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The Association of Halothane-Induced Lipid Peroxidation with the Anaerobic Metabolism of Halothane: An *In Vitro* Study in Guinea Pig Liver Microsomes

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ABSTRACT

The formation of pentane and anaerobic metabolites of halothane (2-chloro-1,1,1-trifluoroethane and 2-chloro-1,1-difluoroethylene) in a mixture of guinea pig liver microsomes and halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) in the presence of NADPH was studied by gas chromatography. Under anaerobic conditions, pentane was formed without halothane and was inhibited by oxygen tension. This anaerobic pentane formation was potentiated 2.5 times by addition of halothane. Halothane-induced pentane formation increased dose-dependently with a halothane concentration of up to 2.1 mmol/liter and then decreased in the presence of increasing concentrations of halothane. Inhibition by a higher substrate was also observed in the formation of anaerobic metabolites of halothane. Antioxidant agents, vitamin E and glutathione, reduced the pentane formation, but did not reduce the anaerobic metabolites of halothane. Metyrapone, an inhibitor of cytochrome P-450, reduced both the pentane and anaerobic metabolites of halothane. These results show holothane-induced lipid peroxidation in association with the anaerobic metabolism of halothane in guinea pig liver microsomes.

Key words: *Halothane, Microsome, Liver, Lipid peroxides, Pentane*

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is a widely used volatile inhalational anesthetic. Its hepatotoxic reaction has been shown to be a great problem for anesthesiologists[3,11]. Halothane is metabolized to 2-chloro-1,1,1-trifluoroethane (CTFE) and 2-chloro-1,1-difluoroethylene (CDFE) in anaerobic conditions[8]. These were metabolized by NADPH-dependent liver microsomal enzyme systems[7]. It is thought that a radical intermediate of halothane such as $^{\bullet}CF_3CHCl$ is produced in association with the anaerobic metabolism of halothane[6], and that this causes lipid peroxidation resulting in hepatocelluar injury.

The possibility that radical intermediates of halothane are produced in the process of anaerobic metabolism have already been investigated *in vivo* and *in vitro*. Fujii et al[6] demonstrated in an *in vivo* study, the presence of radical adducts with N-tert-butyl-alpha-phenylnitrone (BPN) in the liver of a guinea pig by electron spin resonance measurement. Fujii et al[9] also reported that, in an *in vitro* study, radical intermediates of halothane were produced in the course of an anaerobic dehalogenation reaction in a reconstituted rabbit liver microsomal P-450 enzyme system. The radicals of these were trapped by BPN as a form of a BPN-radical adduct.

The question has arisen whether these radicals cause lipid peroxidation or not. Akita et al[2] demonstrated *in vivo* that halothane enhanced microsomal lipid peroxidation occurred in the liver of a guinea pig.

We previously demonstrated that pentane can be used as an index of *in vitro* lipid peroxidation *via* guinea pig liver microsomes with CCl_4[15]. In the present study, we show, by measuring pentane *in vitro*, that lipid peroxidation is enhanced by halothane in the microsomes of the guinea pig liver.

MATERIALS AND METHODS

Adult male guinea pigs weighing 225 g to 275 g were used in this experiment. After a 24-hour fast, the guinea pigs were sacrificed by a blow to the head and the livers were immediately excised. After irrigation with an ice-cold physiologic saline solution through the portal vein, the livers were homogenized with a Potter-Elvehjem teflon homogenizer in 0.05M potassium phosphate buffer (pH 7.4). The homogenates were centrifuged at 8,000 × *g* for 10 min. Supernatants were further centrifuged at 105,000 × *g* for 1 hr. The microsomes obtained were washed in 0.05 M potassium

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phosphate buffer (pH 7.4) by centrifugation at 105,000 × *g* for 1 hr. The washed microsomal fraction was suspended in 0.1 M potassium phosphate buffer (pH 7.4).

Under anaerobic conditions, nitrogen was sealed in a 12.3 ml silicon-capped test tube. The reaction mixture consisted of microsomal suspension in 0.1M potassium phosphate buffer (pH 7.4), 2.1 mmol/liter of NADPH and 4.8 mmol/liter of halothane in a final volume of 3 ml, except where otherwise mentioned. The reaction was started by adding halothane and NADPH and continued at 37°C, for 15 min. After the reaction, 0.5 ml of the gas phase was analyzed by gas chromatography.

Pentane, CTFE and CDFE concentration was measured by gas chromatography[8,15]. The gas chromatograph was a Shimadzu GC-4B model equipped with a flame ionization detector and glass column (5m × 4mm) packed with dioctylphtalate.

Protein was measured by the method employed by Lowry et al[12]. The halothane was obtained from Hoechst Japan Co. (Japan). NADPH was purchased from Boehringer Mannheim (West Germany). Metyrapone was obtained from Ciba Geigy Co. (Japan). All other reagents were of analytical grade.

RESULTS

Since redical intermediates produced in association with dehalogenation of halothane appears to be a initiator of lipid peroxidation, measurements in this experiment were carried out to obtain the information that the formation of pentane is closely correlated with the enzyme in the microsome. Selected experiments are 1) the effect of oxygen concentration on both pentane formation and CTFE and CDFE formation (Fig.1) 2) the time course of the generation of these end products (Fig. 2) 3) the relationship between pentane formation and NADPH which function as an energy-supplier to this reaction (Fig. 3) 4) the relationship between pentane formation and microsomal protein (Fig 4). Furthermore, to confirm the relation between anaerobic dehalogenation of halothane and pentane formation as an index of lipid peroxidation, the additional experiments were performed: 5) the relationship between halothane dose and pentane (Fig. 5), CTFE and CDFE formation (Fig. 6) 6) the effect of antioxidant agents, vitamin E (Table 1) and glutathione (Table 2) on pentane and CTFE and CDFE formation. 7) the effect of metyrapone, inhibitor of dehalogenation of halothane (Table 3) on pentane and CTFE and CDFE formation.

The pentane formation with and without halothane was decreased by increasing oxygen concentrations (left hand graph in Fig. 1). The formation of anaerobic metabolites of halothane (CTFE and CDFE) was also decreased with an increase in the oxygen concentration (right hand graph in Fig. 1). These results imply that the pentane formation was an anaerobic reaction and that the manner of

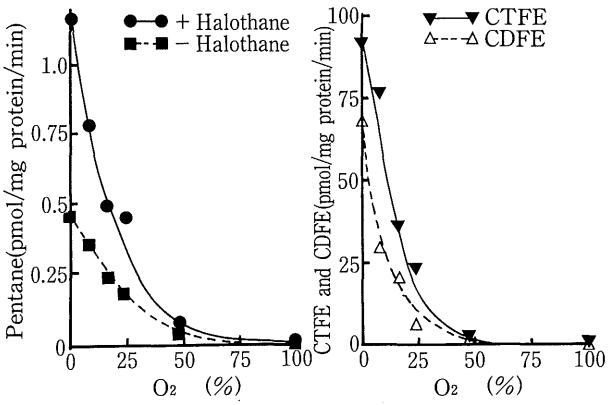

Fig.1. Effect of varying concentrations of oxygen in the gasphase on pentane formation (left) and 2-chloro-1,1,1-trifluoroethane (CTFE) and 2-chloro-1,1-difluoroethylene (CDFE) formation (right). The incubation system consisted of a microsomal suspension (protein concentration 0.40 mg/ml in 0.1 M potassium phosphate buffer, pH 7.4), NADPH (final, 2.1 mmol/liter) and halothane (final, 0.48 mmol/liter) in a final volume of 3 ml. Other experimental conditions are described in Materials and Methods.

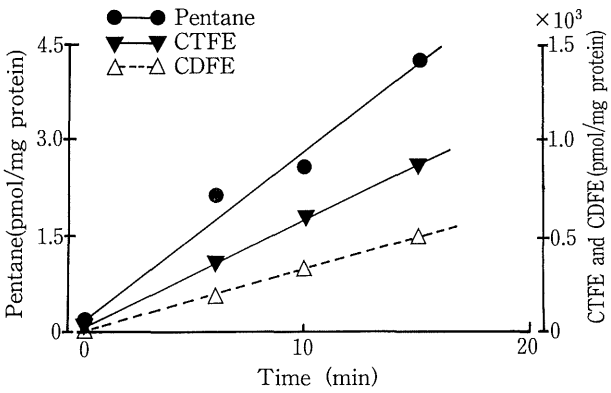

Fig.2. Rate of halothene-induced pentane, 2-chloro-1,1,1-trifluoroethane (CTFE) and 2-chloro-1,1-difluoroethylene (CDFE) formation under anaerobic conditions. The incubation system consisted of a microsomal suspension (protein concentration 0.40 mg/ml in 0.1 M potassium phosphate buffer, pH 7.4), NADPH (final, 2.1 mmol/liter) and halothane (final, 4.8 mmol/liter) in a final volume of 3 ml. Data of halothane induced pentane formatin were referred as to the difference in the amount of pentane formation with and without halothane. Other experimental conditions are described in Materials and Methods.

pentane formation in the relation to the oxygen tension resembles that of anaerobic metabolites of halothane. Since pentane formation without halothane was recognized as shown in the left graph in Fig. 1, the increase in pentane formation induced by halothane should be presented as the difference between the value of pentane with and without halothane. In the following results halothane induced pentane formation is referred as to the difference in the amount of pentane forma-

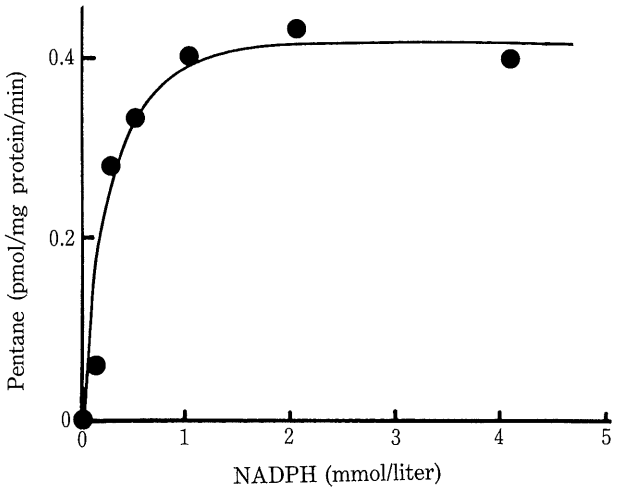

Fig.3. Effect of varying concentrations of NADPH on halothane induced pentane formation under anaerobic conditions. The incubation system consisted of a microsomal suspension (protein concentration 0.45 mg/ml in 0.1 M potassium phsphate buffe, pH 7.4), NADPH (final, 0.1, 0.3, 0.5, 1.1, 2.1, 4.1 mmol/liter) and halothane (final, 4.8 mmol/liter) in a final volume of 3 ml. Data of halothane induced pentane formation were referred as to the difference in the amount of pentane formation with and without halothane. Other experimental conditions are discribed in Materials and Methods.

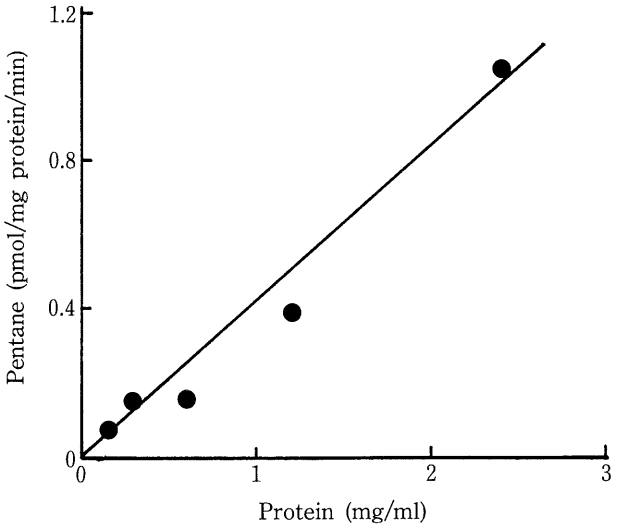

Fig.4. Relation of halothane-induced pentane formation and protein concentration of the microsomal suspension under anaerobic conditions. The incubation system consisted of a microsomal suspension (in 0.1 M potassium phosphate buffer, pH 7.4), NADPH (final, 2.1 mmol/liter) and halothane (final, 4.8 mmol/liter) in a final volume of 3 ml. Data of halothane-induced pentane formatin were referred as to the difference in the amount of pentane formation with and without halothane. Other experimental conditions are described in Materials and Methods.

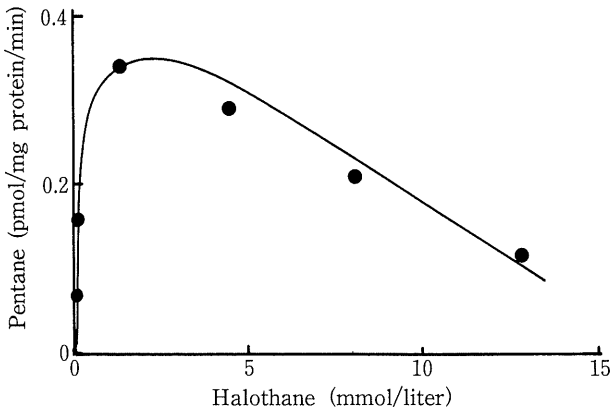

Fig.5. Dose-response relation between halothane concentration and halothane-induced pentane formatin. Halothane (final, 0-12.8 mmol/liter) was added to the incubation system which consisted of a microsomal suspension (protein concentration 0.47 mg/ml in 0.1M potassium phosphate buffer, pH 7.4) and NADPH (final, 2.1 mmol/litter) in a final volume of 3 ml. Data of halothane-induced pentane formatin were referred as to the difference in the amount of pentane formation with and without halothane. Other experimental conditions are described in Materials and Methods.

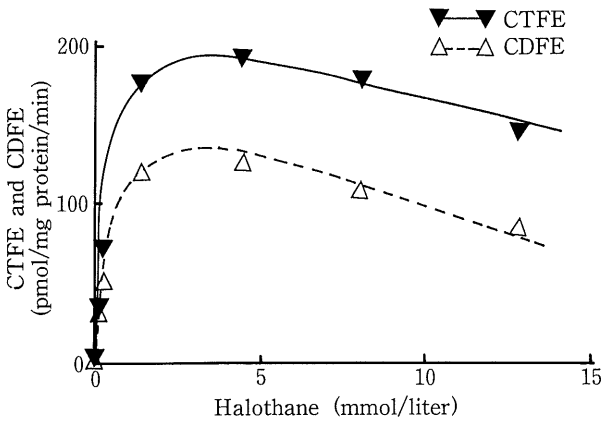

Fig.6. Dose-response relation between halothane concentration and halothane induced 2-chloro-1,1, 1-trifluorotethane (CTFE) and 2-chloro-1, 1-difluoroethylene (CDFE) formation. Halothane (final, 0-12.8 mmol/liter) was added to the incubation system which consisted of a microsomal suspension (protein concentration 0.47 mg/ml in 0.1M potassium phosphate buffe, pH 7.4) and NADPH (final, 2.1 mmol/liter) in a final volume of 3 ml. Other experimental conditions are described in Materials and Methods.

tion with and without halothane.

Fig. 2 shows the time course of pentane formation induced by halothane, CTFE and CDFE formation. There was a linear within 15 min.

Halothane induced pentane formation increased in response to NADPH concentrations and reached a maximum (0.41 pmol/mg protein/min) in the presence of 1.05 mmol/liter NADPH (Fig. 3). Pentane formation induced by halothane required

NADPH like the anaerobic metabolism of halothane[7].

Halothane induced pentane formation showed a linear responce to the protein concentration of the microsomal suspension in the range of 0-2.4 mg/ml protein (Fig. 4).

Pentane formation dose-dependently increased up to a halothane concentration of 1.4 mmol/liter at which the value of pentane reached 0.34 pmol/mg protein/min. Further increase in the application of halothane reduced pentane formation (Fig. 5). A similar change in the production of CTFE and CDFE, anaerobic metabolites of halothane, was also recognized: conspicuous reduction in CTFE and CDFE with an increased doseage of halothane (Fig 6). The manner of pentane formation induced by the change of halothane dose was also similar to that of anaerobic metabolism of halothane.

Table 1. Effect of Vitamin E on pentane, CDFE* and CTFE** formation***

Vitamin E (nmol/liter)	pentane	CDFE	CTFE
	(pmol/mg protein/min) [% inhibition]		
0	0.38[0]	60[0]	60[0]
15	0.20[47]	49[18]	54[10]
30	0.16[59]	44[27]	65[-8]
75	0.01[97]	51[15]	72[20]
100	0.02[95]	41[32]	65[-8]

* 2-chloro-1,1-difluoroethylene
** 2-chloro-1,1,1-trifluoroethane
*** Vitamin E (0, 15, 30, 75, 100 nmol/liter, solved with 10 microliter of etylalchol) was added to the incubation system. The incubation system consisted of a microsomal suspension (protein concentration 0.44 mg/ml in 0.1 M potassium phosphate buffer, pH 7.4), NADPH (2.1 mmol/liter, final), and halothane (4.8 mmol/liter, final) in a final volume of 3ml. Data of halothane-induced pentane formation were referred as to the difference in the amount of pentane formation with and without halothane. Other experimental conditions are described in Materials and Methods.

Both vitamin E (Table 1) and glutathione (GSH), (Table 2) which are antioxidants, sharply reduced halothane induced pentane formation. Without vitamin E, 0.38 pmol/mg protein/min of pentane was formed and reduced to 0.02 pmol/mg protein/min (95% inhibition) with 100 nmol/liter of vitamin E. CDFE and CTFE formation were not reduced by these doses of vitamin E. With no GSH, 0.32 pmol/mg protein/min of pentane was formed, and this was reduced to 0.05 pmol/mg protein/min (84% inhibition) with 5 μmol/liter of GSH. CDFE and CTFE formation were not reduced by these doses of GSH. Pentane formation induced by halothane was inhibited by these antioxidant agents, but anaerobic metabolites of halothane were not inhibited.

Halothane-induced pentane formation (0.41 pmol/mg protein/min) was reduced to 0.06 pmol/mg protein/min (84% inhibition) in the presence of 15.0 mmol/liter of metyrapone (Table 3). The formation of CDFE (81 pmol/mg protein/min) and CTFE (142 pmol/mg protein/min) in the presence of halothane were also reduced by 15.0 mmol/liter of metyrapone (80% and 86% inhibition respectively). Both pentane formation induced by halothane and anaerobic metabolites of halothane were inhibited by the P-450 inhibitor.

Table 2. Effect of GSH on pentane, CDFE* and CTFE** formation***

GSH (μmol/liter)	pentane	CDFE	CTFE
	(pmol/mg protein/min) [% inhibition]		
0.0	0.32[0]	68[0]	86[0]
0.1	0.20[38]	77[-13]	75[13]
0.5	0.11[63]	69[- 1]	82[5]
1.0	0.07[78]	76[-12]	79[8]
2.5	0.04[88]	62[9]	82[5]
5.0	0.05[84]	67[1]	72[16]

* 2-chloro-1,1-difluoroethylene
** 2-chloro-1,1,1-trifluoroethane
*** GSH (0, 0.1, 0.5, 1.0, 2.5, 5.0 μmol/liter) was added to the incubation system. The incubation system consisted of a microsomal suspension. (protein concentration 0.46 mg/ml in 0.1 M potassium phosphate buffer, pH 7.4), NADPH (2.1 mmol/liter, final), and halothane (4.8 mmol/liter, final) in a final volume of 3 ml. Data of halothane-induced pentane formation were referred as to the difference in the amount of pentane formation with and without halothane. Other experimental conditions are described in Materials and Methods.

Table 3. Effect of metyrapone on pentane, CDFE* and CTFE** formation***

Metyrapone (mmol/liter)	pentane	CDFE	CTFE
	(pmol/mg protein/min) [% inhibition]		
0.0	0.37[0]	81[0]	142[0]
0.5	0.26[30]	27[66]	82[42]
10.0	0.06[84]	22[73]	51[64]
15.0	0.06[84]	16[80]	20[86]

* 2-chloro-1,1-difluoroethylene
** 2-chloro-1,1,1-trifluoroethane
*** Metyrapone (0, 5, 10, 15 mmol/liter) was added to the incubation system. The incubation system consisted of a microsomal suspension (protein concentraion 0.46 mg/ml 0.1 M potassium phosphate buffer, pH 7.4), NADPH (2.1 mmol/liter, final) and halothane (4.8 mmol/liter, final) in a final volume of 3 ml. Data of halothane-induced pentane formation were referred as to the difference in the amount of pentane formation with and without halothane. Other experimental conditions are described in Materials and Methods.

DISCUSSION

Pentane is formed from linoleic acid and arachidonic acid by lipid peroxidation reaction[5,10]. The

measurement of pentane is reported to be a good index of lipid peroxidation[4,15,17].

Under anaerobic conditions, a small amount of pentane was formed without halothane. This pentane is thought to be caused by NADPH-dependent microsomal lipid peroxidation[14]. This may be the mechanism of hypoxic liver injury in rats pretreated with phenobarbital[16].

Pentane formation was potentiated by administration of halothane to guinea pig liver microsomes. The formation was reduced by oxygen, and was dependent on the NADPH and protein concentration of the mixture. Inhibition by a higher substrate was observed together with high doses of halothane (more than 2.1 mmol/liter). CTFE and CDFE, anaerobic metabolites of halothane, were also inhibited by the same dose of halothane. These results suggest that halothane induced pentane formation is closely related to the anaerobic metabolism of halothane.

Halothane is dehalogenated under anaerobic conditions, and radical intermediates of halothane ($^{\bullet}CF_3CHCl$) are produced during this reaction[9]. Our results show that pentane formation is potentiated by lipid peroxidation initiated by the free radical cleavage products of halothane, which are anaerobically produced by NADPH-dependent microsomal enzymes.

Vitamin E and GSH, which are antioxidant agents, reduce the pentane formation, but do not affect the formation of CTFE and CDFE. They may reduce the lipid peroxidation reaction by scavenging radicals but do not affect the dehalogenation reaction of halothane. Metyrapone, an inhibitor or cytochrome P-450, reduces both pentane formation and the formation of CTFE and CDFE. It may inhibit the dehalogenation of halothane, so the formation of radical intermediates of halothane is reduced.

It has been shown that the mechanism of liver cell damage caused by CCl_4 is due to the lipid peroxidation which is mediated by radical intermediates ($^{\bullet}CCl_3$)[14]. The way of biotransformation of halothane in the liver is very similar to that of CCl_4 in such a way that radical intermediates ($^{\bullet}CF_3CHCl$) are produced[6-9] in the process of anaerobic dehalogenation of halothane. Since the extent of the lipid peroxidation in the case of CCl_4 is detected by the measurement of pentane[15], it is possible to compare pentane formation in the administration of halothane with that of CCl_4 as an index of lipid peroxidation. The peak value of pentane formation with administration of halothane is 0.34 pmol/mg protein/min (Fig. 5) and that of CCl_4 is 27.6 pmol/mg protein/min[15]. The peak value of pentane formation caused by halothane is approximately 80 times less than that of CCl_4. The potency of holothane to cause lipid peroxidation reaction may seem not matter in clinical use. However, from our results and other reports[1,13], the anaerobic condition, the reduced radical scavenging system and the increased cytochrome P-450 activity may potentiate halothane induced lipid peroxidation. Therefore, in such cases halothane may injure the hepatocyte by lipid peroxidation.

We conclude that the halothane-induced lipid peroxidation reaction in the guinea pig liver microsomes is closely related to the anaerobic metabolism of halothane.

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