalyzed by cDNA-expressed P450 isoforms were determined (fig. 5). Experiments were performed using microsomes from cells expressing CYPs 2A6 or 3A4 and coexpressing P450 reductase. Halothane metab-





**Fig. 4.** Effects of P450 inhibitors on halothane (HAL) metabolism and halothane-dependent malondialdehyde (MDA) formation in human liver microsomes ( $n = 8$ ). Methoxsalen (8MOX) and troleandomycin (TAO) were present at 5 and 100  $\mu\text{M}$ , respectively, and halothane concentrations were saturating. Open and hatched bars represent chlorotrifluoroethane (CTE) and chlorodifluoroethene (CDE). Solid bars depict MDA. \*Significantly different from control ( $P < 0.05$ ) by analysis of variance.

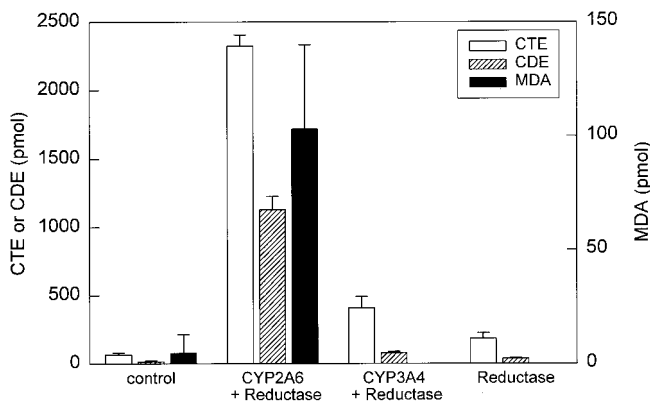


**Fig. 6.** Effects of P450 inhibitors on halothane (HAL) metabolism and halothane-dependent malondialdehyde (MDA) formation catalyzed by coexpressed human CYP2A6 (0.3 nmol) and P450 reductase. Microsomes were preincubated with NADPH and methoxsalen (8MOX) or anti-CYP2A6 antibody, then flushed with nitrogen and incubated with halothane for 60 min. Background values without halothane were subtracted. Open and hatched bars represent chlorotrifluoroethane (CTE) and chlorodifluoroethene (CDE). Solid bars depict MDA. Results are the mean of triplicate incubations. \*Significantly different from control ( $P < 0.05$ ) by analysis of variance.

olism and MDA formation were catalyzed predominantly by CYP2A6. Microsomes containing CYP3A4 and reductase or reductase alone formed a negligible amount of MDA. Halothane metabolism and MDA formation by expressed CYP2A6 were also studied after preincubation with methoxsalen or anti-CYP2A6 monoclonal antibody (fig. 6). Methoxsalen inhibited CDE and CTE production by 87 and 91%, respectively, and inhibited MDA formation by 81%. Anti-CYP2A6 antibody inhibited CDE and CTE productions by 93% and completely inhibited halothane-dependent MDA production.

## Discussion

Malondialdehyde results from the oxidative degradation of fatty acids.<sup>28</sup> As an index of lipid peroxidation,



**Fig. 5.** Halothane-dependent malondialdehyde (MDA) formation catalyzed by microsomes containing cDNA-expressed enzymes. Control microsomes contained vector only. Halothane concentrations were saturating. Background values obtained without halothane were subtracted from those with halothane. Open and hatched bars represent chlorotrifluoroethane (CTE) and chlorodifluoroethene (CDE). Solid bars depict MDA.

the most widely used method for the determination of MDA in biologic materials is based on its reaction with TBA to form a pink complex with an absorption maximum at 532–535 nm. However, numerous aldehydic compounds other than MDA can react with TBA to form a complex that absorbs in the 532- to 535-nm region.<sup>29</sup> Therefore, spectrophotometric assays for MDA express results only as nonspecific TBA-reactive substances. To separate the TBA-MDA adduct from other possible TBA-reactive substances, adduct samples were subsequently analyzed by high-pressure liquid chromatography, providing greater specificity and allowing quantitation of lipid peroxidation specifically as MDA.<sup>26,27</sup>

The results of this investigation clearly demonstrate halothane-dependent lipid peroxidation, assessed by formation of MDA, in human liver microsomes under decreased oxygen tension. Similar to results in previous studies with rat liver microsomes, lipid peroxidation was negligible under normoxic conditions (approximately 1.2 mm oxygen).<sup>11,15–17</sup> Thus, both halothane reduction<sup>7,30</sup> and lipid peroxidation require reduced oxygen tension (peroxidation was slightly enhanced at 10 mmHg and 12  $\mu\text{M}$  oxygen but decreased at higher tensions).<sup>15,17</sup> In addition, similar kinetic profiles between MDA, CDE, and CTE production were observed, as well as parallel effects of cytochrome P450 inhibitors on halothane metabolism and lipid peroxidation catalyzed by human liver microsomes and expressed human CYP2A6. Together, these observations support the hypothesis that lipid peroxidation in human liver microsomes results from halothane reduction under decreased oxygen tensions. This seems to be the first report of halothane-dependent lipid peroxidation in human liver microsomes.

The results also suggest that CYP2A6 is a major catalyst of lipid peroxidation in human liver microsomes. Human liver microsomal MDA formation was inhibited by the CYP2A6 inhibitor methoxsalen but not by the CYP3A4 inhibitor troleanomycin. In addition, expressed CYP2A6 was a better catalyst of MDA formation than was CYP3A4. CYPs 2A6 and 3A4 were the focus of these experiments because previous investigations showed that these two isoforms were the predominant catalysts of human liver microsomal halothane reduction.<sup>7,8</sup> Halothane-dependent lipid peroxidation catalyzed by CYP2A6 is consistent with previous identification of CYP2A6 as the low- $K_m$  isoform responsible for halothane reduction.

Results obtained with rat liver microsomes are consistent with previous evaluations of halothane metabolism and lipid peroxidation in rats but reveal an important species difference in comparison with human liver microsomes. Rat liver microsomal MDA production was increased twofold to fourfold by phenobarbital (CYP2B, 2C, and 3A) or pregnenolone (CYP3A) induction but was unaffected by isoniazid (CYP2E1) induction. Previous investigations also showed that lipid peroxidation in rats *in vivo*, measured by TBA-reactive substances or  $F_2$ -isoprostane production, was enhanced twofold to fourfold by phenobarbital pretreatment but not increased by isoniazid,<sup>19,20</sup> and both phenobarbital and pregnenolone induction resulted in twofold to threefold increases in halothane metabolism by rat liver microsomes, whereas isoniazid had no effect.<sup>9,30,31</sup> These results are consistent with known halothane reduction by rat CYPs 2B1>3A2>2C11 but not 2E1.<sup>32,33</sup> Halothane-dependent MDA formation by human liver microsomes was substantially less than that by induced and even uninduced rat liver microsomes. This species difference may be attributable to the relative absence of CYP2B in human liver, to the fact that the orthologous forms of CYP2A6 in rats exhibits considerable differences in substrate specificity compared with the human form, or to both. Alternatively, or in addition, there may be species differences in the susceptibility of microsomal lipids to peroxidation by the halothane radical, as well as species differences in hepatic lipid composition.<sup>34</sup> Also, MDA formation *per se* during lipid peroxidation is related in part to the number and position of double bonds and lipid chain length.<sup>35</sup> Similar species differences with other models of lipid peroxidation have been observed.<sup>36</sup> Further experiments are needed to elucidate the specific mechanism for the apparent species difference. Regardless of the mechanism, human liver microsomes seem to be a better model than the classically used induced rat liver microsomes for halothane-dependent *in vitro* lipid peroxidation in humans. The species difference in halothane-dependent lipid peroxidation observed *in vitro* is also consistent with the species difference recently observed *in vivo*. Halothane caused a fourfold increase in plasma

isoprostane concentrations (used as the index of peroxidation) in rats<sup>19</sup> but only a 50% increase in humans.<sup>37</sup>

Michaelis-Menten kinetic analysis of human liver microsomal halothane reduction<sup>7,8</sup> and lipid peroxidation shows biphasic kinetics, indicating participation of at least two enzymes. This was confirmed by identifying human halothane reduction metabolism *in vitro* by CYPs 2A6 and 3A4.<sup>7,8</sup> The current investigation suggested a greater role for CYP2A6 than 3A4 in lipid peroxidation, consistent with the former enzyme being the low- $K_m$  isoform; however, CYP3A4-dependent MDA formation was less than expected. Further experiments are necessary to identify definitively the second enzyme participating in halothane-dependent human liver microsomal lipid peroxidation. Nevertheless, CYP2A6 plays a greater role in halothane metabolism and lipid peroxidation. Similarly, additional investigations are required to determine whether halothane-induced lipid peroxidation in humans can be decreased *in vivo*.<sup>37</sup>

In summary, this investigation showed that halothane causes lipid peroxidation in human liver microsomes *in vitro*, identified a major role for CYP2A6 in human microsomal halothane-dependent lipid peroxidation, and showed that inhibition of CYP2A6-catalyzed halothane reduction decreased lipid peroxidation.

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