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Isoflurane Enhances Dechlorination of Carbon Tetrachloride in Guinea-pig Liver Microsomes

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Key words: isoflurane; carbon tetrachloride; chloroform; dechlorination.

Effect of isoflurane on the dechlorination of carbon tetrachloride to chloroform was investigated in the guinea-pig liver microsomes. Under anaerobic conditions, chloroform is produced from carbon tetrachloride through the microsomes in the presence of NADPH, and such production of chloroform was increased by the addition of isoflurane. The K_m for the production of chloroform from carbon tetrachloride was decreased to 86% by isoflurane compared with the control; however the maximum velocity of chloroform production was also decreased to 50%. The formation of the 445 nm band in the mixture of reduced cytochrome P-450 and carbon tetrachloride, and cytochrome P-450 reduction by NADPH were both accelerated by isoflurane, without alteration of NADPH–cytochrome *c* reductase activity. These results indicate that trichloromethyl radical, an intermediate product of carbon tetrachloride, easily combines to the haeme part of cytochrome P-450, whereas the protein part combines to isoflurane after being reduced by NADPH, which results in acceleration of carbon tetrachloride dechlorination under a lower concentration of carbon tetrachloride. These results may have implications for other drugs that are administered during isoflurane anaesthesia.

INTRODUCTION

Isoflurane, 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, is a modern inhaled anaesthetic. This compound is the least metabolized among the florinated inhaled anaesthetics.[1,2] The limited metabolism of isoflurane is mostly the result of alpha carbon oxidation to produce trifluoroacetic acid and inorganic fluoride.[3,4] In fact, a small amount of organic fluoride was detected in the urine of rats that inhaled isoflurane, and the organic fluorine was strongly suspected to be trifluoroacetic acid.[5] This reaction is likely to occur in the liver microsomal mixed-function oxidase system. From urine samples taken over 72 h after anaesthesia, Holaday *et al.* calculated the overall biodegradation rate of isoflurane to be less than 0.2% (w/w) on the basis of organic fluorine. The limited amount of fluoride ion excreted in urine was considered to be so small as to be without biological significance.[1]

The limited metabolism of isoflurane would not be expected to interfere with the biotransformation of other xenobiotics. However, it has been suggested that the aerobic dehalogenation of halothane *in vivo* was significantly inhibited in rats that inhaled isoflurane. In contrast, reductive metabolism of halothane was slightly increased by the inhalation of isoflurane.[6] We have also shown that isoflurane interacts with cytochrome P-450 to increase the production of CDE and CTE, two anaerobic metabolites of halothane. This occurs in guinea-pig liver microsomes in the presence of NADPH, by reducing the K_m for anaerobic dehalogenation of halothane. This suggests that isoflurane induces the activity of the liver microsomal mixed-function oxidase system.[7]

The production of CDE and CTE from halothane is catalysed by the liver mixed-function oxidase system, including cytochrome P-450, under anaerobic conditions. In the reaction, a radical intermediate combines to the haeme part of cytochrome P-450.[8–10] Carbon tetrachloride is also catalysed by the liver mixed-function oxidase system, including cytochrome P-450 to cleave the CCl_3—Cl bond, resulting in a trichloromethyl radical.[11] This radical also combines to the haeme part of cytochrome P-450.[11] Because the mechanism of carbon tetrachloride metabolism seems likely to be the same as anaerobic halothane metabolism, we expect that dehalogenation of carbon tetrachloride is accelerated by isoflurane.

In this study, enhancement of chloroform production from carbon tetrachloride by the microsomal enzyme system in the presence of isoflurane was observed under anaerobic conditions. We observed that isoflurane enhanced the reductive dehalogenation of carbon tetrachloride by the liver microsomal mixed-function oxidase system and have discussed the mechanisms herein.

EXPERIMENTAL

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

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Reagents

Carbon tetrachloride of analytical grade was obtained from Kanto Chem (Tokyo, Japan). Isoflurane prepared for clinical uses was obtained from Dynabot (Osaka, Japan). All other reagents were of analytical grade.

Animals

Male Hartley guinea pigs with body weights of 225–275 g were used. The animals were sacrificed after being starved for 24 h followed by excision of the liver. After perfusing the livers through the portal vein with ice-cold physiological saline, the livers were homogenized in 0.05 M potassium phosphate buffer. Following the centrifugation of the liver homogenate at 9000 *g* for 20 min, the supernatant was centrifuged again at 105 000 *g* for 60 min. The resulting sediment was then suspended in 0.1 M potassium phosphate buffer and used as a liver microsome suspension.

Assay of dehalogenation of carbon tetrachloride

After charging 12.3-ml butyl-rubber-capped test tubes with nitrogen, the mirosome suspension (0.9–1.2 nmol of P-450), nicotinamide adenine dinucleotide phosphate (NADPH: final concentration 2.1 mM), carbon tetrachloride (final concentrations of 38.5, 76.9, 189 and 385 μM) and isoflurane (final concentrations of 0, 37, 74, 184, 370 and 740 μM) were added to a final volume of 1 ml. After 7 min of incubation 37°C, 0.5 ml of the gas phase was taken to measure chloroform by gas chromatography. The K_m value for dehalogenation of carbon tetrachloride was estimated by a double reciprocal plot.

Assay of cytochrome *c* reductase

The reaction system consisted of guinea-pig liver microsomes (0.015–0.02 g liver wet wt.), 0.6 mM KCN and 0.33 mM cytochrome *c* in 0.1 M potassium phosphate buffer (pH 7.4) with and without isoflurane. The reaction was started by the addition of a 30-μl aliquot of 0.01 M NADPH. The changes in absorbance at 550 nm were recorded by a spectrophotometer (Shimadzu UV-300). The experiment was performed at 25°C.

Assay of the cytochrome P-450 reduction rate

In an anaerobic cuvette sealed with a rubber cap, containing 3.0 ml of liver microsomal suspension (0.3 g liver wet wt.) in 0.1 M potassium phosphate buffer (pH 7.4), oxygen-free carbon monoxide was blown for 5 min and then isoflurane (final concentration 1.8 mM) was injected (except in the control cuvette). A 50-μl aliquot of 0.05 M NADPH was injected through the rubber cap to initiate the reaction. The changes in absorbance at 450 nm were recorded by a Shimadzu UV-300 spectrophotometer to measure the CO–cytochrome P-450 complex at various times (P-450v) until the reaction was completed. After removal of the rubber cap, a few crystals of sodium dithionite were added to both the experimental and control cuvette, and the absorbances were measured at both 450 and 490 nm for the estimation of total cytochrome P-450 content (P-450t). The difference between the total cytochrome P-450 content (P-450t) and the amount of CO–cytochrome P-450 complex formation at various time (P-450v) was the unreduced amount of cytochrome P-450. The unreduced amounts of cytochrome P-450 (P-450t–P-450v) were plotted on semilogarithmic paper. The rate constant of the initial phase, *k*, was estimated by computer (Macintosh) using Cricket Graph from the initial phase of logarithmic plots. The half-time, $t_{1/2}$, for the initial phase was calculated from the formula $t_{1/2} = 0.693/k$.[12] The experiment was performed at 25°C.

Assay of the 445-nm absorption band formation rate

In an anaerobic cuvette sealed with a rubber cap containing 3.0 ml of liver microsomal suspension (0.3 g liver wet wt.) in 0.1 M potassium phosphate buffer (pH 7.4), oxygen-free nitrogen was blown for 5 min and isoflurane (final concentration 920 μM) and/or carbon tetrachloride (final concentration 530 μM) were injected into the experimental cuvette through the rubber cap. A 50-μl aliquot of 0.05 M NADPH was injected into the experimental and the reference cuvette through the rubber caps. The changes in absorbance at 445 nm were recorded by a Shimadzu UV-300 spectrophotometer until the reaction was completed. The experiment was performed at 25°C.

Assay of protein

Protein contained in microsomes was measured using the method of Lowry *et al.*[13]

Statistical analysis

The ANOVA and Student's *t*-test was used for statistical analysis of the results, with $P < 0.05$ being considered as significant.

RESULTS

Effects of isoflurane on the dechlorination of carbon tetrachloride

Under anaerobic conditions, carbon tetrachloride produced chloroform in the presence of NADPH, and the production of chloroform was increased due to addition of isoflurane in guinea-pig liver microsomal suspension. The K_m value for the production of chloroform from carbon tetrachloride was 640 μM in the control group, which was decreased to 90 μM by 740 μM isoflurane. However, the maximum velocity of the chloroform production in the control group (0.54 nmol P-450^{-1} min^{-1}) was decreased to 0.26 nmol P-450^{-1} min^{-1} by 740 μM isoflurane (Fig. 1).

Effects of isoflurane on the cytochrome *c* reduction rate

The first step of carbon tetrachloride dechlorination is the reduction of haeme of cytochrome P-450, which

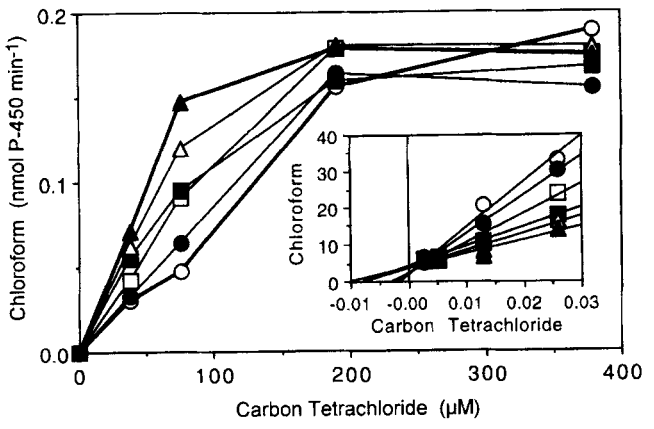

Figure 1. Effects of isoflurane on the formation of chloroform. Reaction system: guinea-pig liver microsomes (0.9–1.2 nM P-450), NADPH (final concentration 2.1 mM), carbon tetrachloride (final concentrations 38.5, 76.9, 189 and 385 μM) and isoflurane (final concentrations 0.37, 74, 184, 370 and 740 μM) in a final volume of 1 ml. After 7 min of incubation at 37°C, 0.5 ml of the gas phase was taken to measure chloroform by gas chromatography. The K_m value for dehalogenation of carbon tetrachloride was estimated by a double reciprocal plot: (○) control; (●) 37 μM isoflurane; (□) 74 μM isoflurane; (■) 185 μM isoflurane; (△) 370 M isoflurane; (▲) 740 μM isoflurane. Each value was the mean of six samples.

forms the complex with carbon tetrachloride by NADPH–cytochrome P-450 reductase. The NADPH–cytochrome P-450 reductase can reduce cytochrome *c* in the presence of NADPH. The NADPH-cytochrome *c* reduction activity was not altered in the presence of isoflurane up to 1.8 mM. Thus, the NADPH-cytochrome P-450 reductase was not affected by isoflurane (Fig. 2).

Effects of isoflurane on the cytochrome P-450 reduction rate

The rate of cytochrome P-450 reduction by NADPH was estimated by recording the formation of the carbon monoxide complex of cytochrome P-450. The formation of the carbon monoxide complex of cytochrome

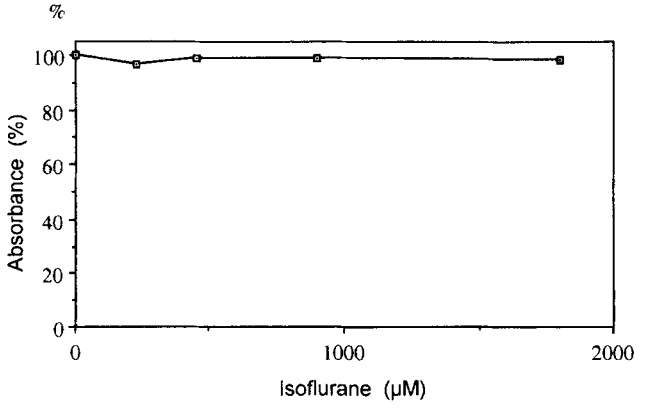

Figure 2. Effects of isoflurane on the cytochrome *c* reductase. The reaction system consisted of guinea-pig microsomes (0.015–0.02 g liver wet w.ml^{-1}), 0.6 mM KCN and 0.33 mM cytochrome *c* in 0.1 M potassium phosphate buffer (pH 7.4), with and without isoflurane. The reaction was started by the addition of a 30-μl aliquot of 0.01 M NADPH. The changes in absorbance at 550 nm were recorded by a spectrophotometer. The experiment was performed at 25°C.

Table 1. Effect of isoflurane on the rate of cytochrome P-450 reduction in guinea-pig liver microsomes[a]

	Rate constant of initial phase (s^{-1})	$t_{1/2}$ (s)
Control	0.014 ± 0.004	49.5
Isoflurane	0.025 ± 0.006	27.7

[a]The reduction rate of cytochrome P-450 in guinea-pig liver microsomal suspension (0.1 g liver wet wt.) in a final volume of 3.0 ml of 0.1 M potassium phosphate buffer (pH 7.4) in the presence of isoflurane (1.8 mM) was measured as described in the experimental section. The rate constant of the initial phase, *k*, was determined by a computer (Macintosh) using Cricket Graph from the initial phase of logarithmic plots. The half-time, $t_{1/2}$, for the initial phase was calculated from the formula $t_{1/2} = 0.693/k$. The data represent the means and standard deviation of six samples. The experiment was performed at 25°C.

P-450 was significantly increased in the presence of isoflurane (Table 1).

Effects of isoflurane on the 445-nm absorption band formation rate

During the formation of chloroform from carbon tetrachloride, an intermediate complex which can be recognized by the spectrophotometer at 445 nm absorption is formed. The 445-nm absorption band formation rate was significantly stimulated by the addition of isoflurane (Fig. 3).

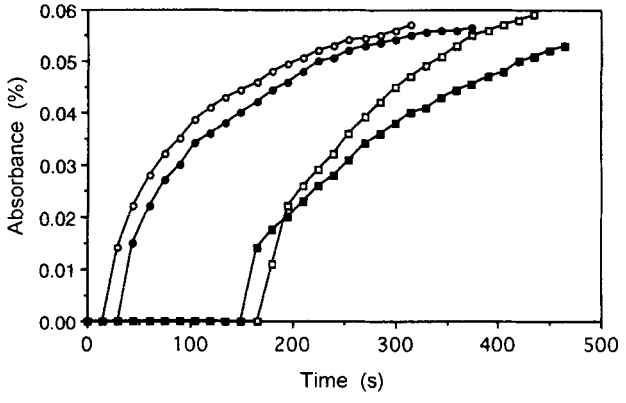

Figure 3. Effects of isoflurane on the 445-nm absorption band formation rate. In an anaerobic cuvette sealed with a rubber cap containing 3.0 ml of liver microsomal suspension (0.3 g of liver wet wt.) in 0.1 M potassium phosphate buffer (pH 7.4), oxygen-free carbon monoxide was blown for 5 min and isoflurane (final concentration 920 μM) and/or carbon tetrachloride (final concentration 530 μM) were injected into the experimental cuvette through the rubber cap. A 50-μl aliquot of 0.05 M NADPH was injected into both the experimental and reference cuvettes through the rubber cap. The changes in absorbance at 445 nm were recorded by a Shimadzu UV-300 spectrophotometer until the reaction was completed: (○, ●) with isoflurane; (□, ■) without isoflurane. The experiment was performed at 25°C.

Destruction of cytochrome P-450 during dehalogenation of carbon tetrachloride

Cytochrome P-450 content was decreased to 71.6 ± 3.8% (mean ± SD; $n = 5$) after 7 min of incu-

bation of microsome with 76.9 μM carbon tetrachloride. Addition of 370 μM isoflurane in the same incubating condition decreased the cytochrome P-450 content to 82.0 ± 3.2% (mean ± SD; $n = 5$).

DISCUSSION

Carbon tetrachloride underwent dehalogenation to chloroform by guinea-pig liver mirosomes in the presence of NADPH, and the production of chloroform was increased by the addition of isoflurane. The K_m value for the production of chloroform from carbon tetrachloride was decreased to 86% of the control value by 740 μM isoflurane, however the maximum velocity of the chloroform production was decreased to 50% of the control value by the same isoflurane concentration (Fig. 1). These results may indicate that the affinity of cytochrome P-450 to carbon tetrachloride increased by isoflurane and induced dechlorination of carbon tetrachloride.

In suspensions of guinea-pig liver microsomes in aerobic and anaerobic conditions, isoflurane or carbon tetrachloride produced an absorption spectrum with a maximum at 390 nm and a minimum at 420 nm, which is a characteristically a type I spectrum. However, the carbon tetrachloride-induced type I spectrum was changed due to the addition of NADPH in anaerobic conditions, and this produces an absorption spectrum with a maximum at 445 nm and a minimum at 420 nm, a characteristically modified type II spectrum (data not shown). The substrates which show type I difference spectra combine with the protein part of P-450, and the substrates which show modified type II difference spectra combine with the haeme part of P-450.[11]

A possible mechamism for the dechlorination of carbon tetrachloride is as follows: oxidized cytochrome P-450 reacts with substrates to form a cytochrome P-450–substrate complex; type I complexes are then reduced by NADPH–cytochrome P-450 reductase; carbon tetrachloride undergoes cleavage of the CCl_3—Cl bond, resulting in formation of a trichloromethyl radical; the trichloromethyl radical changes its binding sight to the haeme part of cytochrome P-450; this complex, which shows a modified type II spectrum, is in turn subsequently decomposed, with the formation of chloroform and oxidized cytochrome P-450.[11]

Chemical compounds that produce type I spectra accelerate the initial rate of NADPH-linked cytochrome P-450 reduction in the rat liver microsomes.[14] On the other hand, Sasame and Gillette reported that the type I substances did not alter the rate of cytochrome P-450 reduction of *p*-nitrobenzoate reduction in mice liver microsomes.[12] Thus, type I substances do not uniformly react to the reduction of cytochrome P-450. From our results, the rate of cytochrome P450 reduction in the guinea-pig liver microsomes was increased by isoflurane, a type I substrate (Fig. 3).

The formation rate of the trichloromethyl radical–cytochrome P-450 complex and cytochrome P-450 reduction after the addition of NADPH were also stimulated by excess of isoflurane (Fig. 3 and Table 1). The alteration in the absorbance spectra at 445 nm after the addition of NADPH reflects the change in either the structure or the kinetic properties of cytochrome P-450.[15,16] These effects due to alterations in the activity of NADPH–cytochrome P-450 reductase are not acceptable, because isoflurane did not alter cytochrome *c* reductase (Fig. 2). Destruction of cytochrome P-450 during metabolism of carbon tetrachloride was prevented by 10.4% by 370 μM isoflurane, however chloroform formation was induced to 250% of control by the same concentration of isoflurane (Fig. 1). These results suggest that the role of isoflurane in preventing cytochrome P-450 destruction was negligible on the enhancement of chloroform formation. The maximum velocity of chloroform production was decreased in the presence of isoflurane (Fig. 1). So, higher concentrations of carbon tetrachloride are expected to interact greater than are lower concentrations with isoflurane on the protein part of cytochrome P-450.

We conclude that isoflurane stimulates the binding between cytochrome P-450 and the trichloromethyl radical, resulting in acceleration of the dechlorination of carbon tetrachloride; these results may have implications for other drugs that are administered during isoflurane anaesthesia.

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