Enzyme Inhibition by Analgesic and Hypnotic Agents on Anaerobic Dehalogenation of Halothane

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Enzyme inhibition on anaerobic dehalogenation of halothane by various analgesic or hypnotic agents was investigated *in vitro* using rat liver microsomal fraction. The production rate of chloro-difluoro-ethylene (CDE) and chloro-trifluoroethane (CTE), anaerobic metabolites of halothane, was measured when various concentrations of analgesic or hypnotic agents (fentanyl, morphine, pentazocine, buprenorphine, ketamine, diazepam, chlorpromazine and hydroxyzine) were supplemented. Inhibitor constant (Ki) of each agent was calculated and compared with each other. The activity of NADPH-cytochrome c reductase (fp_2) and NADH-ferricyanide reductase (fp_1) was measured when each agent was added. The values of inhibitor constants (Ki) for CDE and CTE formation were in the following order from large to small values; morphine (656 μM and 2570 μM), chlorpromazine (49.7 μM and 68.1 μM), ketamine (24.9 μM and 64.4 μM), fentanyl (23.9 μM and 34.6 μM), hydroxyzine (19.2 μM and 50.8 μM), diazepam (17.0 μM and 13.9 μM), buprenorphine (11.2 μM and 22.4 μM), and pentazocine (1.96 μM and 6.67 μM) respectively. Pentazocine inhibited the formation of CDE 300 fold greater than morphine. The activity of fp_2 and fp_1 did not change by the addition of these analgesic or hypnotic agents. These results indicate that various analgesic or hypnotic agents, which are commonly used with halothane in clinical anesthesia, suppress the anaerobic dehalogenation of halothane *in vitro*. They also imply that the suppression of production of halothane metabolites is the result of direct enzyme inhibition on cytochrome P-450, since these agents did not affect the activity of fp_2 and fp_1 which are flavoproteins existing in the microsomal electron transport system. (Key words: halothane, enzyme inhibition, analgesics, hypnotics)

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Chloro-difluoroethylene (CF_2CHCl, CDE) and chloro-trifluoroethane (CF_3CH_2Cl, CTE) are anaerobic metabolites of halothane (2-chloro-2-bromo-1, 1, 1,-trifluoroethane) which were first identified in the expired gas of rabbits inhaling halothane[1]. Subsequent studies have shown that halothane is dehalogenated anaerobically to CDE and CTE under the existence of NADPH and that these reactions require cytochrome P-450 enzyme system[2,3]. These reactions possess clinical importance, since free radicals

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which are produced through these reactions[4] are suggested to be a cause of liver disorder induced by halothane[5]. On the other hand, it is known that metabolism of certain drugs is inhibited by other agents through the mechanism of enzyme inhibition, which occurs on cytochrome P-450. If anaerobic dehalogenation of halothane is inhibited by other drugs, there is a possibility that some combination of agents may prevent liver disorder following exposure to halothane.

The purpose of this study is to manifest how the anaerobic dehalogenation of halothane is affected by various analgesic or hypnotic agents which are commonly used with halothane simultaneously. For this purpose, we compared the degree of enzyme inhibition of cytochrome P-450 by each agent using rat liver microsome *in vitro*.

Materials and Methods

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

1. Animals and Reagents

Male Wistar rats 180–420g in body weight were used. Halothane was obtained from Hoechist, Japan. Fentanyl, pentazocine and ketamine were provided by Sankyo Co., Japan, buprenorphine by Ohtsuka Co., Japan, diazepam by Yamanouchi Co., Japan and hydroxyzine by Pfizer Co., Japan. The other reagents were of analytical grade.

2. Preparation of liver microsome

Untreated rats were sacrificed by decapitation after fasting for 24 hrs. After removal of the liver and perfusion with ice cold saline containing 1 mM of EDTA, it was homogenated with phosphate buffer (0.1 mM of potassium phosphate buffer, pH = 7.5, KPB). The homogenate was centrifuged at 9,000 × g for 15 min, and the supernatant was centrifuged again at 105,000 × g for 60 min to the microsomal fraction. The precipitate was suspended to 0.1 mM KPB (microsomal suspension). All these procedures were carried out in a cold room.

3. Assay of anaerobic dehalogenation of halothane

Cytochrome P-450 was measured by the method of Omura and Sato[6]. Eight hundred μl of microsomal suspension containing 3 nmol of cytochrome P-450 (0.031 mg·ml^{-1}) protein was sealed in a test tube (13.5 ml in volume) and air was replaced by nitrogen gas. Then, 0.1 ml of solution of eight kinds of analgesic or hypnotic agent was supplemented in it. Agents and concentration in the final mixture were as follows: fentanyl (100 μM, 30 μM and 10 μM), morphine (2,000 μM, 1,000 μM and 600 μM), pentazocine (10 μM, 3 μM and 1 μM), buprenorphine (50 μM, 20 μM and 10 μM), ketamine (50 μM, 30 μM and 10 μM), diazepam (30 μM, 10 μM and 5 μM), chlorpromazine (200 μM, 100 μM and 60 μM), and hydroxyzine (30 μM, 20 μM, 10 μM and 6 μM), respectively. After pre-incubation for 7 min at 37°C, 0.1 ml of 50 mM NADPH and various concentrations of halothane were added in the test tube to initiate the reaction. After 10 min, 0.5 ml of the head cap gas was subjected to the gas chromatographical measurement[7] of CDE, CTE, and halothane. The rates of production of CDE or CTE and concentration of halothane were plotted on Lineweaver-Burk's double reciprocal plot with the respective concentration of each analgesic or hypnotic agent. The values of apparent Km and Vmax were calculated by the linear regression line.

Inhibitor constant (Ki) was obtained by measuring the intersection of the interpolated line of the secondary plot which took the concentration of inhibitory agents on the horizontal axis and the "slope" of the linear lines on the foregoing double reciprocal plot (Vmax/Km) on the vertical axis[8].

4. The measurement of activity of microsomal electron transport system

a. Measurement of activity of NADPH-cytochrome c reductase (fp_2)

NADPH-cytochrome c reductase (fp_2) was measured as activity of NADPH-cytochrome

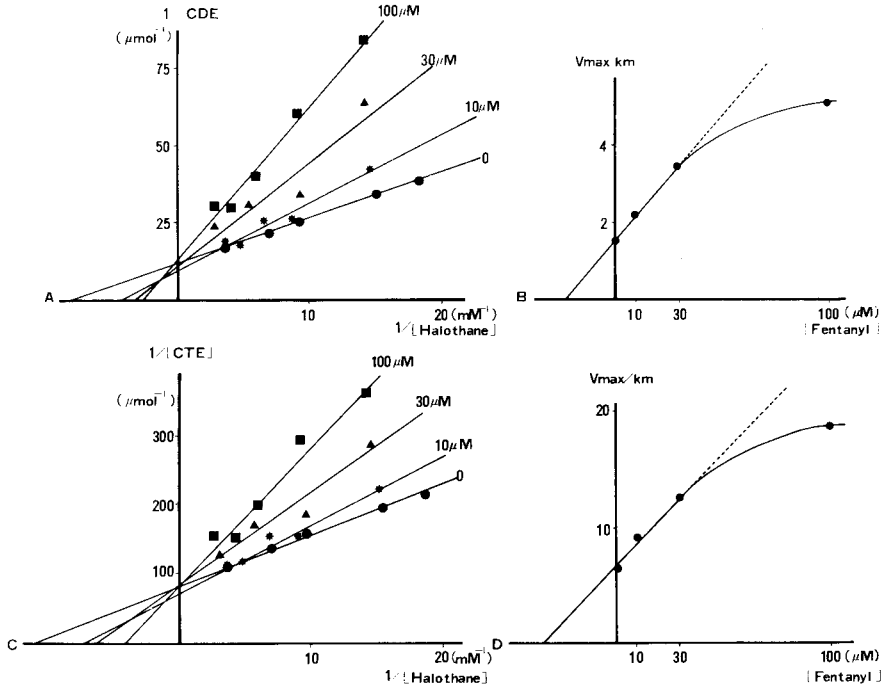

Fig. 1. Enzyme inhibition on anaerobic dehalogenation of halothane by fentanyl. The inhibition of the production of CDE (chloro-difluoro-ethylene) and CTE (chloro-trifluoro-ethane), which are anaerobic metabolites of halothane, is shown when fentanyl was added to rat liver microsomes. The reaction mixture consisted of microsomes (protein 0.031 mg·ml^{-1}) with 3 nmol of P-450, NADPH, halothane and fentanyl (100, 30, 10, 0 μM). (A) shows the relationship between CDE production and halothane concentration on Lineweaver-Burk's double reciprocal plot. The higher the concentration of fentanyl, the steeper are the slopes, indicating that fentanyl suppressed CDE production. (B) shows the secondary plot taking fentanyl concentration on the horizontal axis and the "slope" of double reciprocal plot (Vmax/Km) on vertical axis. Inhibitor constant (Ki) was calculated to be 23.9 μM by the intersection of the interpolated line. (C) shows that CTE production was also inhibited by fentanyl, and Ki was calculated to be 34.6 μM from (D).

P-450 reductase. The mixture of KPB (5.1 ml of 4 mM), KCN (0.3 ml of 12 mM), microsomal suspension (0.1 ml) and cytochrome c (0.5 ml of 4 mM) and each analgesic or hypnotic agents was used. The concentration of analgesic and hypnotic agents in the mixture was as follows; 10 μM of pentazocine, 30 μM of diazepam, 50 μM of buprenorphine, 100 μM of fentanyl, 30 μM of hydroxyzine, 50 μM of ketamine, 200 μM of chlorpromazine or 2 mM of morphine. Protein concentration of this solusion was 0.050 mg·ml^{-1}. The change of absorbance at 550 nm of this suspension with and without each analgesic or hypnotic agent was measured with a spectrophotometer (Shimadzu UV-300) in a semi-microcell. The maximum concentrations of each agent used in the measurement of CDE and CTE were also used. Fp_2 activity was calculated assuming that millimolar molcular absorbance was 21.1 mM^{-1}·cm^{-1} [9].

b. Measurement of activity of NADH-ferricyanide reductase (fp_1)

NADH-cytochrome b_5 reductase (fp_1) was measured as activity of NADH-ferricyanide

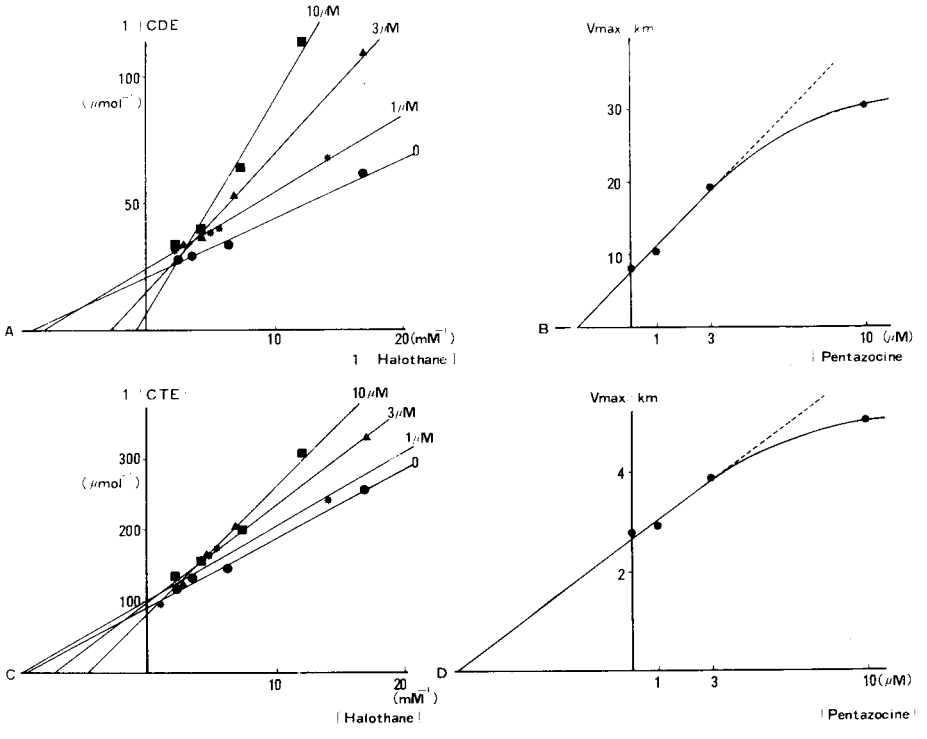

Fig. 2. Enzyme inhibition on anaerobic dehalogenation of halothane by pentazocine. The inhibition of the production of CDE (chloro-difluoro-ethylene) and CTE (chloro-trifluoro-ethane), which are anaerobic metabolites of halothane, are shown when pentazocine was added to rat liver microsomes. The reaction mixture consisted of microsomes (protein 0.031 $mg \cdot ml^{-1}$) with 3 nmol of P-450, NADPH, halothane and pentazocine (10, 3, 1, 0 μM). (A) and (C) are double reciprocal plots indicating that pentazocine also inhibited the production of CDE and CTE. The inhibitor constant (Ki) was calculated to be 1.96 μM and 6.67 μM, respectively, from the secondary plots (B) and (D).

reductase. Immediately after mixing of KPB (5.5 ml of 0.1 M), potassium ferricyanide (0.3 ml of 20 mM), microsomal suspension (0.1 ml) and NADH (30 ul of 60 mM) in a test tube with and without each analgesic or hypnotic agent, a decrease of absorbance per minute at 420 nm was measured with the spectrophotometer. Protein concentration of this solusion was 0.050 $mg \cdot ml^{-1}$. Fp_1 activity was calculated, assuming that ferricyanide molcular absorbance constant was 1.02 $mM^{-1} \cdot cm^{-1}$ [10].

Results

a. Inhibitor constant (Ki) of various agents to CDE and CTE production

Figure 1-A shows the difference of the rate of CDE production in each fentanyl concentration on double reciprocal plot. The higher the fentanyl concentration, the steeper became the slope, showing that fentanyl inhibited the CDE production. As shown in figure 1-B, a linear line was obtained under 30 μM of fentanyl concentration on the secondary plot. Inhibitor constant (Ki) of fentanyl to CDE production was calculated to be 23.9 μM at the intersection of the horizontal axis to the extrapolated linear line. Figure 1-C is the double-reciprocal plot showing that CTE production was also inhibited by fentanyl. Ki of fentanyl to CTE production was calculated to be 34.6 μM from the secondary plot

Table 1. Inhibitor constant (Ki)

Agents	CDE (μM)	CTE (μM)
Pentazocine	1.96	6.67
Buprenorphine	11.2	22.4
Diazepam	17.0	13.9
Hydroxyzine	19.2	50.8
Fentanyl	23.9	34.6
Ketamine	24.9	64.4
Chlorpromazine	49.7	68.1
Morphine	656	2570

Inhibitor constant (Ki) of various agents to anaerobic dehalogenation of halothane *in vitro* are shown. CDE (chloro-difluoro-ethylene) and CTE (chloro-trifluoro-ethane) are anaerobic metabolites of halothane.

(fig. 1-D). As seen in figure 1-A and 1-C, the crossing point of linear lines was found on the vertical axis, suggesting that inhibition can be classified as the competitive type.

Figure 2-A and C are double reciprocal plots, indicating that pentazocine also inhibited the production of CDE and CTE. However, the crossing points of linear lines were located in the first quadrant, suggesting that the inhibition was the type other than fentanyl. Ki values of pentazocine to CDE and CTE production were 1.96 and 6.67 μM, respectively (fig. 2-B, D).

Table 1 shows Ki values of eight species of analgesic and hypnotic agents to CDE and CTE. The pattern of inhibition of these six agents were unable to be classified into any known types of inhibition as was observed in pentazocine. Ki values to CDE production were arranged in the following order from large to small inhibitory effect; pentazocine, buprenorphine, diazepam, hydroxyzine, fentanyl, ketamine, chlorpromazine and morphine. In general, Ki values of the agent to CTE production were larger than that to CDE except diazepam. Ki values to CTE production were arranged in the following order from large to small inhibitory effect; pentazocine, diazepam, buprenorphine, fentanyl, hydroxyzine, ketamine, chlorpromazine and morphine.

Table 2. Activities of NADPH-cytochrome C reductase (fp_2) and NADH-ferricyanide reductase (fp_1)

Agents	fp_2 (unit·ml^{-1})	fp1 (unit·ml^{-1})
Control	17.6	557
Pentazocine	18.4	539
Buprenorphine	17.0	547
Diazepam	17.9	537
Hydroxyzine	18.6	542
Fentanyl	17.8	525
Ketamine	18.2	541
Chlorpromazine	18.9	532
Morphine	17.6	621

Activity of fp_2 and fp_1, which are the components of microsomal electron transport system, are not inhibited by various agents in rat liver microsome. (1 unit = nmol·min^{-1}).

b. The effect of various agents on microsomal electron transport system

Table 2 shows the effects of various agents on the activities of fp_2 and fp_1, indicating that no significant effect was observed on the microsomal electron transport system.

Discussion

This study demonstrated that various analgesic or hypnotic agents, which are commonly used with halothane in clinical anesthesia, suppressed the anaerobic dehalogenation of halothane *in vitro*. It also suggested that the suppressions arose from the direct enzyme inhibition on cytochrome P-450, since these agents did not affect the activities of fp_2 and fp_1 which are flavoproteins existing in the microsomal electron transport system.

Since cytochrome P-450 has two binding sites for inhibitors in a molecule, a competitive inhibition typically observed with Type I agents such as SKF525-A was found in the inhibition of anaerobic dehalogenation of halothane by fentanyl, which was shown by the location of crossing point of

the linear lines on the vertical axis of the double reciprocal plots (figs. 1-A and 1-C). On the other hand, a non-competitive inhibition often observed with Type II agents such as nicotinamide and aniline comes from the conformational chanage of cytochrome P-450 by binding to sites other than catalytic site[11–13]. This type of inhibition could not be clearly shown among the agents tested. In general, it is not always easy to identify the type of inhibition of agents, since the inhibitor often binds both sites of the enzyme in a different extent[14] and in part complex enzyme system is used in the experiment[15]. Unlike fentanyl, inhibitory type of pentazocine and other six agents could not be classified into any known type of inhibition. This comes partly because the reaction was tested in the microsomal fraction which is necessarily a complex enzyme system, and partly because halothane employed was at the relatively low concentration which makes error of values large in the nature of enzymatic reaction. In higher concentration of halothane, the rate of CDE production is inhibited by halothane itself[7]. Therefore, in this experiment, inhibitory type of each agent could not be shown except for fentanyl. More strict assessment of inhibitory type might require the usage of isolated enzyme system. On the other hand, inhibitor constant could be calculated by the secondary plot in the experiment regardless of the type of inhibition. Comparison of "Ki"s among the agents tested can be worthwhile in the choice of agent for halothane anesthesia.

It is noteworthy that pentazocine inhibits the anaerobic dehalogenation of halothane about 300 times stronger than morphine in terms of molar concentration, as Ki to pentazocine was 300 times smaller than that to morphine. Unlike pentazocine or morphine, other agents such as buprenorphine, fentanyl, hydroxyzine, diazepam, chlorpromazine and ketamine inhibit this reaction in the almost same degree in terms of molar concentration.

It is interesting that the order of Ki values of CDE production to various agents is not identical to the one of CTE production. This might be due to the difference of type of inhibition which could not be elucidated in this study. The findings that CDE production was inhibited more than CTE by the agents tested might support the probability regarding the difference of metabolic pathway of CDE and CTE. The latter is formed after accepting one electron and on the other hand, the former is produced after accepting, in addition, another electron[16].

Plummer et al. showed that cimetidine inhibits anaerobic dehalogenation of halothane[17] and Wood et al. suggested simultaneous administration of cimetidine can suppress liver disorder due to halothane[18]. Some reports showed that volatile anesthetics also inhibit halothane metabolism. Isoflurane has been reported to inhibit aerobic metabolism of halothane and not to affect the anaerobic metabolism of it[19]. In contrast to the fact that enzyme induction by various agents such as barbiturate worsens the liver disorder due to halothane[20], analgesic and hypnotic agents used in this study may reduce the risk of liver disorder due to halothane by enzyme inhibition.

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