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Inhibitory Effect of Paraquat on Biotransformation of Halothane in Rabbit Liver Microsomes

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ABSTRACT

Microsomal fractions were prepared from the liver of rabbits to investigate the effects of paraquat (methyl viologen) on generation of metabolites of halothane under the optimal aerobic and anaerobic conditions. Halothane ($CF_3CHClBr$) is known to undergo oxidative and reductive biotransformation in the hepatic mixed function oxidase system including cytochrome-P450 reductase and cytochrome P450. The results showed that paraquat inhibited generation of metabolites of halothane under these conditions. Generation of the aerobic metabolite, trifluoroacetic acid (CF_3COOH), and anaerobic metabolites, 2-chloro-1, 1, 1-trifluoroethane (CF_3CH_2Cl) and 2-chloro-1, 1-difluoroethylene (CF_2CHCl), were inhibited 50% by 4.96 mM and 35.3 mM paraquat, respectively. Possible mechanisms were speculated on to account for the inhibitory effects: one being the impaired formation of halothane-cytochrome P450 complex by addition of paraquat, and the other the diversion of electrons from cytochrome-P450 reductase to generate active paraquat radicals. It is concluded that paraquat inhibits NADPH-dependent biotransformation of halothane catalyzed in mixed function oxidase system.

Key words: *Paraquat, Anesthetic, Halothane, Biotransformation*

The herbicide paraquat (methyl viologen; 1, 1'-dimethyl-4, 4'-bipyridylium dichloride) is highly toxic toward mammals, the cytotoxicity being usually associated with severe damage to the vital organs[2,6,12,18]. Paraquat toxicity is associated with its ability to be cyclically reduced and oxidized and in this process to generate active oxygen species such as O_2^- and OH [14,22,28,30,33]. Paraquat radicals react rapidly with O_2 to give O_2^- ($k_2 = 7.7 \cdot 10^8$ $M^{-1}.s^{-1}$)[7], and its subsequent reactions. These reactive products may be the basis of toxicity[4,14]. Lipid peroxidation of the cell membrane is therefore assumed to be a main feature of the cytotoxicity of paraquat[1,3,6,8]. However, two different lines of evidence have been demonstrated in vitro by using microsomes; inhibited lipid peroxidation[20] and facilitated lipid peroxidation through enhanced generation of O_2^-[22,31]. Involvement of cytochrome P450 in the pathway of lipid peroxidation may account for this discrepancy[1,3,19,27,30].

The biotransformation of drugs may also be modified by paraquat, as they are catalyzed by P450 in the hepatic mixed function oxidase system including cytochrome-P450 reductase and P450. From the standpoint of clinical anesthesia, the interaction between paraquat and halothane ($CF_3CHClBr$), an anesthetic also used for the patients of paraquat of paraquat poisoning, requires investigation. Halothane is known to undergo oxidative biotransformation to the stable end product, trifluoroacetic acid (TFAA; CF_3COOH)[5,16,23], and to undergo reductive biotransformation to form the volatile metabolites, 2-chloro-1, 1, 1-trifluoroethane (CTFE; CF_3CH_2Cl) and 2-chloro-1, 1-difluoroethylene (CDFE; CF_2CHCl)[10,21,24]. We examined the interaction between paraquat and the biotransformation of halothane to clarify the role of P450 in vitro by measuring metabolites of halothane using gas chromatography[5,9,10,24] and ion-exchange chromatography[11,25].

MATERIALS AND METHODS

Chemicals

Deionized water was used to prepare buffer-A (0.1 M potassium phosphate buffer, pH 6.0) and buffer-B (0.1 M potassium phosphate buffer, pH 7.4). Halothane was purchased from Hoechist Japan. NADPH and cytochrome c were from Boehringer Mannheim, West Germany. Paraquat dichloride (methyl viologen) was a generous gift from ICI Japan. Determination of TFAA, CDFE and CTFE was made using authentic materials; TFAA was from Katayama Chemical Japan, and CDFE and CTFE were from PCR incorporated, Florida, USA. Other reagents were of analytical quality.

Preparations

Male, Japanese white rabbits each weighing about 4.0 kg were allowed access to standard laboratory food and water *ad libitum*. All rabbits were starved overnight and were killed by neck dislocation. The liver was weighed and homogenized with ice-cold

buffer-B in a Teflon glass homogenizer. The resulting homogenate was centrifuged at 10000 × *g* for 60 min. The supernatant was then centrifuged at 105000 × *g* for 60 min, and the microsomal pellet was collected. Microsomes were washed by resuspending in buffer-B to the original volume and by resedimenting at 105000 × *g* for 60 min. The microsomal pellet was then stored at −80°C until it was utilized. All operations were performed at 4°C.

Generation of TFAA and analysis

The microsomal pellet was resuspended in buffer-A for the optimal assay conditions for TFAA in vitro as previously described[25]. Briefly, the reaction mixture (final volume of 1.4 ml) containing microsomes (3.57 μM P450), 5.0 mM halothane, various concentrations of paraquat, ranging from 0 mM to 10.0 mM, and NADPH (final concentration of 10.0 mM) was incubated at 37°C for 40 min in a 60 cm^3 glass flask filled with O_2 and sealed with a silicon cap. Generation of TFAA was initiated by addition of NADPH and was terminated by ultrafiltration of microsomal protein using Centriflo™ CF-25 (Amicon, MA, USA) for 60 min at 4°C.

TFAA was analysed by using an ion-exchange chromatographic analyzer (model IC-100, Yokogawa Hokushin Electric, Japan) equipped with an electro-conductimetric detector. An anion exchange resin packed in 4 mm × 250 mm stainless column (SAX-1, Yokogawa Hokushin Electric, Japan) was used for the immobile phase. Column temperature was 40°C. The eluent, containing 2 mM Na_2CO_3 and 2 mM $NaHCO_3$, was used at a flow rate of 2 $ml.min^{-1}$ [17].

Generation of CDFE and CTFE and analysis

For anaerobic studies, the microsomes were resuspended in buffer-B to prepare the same concentrations of incubations as under aerobic conditions[9,10]. Aliquots were added to 15 cm^3 test tubes, in which O_2-free N_2 was purged, sealed with a silicon cap. The mixtures were incubated at 37°C in the presence of paraquat, ranging from 0 mM to 30.0 mM. The reaction was initiated by injection of NADPH.

Both CDFE and CTFE were analyzed 40 min after the addition of NADPH by the head-space gas method[21]; 0.5 cm^3 of head space gas was used for gas chromatographic analysis. Determination of CDFE and CTFE was made using authentic materials, which were run as controls by using heat-denatured microsomes under the same conditions. Aliquots of the head-space of the sample were injected onto a 4 mm × 3 m stainless steel column packed with 60/80 mesh 20% of dioctylphthalate (Gasukuro Kogyo Inc., Japan). The column temperature was 100°C in a gas chromatograph (model GC-4A, Shimadzu, Japan) equipped with a flame ionization detector. The carrier gas was helium at a flow rate of 30 cm^3min^{-1}. CDFE and CTFE eluted from the column at approximately 0.80 and 1.06 min, respectively[9,10]. The sum of CDFE and CTFE was assumed to be the entire amount of anaerobic volatile metabolites generated in this system.

Assays and generation of paraquat radicals

Optical spectroscopy was carried out at 37°C using a recording spectrophotometer (model UV 300, Shimadzu, Japan). The content of P450 was determined by the method of Omura and Sato[26]. The spectral effects of paraquat and halothane on difference spectra of P450 were monitored in the microsomal suspension in buffer-A containing 5.0 μM P450, 10.0 mM paraquat and 10.0 mM halothane under aerobic conditions. The spectral changes induced by paraquat and halothane under anaerobic conditions were also demonstrated using buffer-B in the same reagent concentrations under aerobic condition. Reactions under anaerobic conditions were carried out in cuvettes which allowed purging the reaction volume with O_2-free N_2.

The blue-colored paraquat radicals were determined by measuring absorbance at 600 nm[7] so as to exclude Soret band absorption of P450 around 395 nm, another absorbance of blue-colored paraquat radicals. Anaerobic reactions were carried out in cuvettes which allowed purging the reaction volume with O_2-free N_2. Generation of paraquat radicals was anaerobically measured by adding NADPH (final concentration of 1.0 mM) into the reaction mixture. This contained buffer-B and microsomal protein (0.5 μM P450) at various paraquat concentrations (0.2, 1.0 and 2.0 mM) in a final volume of 3 ml in the presence or absence of 5.0 mM halothane without addition of exogenous iron. The activity of cytochrome-P450 reductase was assayed by measuring the absorbance at 550 nm of NADPH-cytochrome c reductase activity in buffer-B by the method of Yasukochi and Masters[32].

Statistics

From the reciprocal plot of generation of metabolites against the paraquat concentration, 50% inhibitory concentration (IC50) was calculated under both aerobic and anaerobic conditions. Lines drawn through data points were determined by linear regression analysis. Generation of TFAA under aerobic conditions and sum of CDFE and CTFE under anaerobic conditions are described as "per nmol P450" because it is known that they are P450 concentration dependent.

RESULTS

Generation of TFAA was inhibited by paraquat. Determined IC50 was 4.96 mM, which was calculated from the reciprocal plot of generation of TFAA against paraquat concentration under aerobic conditions (Fig. 1). In this figure, lines drawn through data points were determined by linear regression analysis; $Y = 2.5X + 12.4$ ($R = 0.82$, $p<0.01$).

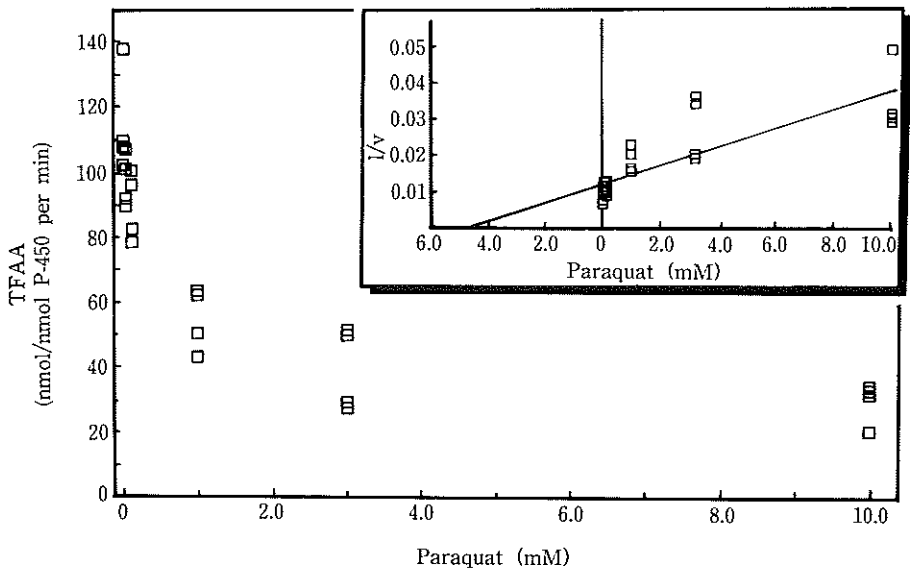


Fig. 1. Inhibitory effect of paraquat on generation of trifluoroacetic acid from halothane under aerobic conditions.
Generation of TFAA was inhibited by paraquat. Four series of data sets are presented. Determined IC50 was 4.96 mM, calculated from the reciprocal plot of generation of TFAA (1/v) against paraquat concentration (right upper corner).

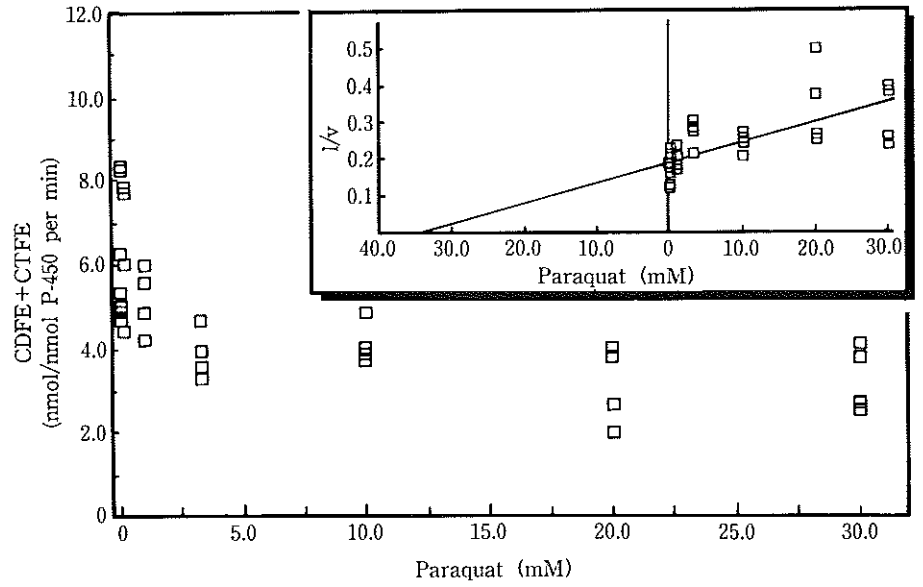


Fig. 2. Inhibitory effect of paraquat on generation of volatile metabolites from halothane under anaerobic conditions.
Generation of volatile metabolites, 2-chloro-1, 1, 1-trifluoroethane (CTFE) and 2-chloro-1, 1-difluoroethylene (CDFE), was inhibited by paraquat. Four series of data sets are presented. Determined IC50 was 35.3 mM, which was derived from the reciprocal plot of generation of volatile metabolites (1/v), CDFE plus CTFE, against paraquat concentration (right upper corner).

Generation of anaerobic volatile metabolites, CDFE plus CTFE, was also inhibited at various paraquat concentrations. Determined IC50 was 35.3 mM, calculated from the reciprocal plot of generation of volatile metabolites against paraquat concentrations under these conditions (Fig. 2). Lines drawn through data points were determined by linear regression analysis; Y = 5.4X + 190.5 (R = 0.70, $p<0.01$) in this figure.

The effects of paraquat and halothane on the difference spectra in the microsomal suspensions under aerobic and anaerobic conditions are shown in Fig. 3. Under aerobic conditions, addition of paraquat induced spectra resembling those of Type I, which suggested the formation of paraquat-P450 complex in microsomal suspensions. Halothane

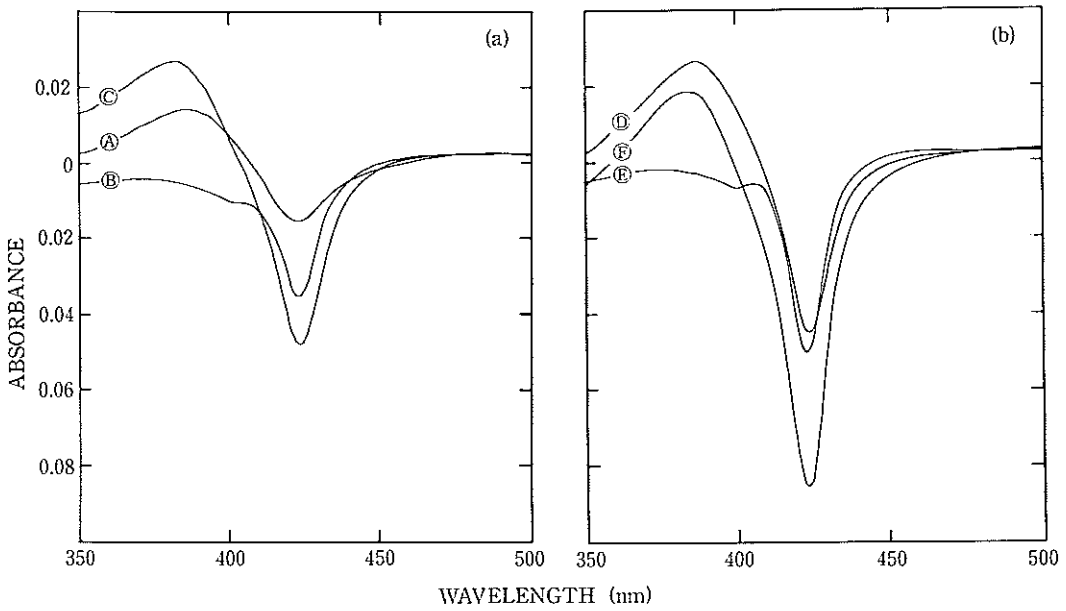


Fig. 3. Difference spectra of cytochrome P450 in the presence of paraquat and halothane under aerobic and anaerobic conditions.
Difference spectra obtained under aerobic condition are presented in (a). Spectra B obtained after addition of paraquat to the microsomal preparation indicated a trough at 423 nm without an apparent peak. Addition of halothane to another microsomal preparation induced type I spectra A with a trough at 423 nm and a peak at 387 nm. Addition of halothane to the paraquat-coexisting preparation indicated spectra C with an obvious trough at 423 nm and a peak at 382 nm, suggesting mixed type spectra of paraquat and halothane.
Difference spectra obtained under anaerobic condition are presented in (b). Spectra E obtained after the addition of paraquat to the microsomal preparation indicated a trough at 422 nm without an obvious peak. Addition of halothane to another microsomal preparation indicated Type I spectra D with a trough at 423 nm and a peak at 387 nm. Spectral changes were observed by addition of halothane to the paraquat-coexisting preparation. Spectra F indicated a trough at 382 nm and a peak at 423 nm, which were postulated as mixed type spectra of paraquat and halothane.

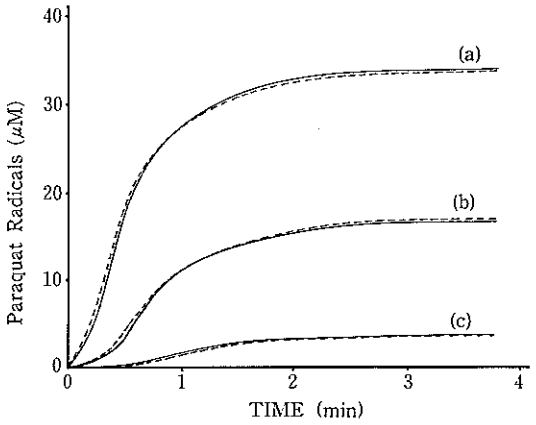


Fig. 4. Generation of paraquat radicals from paraquat under anaerobic conditions.
Generation of paraquat radicals was anaerobically monitored at 600 nm. Paraquat concentrations in the mixture were 0.2 (c), 1.0 (b) and 2.0 (a) mM. Lines indicated halothane in the presence (dotted line) or absence (solid line). No inhibitory effects of halothane on generation of paraquat radicals were observed.

produced Type I spectra which were modified by paraquat. Under anaerobic conditions, spectral changes induced by the addition of halothane and paraquat were similar to those obtained under aerobic conditions, although the absorbance in difference spectra was larger than that under aerobic conditions. It was obvious, therefore, that formation of the halothane-P450 complex was inhibited by addition of paraquat since they might compete with each other for the same molecular portion of P450 under both aerobic and anaerobic conditions.

Under anaerobic conditions, paraquat radicals were generated by addition of NADPH (Fig. 4). In a paraquat-coexisting preparation, generation of paraquat radicals was not affected by halothane. Initial generation of paraquat radicals was 21.4 $mM \cdot min^{-1}$ in the mixture containing 2.0 mM paraquat. Generation of paraquat radicals was dependent on the concentration of paraquat in the mixture. This indicated that the electron diversion from cytochrome-P450 reductase to reduce paraquat ion might take place even under such halothane-coexisting conditions.

Cytochrome-P450 reductase activity, observed by the reduction of cytochrome c, was not inhibited by paraquat but slightly enhanced (Table 1).

DISCUSSION

Paraquat is known to cause cytotoxicity by

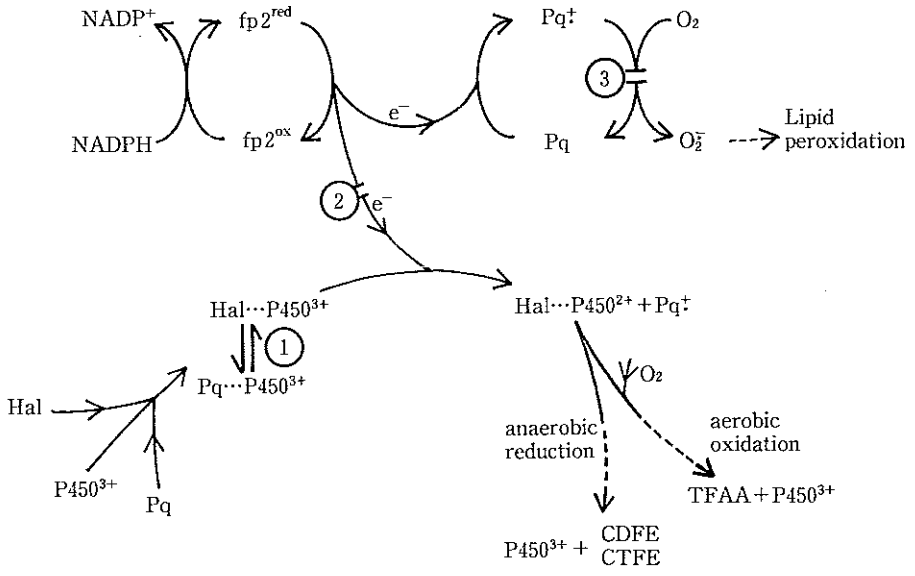
Table 1. Effect of paraquat on NADPH-cytochrome c reductase activity of rabbit liver microsomes.

Addition	Changes in absorbance (μM/min)	% of enhancement
microsomes (–)	0	—
microsomes (+)		
+ NADPH (1.0 mM) alone	3.32	—
+ NADPH (1.0 mM)		
+ paraquat (1.0 mM)	3.55	6.9
+ paraquat (10 μM)	4.13	24.4
+ paraquat (0.1 μM)	4.27	28.6

Slightly enhanced NADPH-cytochrome c reductase activities were demonstrated by addition of paraquat.

producing lipid peroxidation in the cell membrane *in vivo*[3,8]. Paraquat is cyclically reduced and oxidized by microsomal oxido-reductase plus NADPH. During this process, it can generate active oxygen species such as O_2^- and hydroxyl radicals[14,22,28,30,33]. Peroxidation of the microsomal membrane is due to the generation of hydroxyl radicals since lysis of lysosomes are inhibited by hydroxyl radical scavengers[22]. Under *in vitro* conditions paraquat stimulates lipid peroxidation in mouse lung microsomal lipids through enhanced generation of superoxide[3,4]. On the other hand, paraquat strongly inhibits NADPH-dependent lipid peroxidation[22]. The discrepancy is not clear, but it may be due to the differences in species and addition of chelated iron. Klimek et al[19] have demonstrated that paraquat has no effects on non-enzymatic Fe^{2+}-supported lipid peroxidation but strongly inhibits enzymatic NADPH-dependent lipid peroxidation in bovine adrenal cortex mitochondria.

Halothane is aerobically metabolized to generate TFAA[5,23], and anaerobically transformed to form volatile metabolites, CTFE and CDFE[10,21,24,25]. Our study showed that paraquat inhibits biotransformation of halothane aerobically and anaerobically. In contrast, halothane had no effects on the generation of paraquat radicals under anaerobic conditions. Therefore, it is speculated that only paraquat has a major effect on the generation of metabolites. The mechanism of paraquat-induced inhibition of biotransformation of halothane may be better understood by considering the mechanism of inhibited lipid peroxidation by paraquat *in vitro*. As summarized in Scheme 1, we found that paraquat-induced inhibition of biotransformation of halothane occurs in two sequential steps. Paraquat readily accepts electrons from NADPH to be reduced and aerobically generates reactive oxygen species immediately[7,33]. The mixed function oxidase system including cytochrome-P450 reductase and P450 has an important role in this process because activation of paraquat and biotransformation of halothane are both initiated in this system. The electrons from cytochrome-P450 reductase may be



Scheme 1. Proposed reaction sequence for P450 dependent biotransformation of halothane and inhibition steps by paraquat under aerobic and anaerobic conditions. Abbreviations used are; Hal, halothane ($CF_3CHClBr$); Pq, paraquat (methyl viologen); Pq^+, paraquat radicals; TFAA, trifluoroacetic acid (CF_3COOH); CTFE, 2-chloro-1, 1, 1-trifluoroethane (CF_3CH_2Cl) and CDFE, 2-chloro-1, 1-difluoroethylene (CF_2CHCl), fp2; cytochrome-P450 reductase.

diverted for the reduction of paraquat to inhibit biotransformation of halothane, as shown in step 2. Reduction of halothane-P450 complex may be inhibited because the diverted electrons are only for the generation of paraquat radicals, not for reoxidation of paraquat radicals under anaerobic incubation as presented in step 3. However, oxidation of halothane-P450 complex under aerobic preparation may be disturbed as the electrons are consumed for cyclic reduction and oxidation of paraquat to generate reactive oxygen species. It was previously demonstrated that NADPH-dependent reduction of P450 is strongly inhibited by the addition of paraquat and reoxidation of P450 by paraquat is associated with the generation of paraquat radicals, indicating the ability of P450 to reduce paraquat[19,22]. Therefore, the results of this study are consistent with these reports.

The addition of various substrates of the P450 monooxygenase system to liver microsomes causes a characteristic difference spectra due to substrate binding to oxidized P450[13,15]. The spectra are classified into 3 categories, Type I, Type II and reverse Type I, depending on the shape of the induced spectra[29]. In this study, paraquat was found to alter the Type I spectrum induced by halothane, indicating the changes of the spin state of P450. In contrast, the spectrum induced by paraquat was easily altered by addition of halothane, generating a Type I resembling spectrum. Therefore, paraquat may affect the formation of halothane-P450 complex by changing the spin state of oxidized P450, as shown in step 1 in Scheme 1.

Our results indicate that the inhibitory effects of paraquat on biotransformation of halothane involves P450, which regulates generation of metabolites of drugs in the microsomal mixed function oxidase system. In the clinical setting, halothane may be applicable for anesthesia of patients with paraquat-poisoning because it generates a smaller amount of metabolites toxic to the vital organs.

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