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Isoflurane Increases the Anaerobic Metabolites of Halothane

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Key words: anaesthetics; volatile; halothane; isoflurane; metabolism; reductive; halothane; chlorodifluoroethene; chlorotrifluoroethane; guinea pig.

The effect of isoflurane on the anaerobic metabolism of halothane to chlorodifluoroethene (CDE) and chlorotrifluoroethane (CTE) was studied with microsomes of guinea pig liver by gas chromatography. The reaction mixture used to measure the end products of anaerobic metabolism consisted of a microsomal suspension, 3 mM NADPH, halothane and isoflurane (except in control groups) in 0.1 M potassium phosphate buffer solution (pH 7.4). The K_m values for CDE formation were 601.61 ± 266.91, 254.22 ± 86.58, 257.92 ± 129.11, 268.55 ± 125.66 and 319.22 ± 86.76 μM (mean ± SD, n = 5) at 0 mM (0%), 0.12 mM (0.26%), 0.29 mM (0.64%), 0.58 mM (1.30%) and 1.16 mM (2.59%) isoflurane, respectively. The K_m values for CTE formation were 1204.74 ± 551.64, 553.75 ± 177.89, 521.14 ± 249.77, 560.67 ± 229.61 and 711.05 ± 317.13 μM (n = 5) at 0 mM (0%), 0.12 mM (0.26%), 0.29 mM (0.64%), 0.58 mM (1.30%) and 1.16 mM (2.59%) isoflurane, respectively. In contrast, the V_{max} values for CDE and CTE formation at these isoflurane concentrations were not significantly different than in the control groups. In this study the production of CDE and CTE was significantly ($P < 0.05$) increased by isoflurane, at concentrations up to 0.58 mM (1.30%).

INTRODUCTION

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is a widely used volatile inhalation anaesthetic, but the incidence of hepatotoxicity after halothane anaesthesia is a problem for anaesthesiologists.[1,2] Halothane is metabolized through two different metabolic pathways, an oxidative pathway and a reductive pathway, depending on the oxygen tension. Both of these pathways involve the hepatic microsomal cytochrome P-450 enzyme system and NADPH oxidation. Halothane is metabolized to the non-volatile urinary metabolites trifluoroacetic acid and bromide ion under oxidative metabolism.[3] The reductive pathway causes the production of 2-chloro-1,1,1-trifluoroethane (CTE) and 2-chloro-1,1-difluoroethylene (CDE), which are volatile metabolites of halothane.[4] These anaerobic reactions are catalysed by a P-450-dependent mixed-function oxidase system in liver microsomes via radical intermediates.[5] It has been suggested that the intermediate product of halothane biodegradation at a low oxygen tension may play a major role in halothane-induced hepatic injury. Therefore, one possible mechanism of halothane hepatotoxicity is the covalent binding of anaerobic metabolites to microsomes and then lipid peroxidation of microsomal lipids.[6]

Isoflurane (1-chloro-2,2,2,-trifluoroethyl difluoromethyl ether), a methyl ethyl ether, is relatively new but also a widely used volatile inhalation anaesthetic. It is an isomer of enflurane but does not produce the electroencephalographic abnormalities characteristic of enflurane.[7] Its physicochemical stability is reflected in its resistance to biodegradation: 95% of isoflurane administered is exhaled and less than 0.2% only is biodegraded to metabolites that are excreted in urine as fluoride and trifluoroacetic acid,[8] where as it is recognized that 12–25% of the absorbed halothane is metabolized in the body. The near-absence of isoflurane metabolism suggests that isoflurane is not nephrotoxic or hepatotoxic because the importance of anaesthetic metabolism lies in the association between metabolism and liver and kidney toxicity. Data from animal and human studies support this predicted lack of toxicity.

The degree of isoflurane metabolism is so small as to be without biological significance and it would not be expected to interfere with the biotransformation of other xenobiotics. However, Fiserova-Bergerova reported that isoflurane can significantly inhibit the oxidative metabolism of halothane and increases its reductive metabolism *in vivo*, as indicated by reduced concentrations of total non-volatile fluorine in tissues and increased concentrations of volatile metabolites in the liver, respectively.[9]

The purpose of this study is to clarify the effect of isoflurane on the anaerobic metabolism of halothane in liver microsomes of guinea pigs, and help to assess the role of isoflurane in lipid peroxidation and hepatic damage after halothane anaesthesia.

EXPERIMENTAL

This study was carried out according to the Guideline on Experimentation in Research Facilities for Laboratory Animal Science, School of Medicine, Hiroshima University.

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Materials

Halothane was obtained from Hoechst Japan Co., NADPH was purchased from Boehringer Mannheim (Germany) and isoflurane was provided by Dainabot Co., Japan; CDE and CTE were purchased from PCR Research Chemical (USA) and were used to determine retention times and to construct calibration curves. All other reagents were of analytical grade.

Animals

Adult male Hartley guinea pigs were used, each weighing ca. 250 g. The guinea pigs were sacrificed by a blow to the head and the livers were excised immediately. After irrigation with ice-cold physiological saline solution through the portal vein to remove blood, the livers were homogenized in 0.1 M potassium phosphate buffer (pH 7.4). The homogenates were centrifuged at 9000 *g* for 20 min and then the supernatants were centrifuged further at 105000 *g* for 60 min. The microsomal pellets obtained from the centrifugation were then rinsed with 0.1 M potassium phosphate buffer (pH 7.4) to remove cytoplasm. The rinsed microsomal fractions were then suspended in 0.1 M potassium phosphate buffer (pH 7.4) to prepare for the reaction mixture. All the above procedures were carried out at 4°C.

Incubation systems

For anaerobic conditions, the air in a 14-ml test tube was replaced with nitrogen and the test tube was sealed with a parafilm-covered rubber cap; the film was made by the American Can Company (USA). The reaction mixture consisted of a microsomal suspension obtained from 0.1 g of liver (0.9 ml), 3 mM NADPH (0.1 ml), halothane (0.13 mM, 0.2 mM, 0.34 mM, 0.67 mM and 1.35 mM) and isoflurane (0 mM (control group), 0.12 mM (0.26%), 0.29 mM (0.64%), 0.58 mM (1.30%) and 1.16 mM (2.59%)). The final volume was 1 ml. Every group had five replicates. After incubation at 37°C for 10 min the reaction was started by adding NADPH and isoflurane (except for the control groups) to the microsomal suspension and then halothane was added by microsyringe and the reaction was allowed to continue at 37°C for 10 min. Then 0.5 ml of gas from the test tube was analysed by gas chromatography. Cytochrome P-450 was measured spectrophotometrically following the procedure of Omura and Sato.[10]

Analysis of CDE and CTE

The gas chromatograph was a Shimadzu GC-4B equipped with a flame ionization detector to measure CDE and CTE. Gas samples (0.5 ml) from the incubation systems were injected into the gas chromatograph using a gas-tight glass syringe. The operating conditions for gas chromatography were slightly modified following the method of Mukai *et al.*[11]

Statistical analysis

The formation of volatile metabolites was determined by calculating peak heights obtained from gas chromatography with calibration of known concentrations of authentic CDE and CTE. Then the K_m (concentration of substrate resulting in half maximum velocity) and the V_{max} (maximum velocity) for CDE and CTE formation were calculated by plotting the Lineweaver–Burk plots using a Kaleida® graph (Version 2.1.3; Synergy Software, PA, USA) for the Macintosh® (Apple, CA, USA), where 1/halothane concentration was plotted on the *x*-axis and 1/production of CDE and CTE in different concentrations of isoflurane was plotted on the *y*-axis. Means and standard deviations were computed for all data in each group. The data were analysed with Student's *t*-test (unpaired) by computer program Stat view® 4.0 (Abacus Concepts Inc., CA, USA) for the Macintosh® (Apple, CA, USA); $P < 0.05$ was taken to indicate a significant difference.

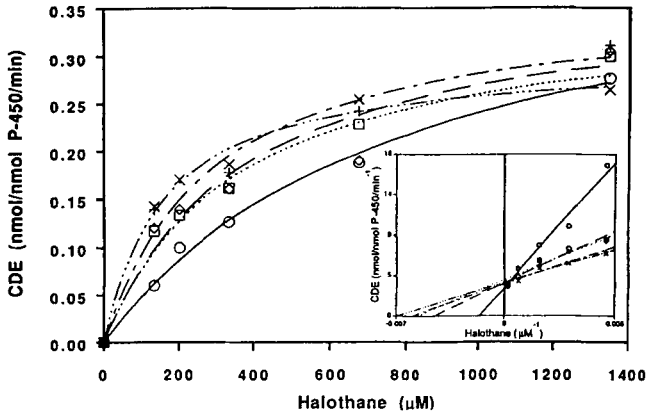

Figure 1. The 1-ml volume of the final reaction mixture consisted of a hepatic microsomal suspension obtained from 0.1 g of liver (0.9 ml), 3 mM NADPH (0.1 ml), halothane (0.13 mM, 0.2 mM, 0.34 mM, 0.67 mM and 1.35 mM) and isoflurane (0 mM (control group), 0.12 mM, 0.29 mM, 0.58 mM and 1.16 mM). The reaction was started and continued for 10 min after incubation at 37°C for 10 min. Then 0.5 ml of gas from the test tube was analysed by gas chromatography. We calculated the K_m and the V_{max} for CDE formation by Lineweaver–Burk plots, where 1/halothane conc. was plotted on the *x*-axis and 1/production of CDE in different concentrations of isoflurane was plotted on the *y*-axis: (——○), (– –□), (· · ·◇), (– · · ·–×) and (— – —+) represent 0 mM (control), 0.12 mM, 0.29 mM, 0.58 mM and 1.16 mM isoflurane, respectively.

RESULTS

The formation of CDE and CTE increased gradually as the concentrations of halothane increased from 0.13 mM to 1.35 mM. At each halothane concentration, the formation of CDE and CTE increased significantly with increasing isoflurane, compared with the formation of those products in the control groups that received 0 mM isoflurane (Figs 1 and 2).

The K_m and V_{max} for the formation of CDE and CTE were determined. The K_m values for CDE formation were 601.61 ± 266.91, 254.22 ± 86.58, 257.92 ± 129.11, 268.55 ± 125.66 and 319.22 ± 86.76 μM (mean ± SD, $n = 5$) at 0 mM (0%), 0.12 mM (0.26%), 0.29 mM (0.64%), 0.58 mM (1.30%) and 1.16 mM (2.59%) isoflurane, respectively. The K_m values for CTE formation were 1204.74 ±

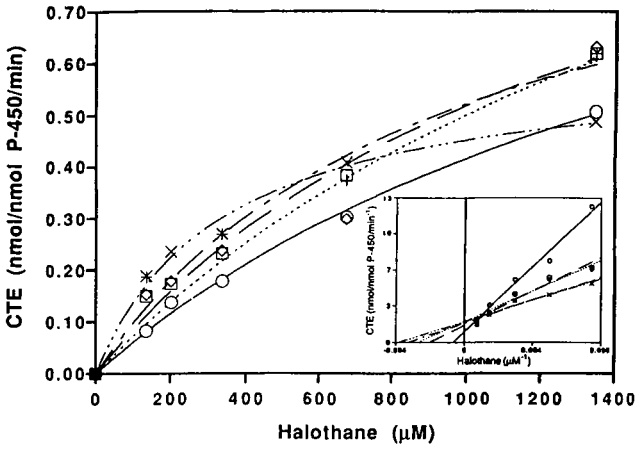

Figure 2. The 1-ml volume of the final reaction mixture consisted of a hepatic microsomal suspension obtained from 0.1 g of liver (0.9 ml), 3 mM NADPH (0.1 ml), halothane (0.13 mM, 0.2 mM, 0.34 mM, 0.67 mM and 1.35 mM) and isoflurane (0 mM (control group), 0.12 mM, 0.29 mM, 0.58 mM and 1.16 mM). The reaction was started and continued for 10 min after incubation at 37°C for 10 min. Then 0.5 ml of gas from the test tube was analysed by gas chromatography. We calculated the K_m and the V_{max} for CTE formation by Lineweaver–Burk plots, where 1/halothane conc. was plotted on the *x*-axis and 1/production of CTE at different concentrations of isoflurane was plotted on the *y*-axis (——○), (– –□), (· · · ◇), (– · · · –×) and (— – —+) represent 0 mM (control), 0.12 mM, 0.29 mM, 0.58 mM and 1.16 mM isoflurane, respectively.

551.64, 553.75 ± 177.89, 521.14 ± 249.77, 560.67 ± 229.61 and 711.05 ± 317.13 μM (n = 5) at 0 mM (0%), 0.12 mM (0.26%), 0.29 mM (0.64%), 0.58 mM (1.30%) and 1.16 mM (2.59%) isoflurane, respectively. Up to 0.58 mM (1.30%) isoflurane the K_m values for CDE and CTE were significantly ($P < 0.05$) lower than in the control groups, but at 1.16 mM (2.59%) isoflurane the changes were not significant (Fig. 3).

The V_{max} values for CDE formation were 0.429 ± 0.112, 0.359 ± 0.043, 0.374 ± 0.084, 0.391 ± 0.069 and 0.418 ± 0.083 nmol (nmol P-450)$^{-1}$ min^{-1} (mean ± SD, n = 5) at 0 mM (0%), 0.12 mM (0.26%), 0.29 mM (0.64%), 0.58 mM (1.30%) and 1.16 mM (2.59%) isoflurane, respectively. The V_{max} values for

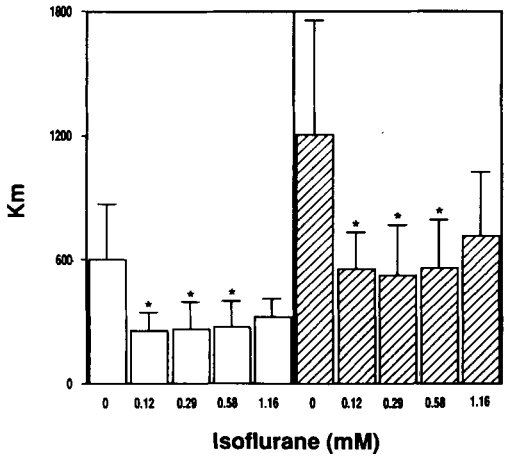

Figure 3. Mean K_m (±SD) for CDE and CTE formations were plotted on the *y*-axis: (□) K_m for CDE formation, (▨) K_m for CTE formations.* Significant differences ($P < 0.05$) compared with the control groups. Each bar represents the mean of five experiments.

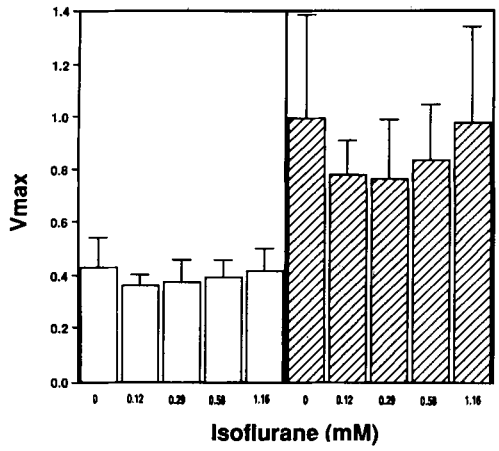

Figure 4. Mean V_{max} (±SD) for CDE and CTE formations were plotted on the *y*-axis: (□) V_{max} for CDE formation, (▨) V_{max} for CTE formation. No significant differences compared with the control groups were observed. Each bar represents the mean of five experiments.

CTE formation were 0.995 ± 0.391, 0.781 ± 0.131, 0.768 ± 0.224, 0.840 ± 0.211 and 0.978 ± 0.360 nmol (nmol P-450)$^{-1}$ min^{-1} (n = 5) at 0 mM (0%), 0.12 mM (0.26%), 0.29 mM (0.64%), 0.58 mM (1.30%) and 1.16 mM (2.59%) isoflurane, respectively. There were no significant differences in the V_{max} values for CDE and CTE formation at the different isoflurane concentrations compared with the control groups (Fig. 4).

DISCUSSION

In this study we found that the formation of both CDE and CTE was significantly increased in the presence of isoflurane.

The K_m values for CDE and CTE formation were low up to 0.58 mM (1.30%) isoflurane (Fig 3). However, there was almost no difference in the V_{max} values for CDE and CTE formation due to the addition of isoflurane (Fig. 4). These data may reflect the activation of anaerobic dehalogenation of halothane caused by an increased affinity of halothane to cytochrome P-450.

According to our results, K_m and V_{max} can be used to identify changes in the formation of CDE and CTE, and thus we strongly support the hypothesis that isoflurane accelerates the anaerobic metabolism of halothane. These effects were not strictly dose dependent. At isoflurane concentrations up to 0.58 mM the K_m values for CDE and CTE formation were significantly lower than in the control groups, but at 1.16 mM (2.59%) isoflurane the differences in K_m were not significant (Fig. 3).

Halogenated inhalation anaesthetics are believed to be metabolized by various enzymes belonging to the hepatic mixed-function oxidase system.[12] Halothane has a high affinity for binding to cytochrome P-450 under anaerobic conditions[13] and cytochrome P-450 catalyses both the oxidative and reductive metabolism of halothane.[14,15] From *in vivo* studies of animals, Fiserova-Bergerova found that inhibition of oxidative

metabolism and promotion of reductive halothane metabolism by isoflurane results from the interference of isoflurane with the oxygen-generating system.[9] In contrast, in our *in vitro* experiment reductive halothane metabolism was promoted by isoflurane when oxygen was absent.

Under anaerobic conditions, a radical intermediate such as $\cdot CF_3CHCl$ is produced by halothane metabolism. It has been suggested that this free radical increases binding to phospholipids of the endoplasmic reticulum and thus results in loss of structural integrity of the endoplasmic reticulum and then hepatic necrosis.[16,17] It can be presumed, therefore, that reductive metabolites of halothane initiate microsomal lipid peroxidation.

In the present study K_m was lowered in the presence of isoflurane, which shows that isoflurane increases the affinity of halothane for binding to cytochrome P-450 under anaerobic conditions. This indicates that isoflurane interferes with the halothane–cytochrome P-450 complex. Isoflurane may act on cytochrome P-450 directly, or on the halothane–cytochrome P-450 complex or on both.

Further studies are required to clarify the mechanism by which isoflurane interferes with the halothane–cytochrome P-450 complex. Whatever the mechanism, it is clear that isoflurane increases the affinity of halothane for cytochrome P-450 during reductive halothane metabolism and thus isoflurane activates the anaerobic dehalogenation of halothane, which may eventually increase lipid peroxidation and hepatic damage.

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REFERENCES

1. G. L. Brody and R. B. Sweet, Halothane anesthesia as a possible cause of massive hepatic necrosis. *Anesthesiology*, **24**, 29–37 (1963).
2. J. Lindenbaum and E. Leifer, Hepatic necrosis associated with halothane anesthesia. *N. Eng. J. Med.* **268**, 525–530 (1963).
3. K. Rehder, J. Forbes, H. Alter, O. Hessler and A. Stier. Halothane biotransformation in man: a quantitative study. *Anesthesiology* **28**, 711–715 (1967).
4. K. Fujii, M. Morio and H. Kikuchi, A possible role of cytochrome P-450 in anaerobic dehalogenation of halothane. *Biochem. Biophys. Res. Commun.* **101**, 1158–1163 (1981).
5. K. Fujii, N. Miki, M. Kanashiro, R. Miura, T. Sugiyama, M. Morio, T. Yamano and Y. Miyake. A spin trap study on anaerobic dehalogenation of halothane by a reconstituted liver microsomal cytochrome P-450 enzyme system. *J. Biochem.* **91**, 415–418 (1982).
6. S. Akita, M. Kawahara, T. Takeshita, M. Morio and K. Fujii, Halothane-induced hepatic microsomal lipid peroxidation in guinea pigs and rats. *J. Appl. Toxicol.* **9**, 9–14 (1989).
7. H. W. Linde, V. E. Lamb, C. W. Quimby, J. Horniand and J. E. Eckenhoff. The search for better anesthetic agents: clinical investigation of ethrane. *Anesthesiology* **32**, 555–559 (1970).
8. D. A. Holaday, V. Fiserova-Bergerova, I. P. Latto and M. A. Zumbiel, Resistance of isoflurane to biotransformation in man. *Anesthesiology* **43**, 325–332 (1975).
9. V. Fiserova-Bergerova, Inhibitory effect of isoflurane upon oxidative metabolism of halothane. *Anesth. Analg.* **63**, 399–404 (1984).
10. T. Omura and R. Sato, The carbonmonoxide binding pigment of liver microsomes. *J. Biol. Chem.* **239**, 2370–2378 (1964).
11. S. Mukai, M. Morio, K. Fujii and C. Hanaki, Volatile metabolites of halothane in the rabbit. *Anesthesiology* **47**, 248–251 (1977).
12. K. M. Ivanetich, S. A. Lucas and J. A. Marsh, Enflurane and methoxyflurane: their interaction with hepatic cytochrome P-450 in vitro. *Biochem. Pharmacol.* **28**, 785–792 (1979).
13. W. Nastainczyk and V. Ullrich, *The Interaction of Halothane with Liver Microsomal Cytochrome P-450, Microsomes and Drug Oxidations*, ed. by V. Ullrich, pp.254–260. Pergamon Press, New York (1976).
14. B. R. Brown and I. G. Sipes. Biotransformation and hepatotoxicity of halothane. *Biochem. Pharmacol.* **26**, 2091–2094 (1977).
15. R. M. Maiorino, I. G. Sipes, A. J. Gandolfi, B. R. Brown and R. C. Lind, Factors affecting the formation of chlorotrifluoroethane and chlorodifluoroethylene from halothane. *Anesthesiology* **54**, 383–389 (1981).
16. J. R. Trudell, B. Bernhard and A. J. Trevor, Reductive halothane metabolism by human and rabbit cytochrome P-450, binding of 1-chloro-2,2,2-trifluoroethyl radical to phospholipids. *Mol. Pharmacol.* **21**, 710–717 (1982).
17. S. Akita, M. Morio, M. Kawahara, T. Takeshita, K. Fujii and M. Yamamoto. Halothane induced liver injury as a consequence of enhanced microsomal lipid peroxidation in guinea pigs. *Res. Commun. Chem. Pathol. Pharmacol.* **61**, 227–243 (1988).