# Changes of Hepatic Tissue Phospholipid Peroxidation, Malondialdehydes, and Antioxidative Enzyme Activities in Dogs with Halothane Inhaltation

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ABSTRACT. To elucidate the pathogenesis of halothane-induced hepatopathy, the changes of hepatic tissue phospholipid peroxidation, malondialdehydes (MDAs), and antioxidative enzyme activities were examined in the portal vein arterialized dogs with halothane inhalation. In group A, which was given halothane inhalation under the hepatic blood flow volume less than 10% of pre-operation volume designated as a hypoxic condition, peroxidized phosphatidylcholine (PC), and free and protein-bound MDA levels significantly increased after inhalation. Although the level of protein bound MDA in group C, given hypoxic condition alone, also increased during the experimental period, the response of this was smaller than that in group A, suggesting that the halothane inhaltation enhanced free radical generation under the hypoxic condition. In contrast, no significant changes of these levels were observed in groups B and D, both of which were supplied with sufficient hepatic oxygen as the normoxic condition. In addition, the significant negative correlations between hepatic oxygen supply and total or protein-bound MDA were observed in only halothane inhaled group. These findings suggested that the cause of halothane-induced hepatopathy is closely related to free radicals mainly generated from halothane anaerobic metabolism under the hypoxic condition. — KEY WORDS: canine, halothane, lipid peroxidation, liver, oxygen supply.

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Hepatopathy is one of the most important complications associated with halothane anesthesia in dogs. Since halothane inhalation especially under the hypoxic condition and treatment with drugs, which reduce hepatic blood flow, usually induces hepatic disorder [10, 13, 15, 18] but not under the sufficient oxygen supply to liver [25], hepatic oxygen supply is one of the factor for developing halothane-induced hepatopathy. Recently, it has been reported that this hepatopathy might be caused by free radicals generated from an anaerobic dehalogenation of halothane by the hepatic microsomal cytochrome P-450 and/or from the enhancement of anaerobic energy metabolism [2, 4, 8, 14, 30]. Free radicals induce membrane phospholipid peroxidation, by which membrane permeavility and functions are altered. Malondialdehydes (MDAs) are major end-products of lipid peroxides that also damage membrane functions due to the aggregation of membrane proteins, inactivation of enzymes, and alteration of ion transport [7, 12].

On the other hand, halothane directly reduces liver blood flow volume followed by hepatic hypoxia [21]. In the hypoxic condition, free radical generation is remarkably enhanced in accompaning with a decrease of aerobic energy metabolism, including nucleotide and prine metabolisms [29]. Hepatic oxygen supply depends on liver blood flow volume consisting of hepatic artery and portal vein, which is easily affected by various factors such as operation, cirrhosis, and chronic liver diseases [9, 10, 17]. Therefore, it is necessary for elucidating the cause of halothane-induced hepatopathy *in vivo* to use a liver perfusion system under the controlled hepatic oxygen supply. Portal arterialization is an available technique to control hepatic oxygen supply, because hepatic blood flow depends only on portal vein [33].

The present study deals with the changes of peroxidized phospholipids, MDAs, and also antioxidative enzyme activities in portal arterialized dogs with halothane inhalation.

## MATERIALS AND METHODS

*Animals*: Fifteen clinically healthy mongrel dogs (1 to 3 years old, weighing 9 to 15 kg) were kept over a week on a commercial diet and water *ad libitum*. After the habituation, these dogs were randomly divided into 4 groups as described in experimental design.

*Portal vein arterialization (PA)*: After fasting for 24 hr, each dog was premedicated with glycopyrolate (0.005 mg/kg, S.C.) to induce anesthesia with thiamylal sodium (25 mg/kg, i.v.). Intratracheal intubation was performed before maintaining anesthesia with nitrous oxide and oxygen (2:1 v/v) under the intravenous administration of 0.1% ketamine hydrochloride in normal saline at the rate of approximately 50 m*l*/kg/hr. The respiration was controlled by injection of suxamethonium chloride (0.2 mg/kg, i.v.) during the experimental period. The operation for PA with a ligation of hepatic artery in addition to portal vein anastomosis (PVA) was carried out in accordance with the method reported previously [33]. After the construction of PA, about 1.5 *l* of supplementary heparinized (10 U/m*l*) arterial

blood collected from other dogs was perfused for controlling hepatic blood flow volume.

*Experimental design*: The hepatic blood flow volume was maintained in approximately 60 min to the planned volume from pre-operation total hepatic blood flow volume determined by a electromagnetic flow meter (MVF-3000, NIHON KOHDEN, Japan). The time when blood flow was stabilized and started halothane inhalation, was designated as the preperfusion or inhalation.

The PA dogs were divided into 4 groups: One point five % of halothane inhalation under the hypoxic condition (group A) or normoxic condition (group B), and without halothane inhalation under the hypoxic or normoxic condition (groups C and D, respectively). In brief, group A (5 dogs) were inhaled 1.5% halothane under the hepatic blood flow volume less than 10% of pre-operation total hepatic blood flow volume, group B (4 dogs) were inhaled under the volume more than 50% of that, and group C (3 dogs) and group D (3 dogs) were not inhaled under the volume less than 10% and more than 50% of that, respectively.

*Samples*: Blood samples were collected from arterialized portal vein and hepatic vein at 30 min, 1, 2 and 3 hr after the perfusion or inhalation for determination of the hepatic oxygen supply and consumption calculated from the data of blood gas analysis by the formula described by Hughes *et al,* [15]. At the same time, small pieces of liver tissue samples were obtained to measure peroxidized phospholipid and MDA levels, and antioxidative enzyme activities.

*Sample preparation and determination for peroxidized phospholipid and MDAs*: Approximately 2 g of the liver specimen was washed with ice-cold saline to remove blood. One hundred miligram of the liver tissue was added with 2.5 m*l* of 0.1 M phosphate buffer (pH 7.4, containing 10 mM ethylenediamine-tetraacetic acid: EDTA) and homogenized with a Potter-Elvehjem homogenizer on ice for 1 min. From 1.2 m*l* of the homogenate, whole lipids and free MDA were extracted by the method of Bligh and Dyer [5]. For protein-bound MDA, the homogenate was alkalized before extraction. The high performance liquid chromatography analysis was carried out for determination of peroxidized phospholipids in accordance with the method of Matsuki *et al*. [19, 20].

*Sample preparation and determination for antioxidative enzyme activities*: For superoxide dismutase (SOD), 50 mg of the liver tissue was added with 5.0 m*l* of 0.05 M potassium phosphate buffer (pH 7.8) and homogenized with a Potter-Elvehjem homogenizer on ice for 1 min. The homogenate was centrifuged at 3,000 rpm for 10 min and the supernatant was collected. Total and Mn-SOD activities were determined with the nitro blue tetrazolium (NBT) method by Oberly and Spitz [22]. For glutathione peroxidase (GSH-px), 100 mg of the liver tissue was added with 5.0 m*l* of 0.05 M sodium phosphate buffer (pH 7.4) containing 0.5 mM EDTA and homogenized with a Potter-Elvehjem homogenizer on ice for 1 min. The GSH-px activity was determined by the method of Jensen and Glausen [16].

*Statistical analysis*: Statistical significance was analyzed by paired *t*-test.

## RESULTS

The changes of peroxidized phospholipid index (ratio), MDA levels, and antioxidative enzyme activities after the perfusion or halothane inhalation in 4 groups are shown in Tables 1, 2, and 3.

*Peroxidized phospholipids*: Percent changes of the hepatic tissue peroxidized phosphatidylethanolamine (PE) and phosphatidylcholine (PC) after the perfusion or inhalation are shown in Fig. 1. In group A, peroxidized PC levels tended to increase after inhalation, while no change of peroxidized PE levels was observed throughout the experimental period. In contrast, peroxized PE levels showed a decreasing tendency and a significant low level at 3 hr after the inhalation compared with the pre value in group B. No change of peroxidized PC levels was observed in group B. In both groups C and D, there were significant changes neither in peroxidized PE nor PC levels during the experimental period.

Table 1. Changes of the peroxidized phospholipids ratio (%) in hepatic tissue

| class | group | pre | 0.5 | 1 | 2 | 3 (hr) |
|---|---|---|---|---|---|---|
| PE | A | 1.13 ± 0.33 | 1.25 ± 0.31 | 1.33 ± 0.34 | 1.22 ± 0.47 | 1.27 ± 0.39 |
| | B | 1.62 ± 0.61 | 1.33 ± 0.17 | 1.20 ± 0.48 | 1.46 ± 0.55 | 1.14 ± 0.41 |
| | C | 1.27 ± 0.52 | 1.30 ± 0.55 | 1.13 ± 0.38 | 1.23 ± 0.44 | 1.03 ± 0.45 |
| | D | 1.84 ± 0.86 | 1.60 ± 1.14 | 1.36 ± 0.82 | 1.54 ± 0.88 | 1.68 ± 1.20 |
| PC | A | 0.95 ± 0.24 | 1.40 ± 0.45 | 1.29 ± 0.21 | 1.42 ± 0.36 | 1.35 ± 0.25 |
| | B | 1.68 ± 0.42 | 1.57 ± 0.39 | 1.56 ± 0.25 | 1.76 ± 0.55 | 1.78 ± 0.45 |
| | C | 0.93 ± 0.28 | 1.17 ± 0.03 | 1.05 ± 0.11 | 0.97 ± 0.18 | 1.12 ± 0.08 |
| | D | 1.81 ± 0.43 | 1.83 ± 0.37 | 1.82 ± 0.44 | 1.72 ± 0.51 | 1.81 ± 0.59 |

Each value is the ratio of peak area detected at 233 nm and 206 nm and given as a mean ± SD. Group A: 1.5% halothane inhalation under the hepatic blood flow volume less than 10% of pre-operation value. Group B: 1.5% halothane inhalation under the hepatic blood flow volume more than 50%. Group C: non-halothane inhalation under the volume less than 10%. Group D: non-halothane inhalation under the volume more than 50%.

Table 2. Changes of the total, free and protein-bound MDA concentrations (nmol/g wet tissue) in hepatic tissue

| | group | pre | 0.5 | 1 | 2 | 3 (hr) |
|---|---|---|---|---|---|---|
| total MDA | A | 19.2 ± 7.96 | 18.3 ± 6.50 | 25.0 ± 12.5 | 27.5 ± 12.8 | 25.4 ± 11.5 |
| | B | 16.5 ± 7.20 | 16.9 ± 7.74 | 16.3± 6.70 | 19.0 ± 7.92 | 17.7 ± 6.16 |
| | C | 20.2 ± 5.23 | 21.3 ± 5.27 | 23.8 ± 4.52 | 21.3 ± 2.58 | 23.8 ± 2.72 |
| | D | 13.1 ± 1.21 | 14.7 ± 2.77 | 18.0 ± 4.33 | 16.1 ± 1.85 | 14.4 ± 2.48 |
| free MDA | A | 7.09 ± 3.88 | 6.47 ± 2.55 | 9.67 ± 6.12 | 11.0 ± 6.40 | 10.2 ± 8.78 |
| | B | 5.11 ± 2.68 | 5.41 ± 2.82 | 5.32 ± 2.19 | 6.78 ± 2.63 | 6.22 ± 3.84 |
| | C | 8.78 ± 2.95 | 8.69 ± 1.81 | 9.55 ± 1.51 | 9.77 ± 3.33 | 10.2 ± 0.78 |
| | D | 5.22 ± 1.84 | 5.01 ± 1.17 | 6.65 ± 1.12 | 5.70 ± 0.90 | 5.15 ± 2.40 |
| protein-bound MDA[a)] | A | 12.1 ± 4.65 | 11.8 ± 4.69 | 15.4 ± 6.84 | 16.4 ± 7.17 | 20.1 ± 12.1 |
| | B | 11.4 ± 5.67 | 11.5 ± 5.92 | 11.0 ± 4.85 | 12.2 ± 5.52 | 11.6 ± 5.18 |
| | C | 11.4 ± 3.13 | 12.6 ± 3.80 | 14.2 ± 3.07 | 11.5 ± 2.42 | 13.6 ± 2.39 |
| | D | 7.91 ± 1.18 | 9.73 ± 1.86 | 11.4 ± 3.28 | 10.4 ± 2.50 | 9.43 ± 1.41 |

Each value is given as a mean ± SD. a) Calculated from (total MDA)–(free MDA). The treatment of each group was described in Table 1.

Table 3. Changes of the total SOD, Mn SOD and GSH-px activities (U/mg protein) in hepatic tissue

| | group | pre | 0.5 | 1 | 2 | 3 (hr) |
|---|---|---|---|---|---|---|
| total SOD | A | 30.0 ± 10.7 | 25.6 ± 7.33 | 24.6 ± 9.07 | 22.8 ± 3.44 | 26.2 ± 13.4 |
| | B | 37.0 ± 17.7 | 37.7 ± 22.3 | 35.9 ± 16.7 | 33.8 ± 6.75 | 31.9 ± 16.5 |
| | C | 29.5 ± 4.23 | 23.9 ± 3.10 | 16.5 ± 2.63 | 21.8 ± 3.33 | 26.4 ± 4.96 |
| | D | 49.0 ± 11.5 | 39.1 ± 6.49 | 43.3 ± 8.71 | 30.5 ± 4.08* | 46.9 ± 14.0 |
| Mn SOD | A | 16.8 ± 8.76 | 15.6 ± 5.70 | 15.6 ± 5.87 | 13.4 ± 2.72 | 12.9 ± 2.83 |
| | B | 21.8 ± 14.3 | 19.4 ± 11.2 | 19.9 ± 8.62 | 19.1 ± 5.63 | 22.2 ± 10.1 |
| | C | 15.9 ± 5.48 | 15.2 ± 2.87 | 12.1 ± 2.51 | 15.6 ± 2.93 | 21.9 ± 2.84 |
| | D | 21.2 ± 6.21 | 23.7 ± 10.0 | 18.5 ± 3.57 | 20.0 ± 9.33 | 24.5 ± 13.2 |
| GSH-px | A | 16.6 ± 8.10 | 17.8 ± 11.9 | 19.2 ± 10.6 | 14.7 ± 6.84 | 13.6 ± 6.79 |
| | B | 37.6 ± 13.1 | 49.5 ± 21.1 | 43.9 ± 21.0 | 45.1 ± 40.0 | 45.1 ± 26.7 |
| | C | 38.8 ± 7.68 | 34.6 ± 1.31 | 28.8 ± 6.35 | 24.0 ± 3.39* | 35.5 ± 5.52 |
| | D | 30.5 ± 9.70 | 32.6 ± 7.23 | 23.7 ± 6.35 | 30.0 ± 0.58 | 40.0 ± 11.1 |

Each value is given as mean ± SD. *: Significant difference compared with the pre value by paired *t*-test ($p<0.05$). The treatment of each group was described in Table 1.

*Malondialdehydes* (*MDAs*): Figure 2 shows percent changes of the free and protein-bound MDA levels after the perfusion or inhalation. In group A, both levels of the free and protein-bound MDA significantly increased at 1 and 2 hr after the perfusion or inhalation, whereas only free MDA level showed an increasing tendency at 2 and 3 hr after the inhalation in group B. In group C, the level of protein-bound MDA significantly increased at 30 min and 1 hr after the perfusion, however, no significant changes of both levels were observed in group D.

*Antioxidative enzyme activities*: Figure 3 shows percent changes of the antioxidative enyzme (total and Mn-SOD, and GSH-px) activities of hepatic tissue in 4 groups. In group C, total SOD activity tended to decrease with a significantly lower level compared with pre value at 1 hr after the perfusion. In the remaining 3 groups, no significant changes were observed during the experimental period. In addition, no significant change of the hepatic tissue GSH-px activity was observed in 4 groups.

*Correlation between hepatic oxygen supply or consumption and MDAs*: The correlations between hepatic oxygen supply or consumption (Table 4) and MDAs (total, protein-bound, and free MDA) were examined in halothane inhalation and non-inhalation groups (Figs. 5 and 6, respectively). Significantly negative correlations between percent changes of the oxygen supply and total MDA, and also protein-bind MDA were observed in the halothane inhalation group, however, no correlation was detected among them in non-inhalation groups. Between oxygen consumption and total MDA or protein-bound MDA, significantly negative correlations were also detected in halothane inhalation groups, but not in non-inhalation

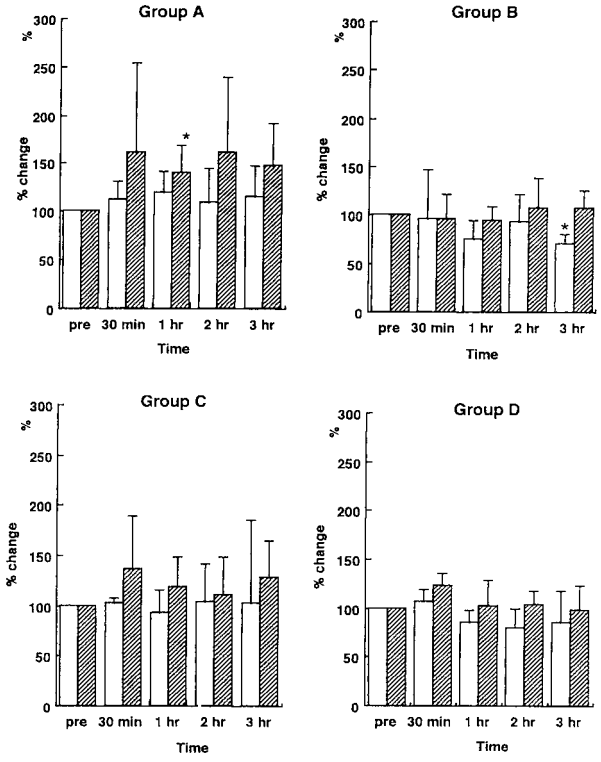


Fig. 1. Percent changes of the peroxidized phospholipids in hepatic tissue. The treatment of each group was described in Table 1. □: peroxidized phosphatidylethanolamine ratio, ▨: peroxidized phosphatidylcholine ratio. *: significant difference ($p<0.05$) compared with each pre value by the paired *t*-test.

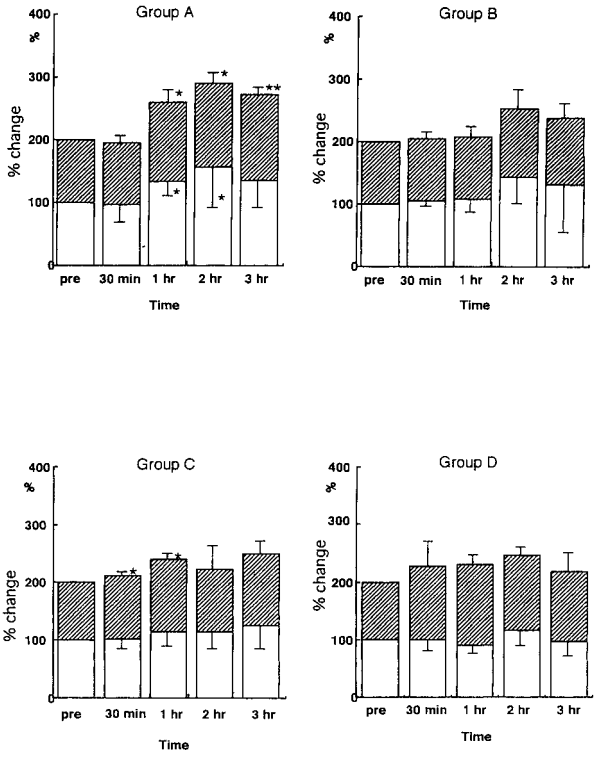


Fig. 2. Percent changes of free ( □ ) and protein-bound MDA ( ▨ ) in hepatic tissue. The treatment of each group was described in Table 1. * and **: significant difference compared with each pre value by the paired *t*-test. ($p<0.05$ and $p<0.01$, respectively).

groups.

## DISCUSSION

It has been widely accepted that hepatopathy was commonly induced by halothane under the hypoxic condition [13, 21, 26]. In the hypoxic condition, free radical generations are remarkably enhanced by the activation of the anaerobic metabolic pathway for nucleotides and purines [24, 32]. In addition, the lipid peroxidation was induced under the hypoxic condition, indicating an increase of MDAs level [34]. Omar *et al*. [23] also reported that the increase of MDAs in hepatic tissue during the ischemic condition. In this study, peroxidized PC level tended to increase after the halothane inhalation in group A, which was given hypoxic condition by the hepatic blood flow volume less than 10% of pre-operation volume. In contrast, no changes of these levels were observed during the experimental period in group B with halothane inhalation and group D without, both of which were kept under the sufficient oxygen supply to liver designated as the normoxic condition. Although protein-bound MDA level in group C, given hypoxic condition alone, increased after the perfusion, the response of this was smaller than that in group A. Becker *et al*. [3] demonstrated that decrease of aerobic energy metabolism, which induced free radical generations, was significantly enhanced by the treatment with halothane in murine hepatocytes *in vitro*. Therefore, these results obtained in this study suggested that halothane inhalation enhances free radical generations related to the anaerobic energy metabolism under the hypoxic condition *in vivo*.

On the other hand, halothane shows a competitive effect on oxygen binding to cytochrome P-450, especially under the hypoxic condition, followed by the induction of anaerobic halothane dehalogenation. Since intermediate metabolites of halothane have a free radical activity, the phospholipids peroxidation is induced in cell membranes [1]. The subsequent cell death is developed by the activation of phospholipase A2 ($PLA_2$), by which peroxidized phospholipids are degradated to low molecular end products MDAs [27]. In this study, free and protein-bound MDA levels significantly increased at 1 and 2 hr after the inhalation in group A. Matsuki *et al*. [20] reported that the accumulation of peroxidized phospholipids in equine skeletal muscle following exercise contributed to increase protein-bound MDAs. The peroxidized PE tended to decrease after the inhalation in group B, showing significantly lower at 3 hr compared to the pre value. Since

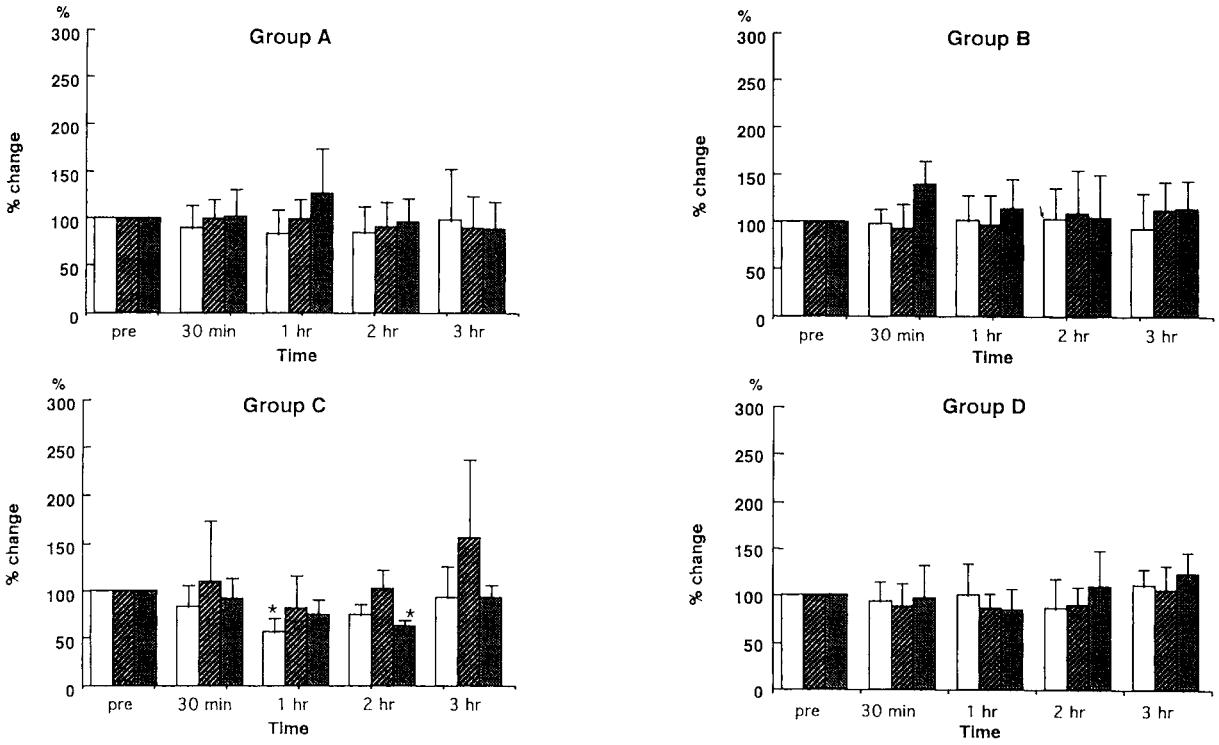


Fig. 3. Percent changes of the total SOD (□), Mn-SOD (▨), and GSH-px (■) in hepatic tissue. The treatment of each group was described in Table 1. *: significant difference ($p<0.05$) compared with each pre value by the paired *t*-test.

Table 4. Changes of the oxygen supply and consumption (ml/min·100 g tissue) in hepatic tissue

| | group | pre | 0.5 | 1 | 2 | 3 (hr) |
|---|---|---|---|---|---|---|
| $O_2$ supply | A | 6.80 ± 3.35 | 1.96 ± 0.91* | 2.07 ± 0.90* | 1.92 ± 0.96* | 1.64 ± 0.98* |
| | B | 9.39 ± 3.15 | 10.2 ± 4.64 | 10.7 ± 5.39 | 10.5 ± 5.38 | 10.0 ± 5.32 |
| | C | 11.2 ± 2.01 | 1.55 ± 0.26* | 1.57 ± 0.11* | 1.58 ± 0.17* | 1.44 ± 0.11* |
| | D | 18.2 ± 6.02 | 18.7 ± 6.84 | 18.4 ± 6.17 | 17.8 ± 4.61 | 16.5 ± 3.21 |
| $O_2$ consumption | A | 1.97 ± 0.80 | 1.04 ± 0.61* | 1.01 ± 0.66** | 0.91 ± 0.58** | 0.84 ± 0.54* |
| | B | 3.01 ± 2.65 | 3.15 ± 2.68 | 3.38 ± 2.15 | 3.39 ± 2.37 | 3.06 ± 2.12 |
| | C | 2.63 ± 0.85 | 0.64 ± 0.07 | 0.44 ± 0.21 | 0.65 ± 0.34 | 0.58 ± 0.29 |
| | D | 5.11 ± 1.24 | 5.39 ± 0.75 | 4.73 ± 0.11* | 5.05 ± 0.82 | 4.06 ± 1.14 |

Each value is given as a mean ± SD. * and **: Significant difference compared with pre value by the paired *t*-test ($p<0.05$ and $p<0.01$, respectively). The treatment of each group was described in Table 1.

the level of peroxidized PE, which is abundant in the inside layer of membrane, is more susceptible to the degradation by $PLA_2$ [6, 31], peroxidized PE was converted to free MDAs, which showed an increase tendency after the inhalation in group B.

No significant correlations between oxygen supply or consumption and free MDA was observed in this study, probably because free MDA is rapidly converted to the protein-bound MDA by mitochondrial enzymes [4, 28]. However, significantly negative correlations ($p<0.01$) between hepatic oxygen supply and total or protein-bound MDA were observed in only halothane inhaled groups like as reported *in vitro* study by Gut and Huwyler [11]. Therefore, the generation of free radicals in halothane inhalation under the hypoxic condition was mainly caused by the halothane anaerobic metabolism. On the other hand, SOD activities tended to decrease in groups A and C after the perfusion or inhalation, whereas no significant decrease of GSH-px activity was observed in group A in this study. Marubayashi *et al.* [18] suggested that a decrease of SOD activity indicated an elimination of free radicals, however Sato *et al.* [26] reported that the GSH-px has no effect on the elimination of free radicals generated from the anaerobic energy metabolism under the hypoxic condition. Therefore, free radicals were also generated by the decrease of energy metabolism in addition to halothane-related one.

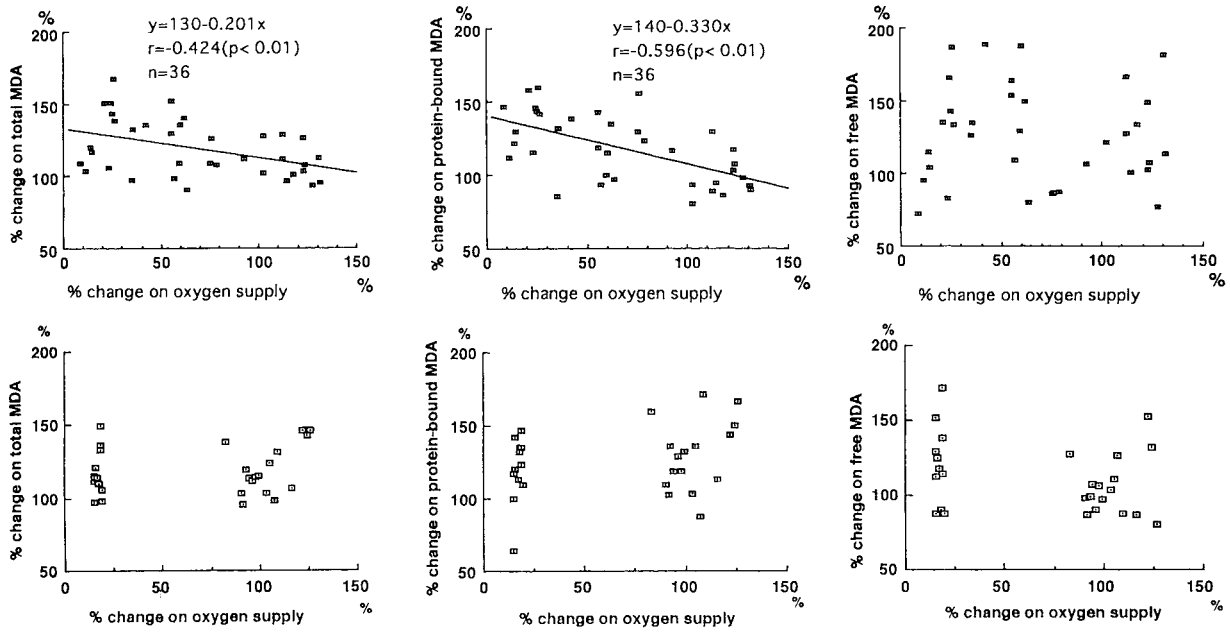


Fig. 4. Correlations between percent change of oxygen supply and MDAs with (upper side) and without (lower side) halothane anesthesia.

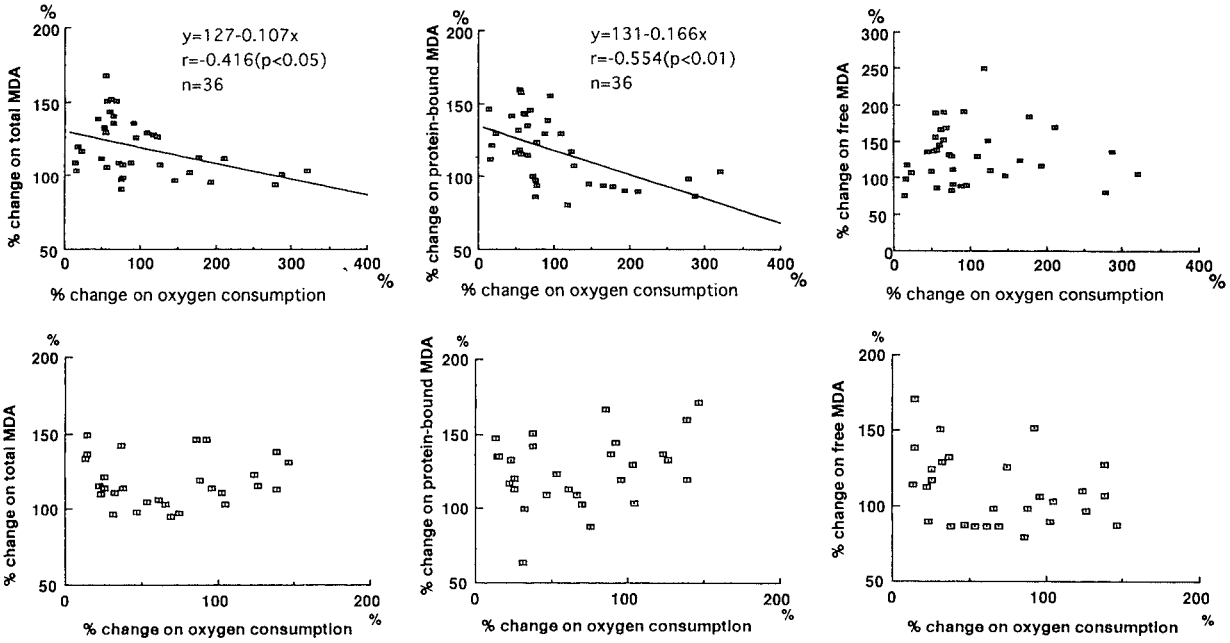


Fig. 5. Correlation between percent change of oxygen consumption and MDAs with (upper side) and without (lower side) halothane anesthesia.

In conclusion, the cause of halothane-induced hepatopathy is closely related to free radicals generated from the anerobic halothane metabolism in addition to those from the anaerobic energy metabolism.

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