Hepatic Microsomal Lipoperoxidation and Inhalation Anesthetics: A Biochemical and Morphologic Study in the Rat

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Hepatic microsomal lipoperoxidation in vivo is stimulated by anesthetic concentrations of halothane (Fluothane) and chloroform in phenobarbital-pretreated rats. Diethyl ether and fluroxene (Fluoromar) do not share this property in the rat. Studies in vitro show no enhancement of lipoperoxidation, but in this circumstance metabolism of the anesthetics is minimal. It is speculated that enhancement of metabolism of halothane and chloroform by phenobarbital induction is capable of fostering a peroxidative breakdown of hepatic microsomal polyenoic lipid esters. (Key words: Lipoperoxidation; Hepatic microsomes; Diene conjugates; NADPH cytochrome c reductase; Halothane; Fluroxene; Diethyl ether; Chloroform.)

Halothane (Fluothane) has been widely used as an inhalation anesthetic since it was introduced to practice. Occasional clinical reports attest to sequelae of various degrees of hepatic injury, sometimes leading to hepatic necrosis and death following administration of this agent. Major obstacles to the study of this toxic effect are the unpredictable and sporadic nature of attack and the inability to produce biochemical or morphologic evidence of hepatic damage with acute administration of halothane in the laboratory animal. Whether halothane possesses the basic ability to foster hepatic damage by a toxic action in certain circumstances remains a mystery. Since microsomal lipoperoxidation has been postulated as a primary event in carbon tetrachloride hepatotoxicity, the present experiments were conducted to explore this phenomenon with commonly-used anesthetics. Chloroform, an anesthetic with well-recognized hepatotoxic effects, was compared with halothane, fluroxene (Fluoromar), and diethyl ether.

Methods

Studies in Vivo

Lipoperoxidation Studies. Male Sprague-Dawley rats (180–200 g) were used for the majority of these studies, although a group of female rats was studied to insure that the observations were not sex-specific. Animals were divided into two groups: 1) induced rats, those pretreated with phenobarbital 75 mg/kg ip for five days prior to experiments; and 2) non-treated control animals. These animals had access to Purina Rat Chow and water ad lib until the time of the experiments. All animals were anesthetized in a large plexiglass chamber with a standard anesthesia machine (Vetritol) calibrated by gas chromatography. SKF 525-A (2-diethylaminoethyl-2,2-diphenylvalerate HCl) was administered to 12 phenobarbital-pretreated rats ip 30 minutes before halothane anesthesia. The dosage of this microsomal enzyme-inhibiting agent was either 50 mg/kg (six rats) or 150 mg/kg (six rats). DPPD (N,N'-diphenyl-p-phenylenediamine), a potent antioxidant, was administered ip to another group of six induced rats 30 minutes prior to halothane anesthesia. Anesthetic concentrations in oxygen were: halothane, 1 per cent; chloroform, 1 per cent; fluroxene, 4.4 per cent; diethyl ether, 3.8 per cent. These anes-
Lipid peroxidation was administered for two hours, and the concentrations used (approximately 1.3 MAC) produced narcosis in all animals. The overall acute anesthetic mortality rate was less than 10 per cent, with no difference between control and induced groups.

Animals were sacrificed immediately after two hours of anesthesia and 6 g (wet weight) of liver removed for study. The livers were homogenized in cold 0.25 M sucrose, pH 7.4, and then centrifuged at 10,000 × g for 30 minutes. The supernatant fraction was subsequently centrifuged at 105,000 × g for 60 minutes, and the microsomal pellet rinsed with 1.15 per cent KCl at 2°C. The microsomes were then resuspended in 1.15 per cent KCl to a final concentration of 20 mg protein/ml, determined by the biuret method. The degrees

![Graph showing diene conjugate formation](image)

**PHENOBARBITAL PRETREATED RATS**
75 mg/kg ip/24 h. FOR 5 DAYS
PRIOR TO 1% HALOTHANE FOR 2 h.

**CONTROL RATS, 1% HALOTHANE**
FOR 2 h. n = 3

**MEAN ± SE.**

![Graph showing spectral data](image)

**Fig. 1.** Representative diene conjugate absorption curve for lipid extracts of microsomes from animals pretreated with phenobarbital and anesthetized with halothane (solid line). Control animals anesthetized with halothane showed only baseline absorption of triglycerides (dashed line) and did not differ in this respect from control non-anesthetized or phenobarbital-pretreated non-anesthetized preparations.

**Fig. 2.** Diene conjugate formation (±SE) in hepatic microsomes of phenobarbital-pretreated animals. All anesthetics were administered for two hours. The degree of lipoperoxidation is the difference between the control value (0.138 ± 0.006) and the values from anesthetized animals. Halothane and chloroform produced diene conjugate at the P < 0.001 level of significance compared with induced non-anesthetized animals.

... of lipoperoxidation of chloroform-methanol extracts of the microsomes were determined by measuring ultraviolet absorption of diene conjugates (products of lipoperoxidation) at 245 mλ, in a Zeiss spectrophotometer. The method used was that described by Klaassen and Plaa. The figure illustrates typical spectrophotometric curves of lipid extracts of microsomes examined in the ultraviolet spectrum region 240-280 mλ. The absorption curve of microsomal lipid extracts from control animals anesthetized with halothane results primarily from absorption of ultraviolet light by normal triglycerides. The optical density difference (ΔOD) between the two curves represents diene conjugates formed in hepatic microsomes of phenobarbital-pretreated animals anesthetized with halothane. (The extinction coefficient or ε value of diene conjugates ranges from ~10,000 to 30,000, whereas triglycerides have a far lower ε value).

**Morphologic Studies.** In another experiment, a control group of three normal animals and a group of three animals pretreated with phenobarbital (75 mg/kg ip) for five days were anesthetized with 1 per cent halothane in oxygen for two hours. These rats were sacrificed 24 hours after anesthesia and the...
Studies in Vitro

Malonaldehyde Formation. Malonaldehyde (MA) formation was used as an index of lipoperoxidation in vitro. Malonaldehyde is a product of arachidonic and linoleic acid peroxidation. MA formation, peroxide formation, and diene conjugation have been found to be linear when plotted against each other.\(^9\) Hepatic microsomes were prepared by techniques described in the in-citro methods section. The surfaces of the microsomal pellets were thoroughly rinsed with 50 mM tris-HCl buffer, pH 7.4, to remove adhering sucrose, which interferes with MA determination. Microsomes were then resuspended to a concentration of 20 mg protein/ml in tris-HCl buffer. Both enzymatic (NADPH-linked) and nonenzymatic (ascorbate-linked) lipoperoxidation were studied.

Incubations were carried out in the presence of air in a Dubnoff metabolic incubator at 37 C. Incubation mixtures for NADPH-linked peroxidation contained 0.2 ml microsomes (4 mg protein), glucose-6-phosphate (40 μmol), glucose-6-phosphate dehydrogenase (2 IU), NADP (2 μmol), nicotinamide (50 μmol), MgCl\(_2\) (15 μmol) and tris-HCl buffer were added to produce a final volume of 2.6 ml. The buffer had been previously equilibrated with halothane, chloroform, fluoroxyne, or diethyl ether such that the final concentration of every anaesthetic in the beakers was 3 mM. The effects of 1 and 3 mM trifluoracetic acid, a primary metabolite of halothane\(^11\) and fluoroxyne,\(^12\) were also studied. Enzymatic lipoperoxidation was initiated by adding 10 μl of a solution containing 1 M ADP and 6 mM FeCl\(_3\) to each beaker at time zero. Reactions were terminated at five minutes by adding 0.1 ml 90 per cent trichloroacetic acid. Ascorbate-linked peroxidation was initiated by pipetting 0.1 ml of freshly prepared 1 mM ascorbic acid into beakers containing 0.2 ml microsomes (4 mg protein) and 2.0 ml tris-

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Dose (mg/kg ip)</th>
<th>Optical Density (245 μm) ± SE</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0.277 ± 0.016 (n = 12)</td>
</tr>
<tr>
<td>SKF 525-A</td>
<td>50</td>
<td>0.214 ± 0.009 (n = 6)†</td>
</tr>
<tr>
<td>SKF 525-A</td>
<td>150</td>
<td>0.194 ± 0.007 (n = 6)‡</td>
</tr>
<tr>
<td>DPPD</td>
<td>1.5</td>
<td>0.152 ± 0.037 (n = 6)‡</td>
</tr>
</tbody>
</table>

* All studies were performed in animals pretreated with phenobarbital. "n" represents the number of animals per experiment.
† Significant difference from non-inhibited group, \(P < 0.005\).
‡ Significant difference from non-inhibited group, \(P < 0.001\).
HCl buffer. Anesthetic and trifluoroacetic acid concentrations were the same as in the NADPH-linked peroxidation studies. Neither ADP-Fe- nor an NADPH-generating system was utilized in ascorbate-linked peroxidation studies. After five minutes of incubation at 37°C this reaction was terminated by adding 0.1 ml 90 per cent trichloroacetic acid.

MA was determined colorimetrically with the thiobarbiturate method described by Ernster and Nordenbrand. The intensity of the orange-red color was measured with a Zeiss spectrophotometer at 535 mμ. An extinction coefficient of 1.56 × 10³ M⁻¹ cm⁻¹ was used to calculate the amount of MA formed.

NADPH Cytochrome c Reductase Activity

The hepatic microsomal flavoprotein, NADPH cytochrome c reductase, has been implicated as a primary enzyme involved in NADPH-linked enzymatic lipoperoxidation. Therefore, the acute effects of halothane, chloroform, diethyl ether, fluoroxene, and trifluoroacetic acid on activity of this enzyme were investigated. Hepatic microsomes were prepared as described previously; however, potassium phosphate buffer, 50 mM, containing 0.1 mM EDTA was used instead of tris-HCl buffer. Microsomes, 0.5 ml (3 mg protein), cytochrome c, Sigma Type III (150 μmol), and 1.0 ml phosphate-EDTA buffer were added to spectrophotometric cuvettes at room temperature. These closed cuvettes were equilibrated with the four anesthetics in buffer to yield a final concentration of 3 mM. Trifluoroacetic acid in buffer was added to the cuvettes to reach 3 mM concentrations. To one cuvette (blank), 0.5 ml buffer was added, and to the other (reaction cuvette), chemically-reduced NADPH (0.3 μmol) in 0.5 ml buffer. The AOD between these cuvettes at 550 mμ was recorded at 15 seconds and converted to μmol cytochrome c reduced/mg protein/min.

Table 2. The Effects of Anesthetics on NADPH-linked Malonaldehyde Formation in vitro

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>n</th>
<th>MA Formation ± SE (μg/mg Microsomal Protein/Min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td></td>
<td>4.22 ± 0.28</td>
</tr>
<tr>
<td>Halothane</td>
<td>3 mM</td>
<td>7</td>
<td>1.06 ± 0.32‡</td>
</tr>
<tr>
<td>Ether</td>
<td>3 mM</td>
<td>5</td>
<td>4.16 ± 0.58</td>
</tr>
<tr>
<td>Chloroform</td>
<td>3 mM</td>
<td>4</td>
<td>2.70 ± 0.33‡</td>
</tr>
<tr>
<td>Fluoroxene</td>
<td>3 mM</td>
<td>4</td>
<td>3.21 ± 0.67</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>1 mM</td>
<td>4</td>
<td>4.82 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>3 mM</td>
<td>4</td>
<td>4.73 ± 0.42</td>
</tr>
</tbody>
</table>

*Experiments in all cases were performed with hepatic microsomes from rats pretreated with phenobarbital. "n" represents the number of animals per experiment.

† Significant difference from control, P < 0.001.
‡ Significant difference from control, P < 0.003.

Table 3. The Effects of Anesthetics on Ascorbate-linked Malonaldehyde Formation in vitro

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>n</th>
<th>MA Formation ± SE (μg/mg Microsomal Protein/Min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td></td>
<td>1.01 ± 0.02</td>
</tr>
<tr>
<td>Halothane</td>
<td>3 mM</td>
<td>5</td>
<td>0.71 ± 0.05‡</td>
</tr>
<tr>
<td>Ether</td>
<td>3 mM</td>
<td>4</td>
<td>1.09 ± 0.06</td>
</tr>
<tr>
<td>Chloroform</td>
<td>3 mM</td>
<td>5</td>
<td>0.79 ± 0.08‡</td>
</tr>
<tr>
<td>Fluoroxene</td>
<td>3 mM</td>
<td>5</td>
<td>0.66 ± 0.08</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>1 mM</td>
<td>5</td>
<td>1.16 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>3 mM</td>
<td>5</td>
<td>1.41 ± 0.10‡</td>
</tr>
</tbody>
</table>

*Experiments in all cases were performed with hepatic microsomes from rats pretreated with phenobarbital. "n" represents the number of animals per experiment.

† Significant difference from control value, P < 0.001.

Results

Studies in Vivo

Lipoperoxidation Studies. None of the four anesthetics studied caused diene conjugate formation in control animals. The optical density values for these non-induced animals ±SE were: non-anesthetized, 0.138 ± 0.006 (n = 12); halothane, 0.136 ± 0.009 (n = 6); chloroform, 0.126 ± 0.008 (n = 3); diethyl ether, 0.106 ± 0.005 (n = 5). Lipoperoxidation was produced by chloroform and halothane anesthesia in phenobarbital-pretreated animals, as illustrated in figure 2. The time course of diene conjugate formation in induced animals anesthetized with 1 per cent halothane for two hours or less is illustrated in figure 3. Large increases occur within 30 minutes of inhalation and peak at 60 minutes. The ephemeral nature of diene conjugates is shown by a re-
turn to control values 16 hours after halothane anesthesia. Phenobarbital-pretreated female rats had doubled optical density values after halothane anesthesia, demonstrating that the effect is not sex-specific. Diene conjugate formation was observed in induced rats anesthetized with fluroxene, but the change was not significant. Injection of trifluoroacetic acid (1 mg/kg ip in saline solution) did not stimulate diene conjugate formation in rats.

Table 1 shows that diene conjugate formation produced by halothane in induced rats can be inhibited by preanesthetic treatment with SKF 525-A or DPPD. SKF 525-A was dissolved in saline solution and injected ip. DPPD was dissolved in 0.2 ml acetone and administered ip. Acetone per se does not inhibit halothane-produced lipoperoxidation. Pure acetone, 0.2 ml ip, 30 minutes prior to halothane anesthesia in induced rats produced an optical density value of 0.251 ± 0.02 (n = 3), which is no different from the value observed in induced rats not given acetone.

Morphologic Studies. Liver sections from phenobarbital-pretreated animals showed the increased mitotic activity and peripheral deposits of glycogen normally associated with the induction phenomenon.17 Livers of animals pretreated with phenobarbital and anesthetized with halothane revealed altered morphology, although the changes were minimal. Changes consisted of periportal and centrilobular vacuolization, rare pyknosis, cytoplasmic homogenation, and increased mitotic activity. Necrosis was not seen in any section.

Studies in Vitro

Lipoperoxidation. The effects of anesthetics and trifluoroacetic acid on NADPH-linked (enzymatic) MA formation are shown in table 2; those of ascorbate-linked (nonenzymatic) MA formation in table 3.

NADPH Cytochrome c Reductase Activity. The results of this study are shown in figure 4.

Discussion

Halothane and chloroform stimulate lipoperoxidation in vivo in phenobarbital-pretreated rats, whereas diethyl ether and fluroxene do not. This is of interest because the former two agents have been implicated in production of hepatic damage in man, whereas the latter two have not been as suspect.15 The lack of effect of fluroxene in the rat is somewhat surprising, since the vinyl radical of this agent is potentially reactive. Formation of lipoperoxides is illustrated in figure 5. Lipoperoxidation begins by accumulation of free radicals, during which antioxidants are consumed and hydrogen is abstracted from polyenoic fatty acids. Free radicals eventually reach a concentration such that after this period of induction of the reaction, rapid oxygen absorption ensues and peroxy radicals are formed concomitant with double-bond rearrangement.19 The peroxy radical in turn abstracts hydrogen
from another fatty acid chain to form a new radical. Carbonyl residues and dienes are produced. Tam and McCay\textsuperscript{19} found these peroxide functions are associated with the $\beta$-position polyenoic fatty acids, primarily arachidonie, linoleic, and linolenic fatty acid esters of phosphatidylcholine and phosphatidylethanolamine.

Hashimoto \textit{et al.}\textsuperscript{21} and Recknagel and Ghoshal\textsuperscript{22} propose that the hepatotoxicity and the metabolism of the halogenated hydrocarbon, carbon tetrachloride (CCl$_4$), are closely linked. These authors state that the metabolism of CCl$_4$ produces free radicals which promote lipoperoxidation. Peroxidative-chain propagation eventually causes destruction of lipid-rich membranes, altering the functional capacity of the endoplasmic reticulum. Triglycerides subsequently accumulate, and fatty degeneration and necrosis of the liver ensue. This mechanism would appear to be reinforced by the work of Garner and McLean\textsuperscript{23} who demonstrated that induction of drug-metabolizing enzymes by phenobarbital pretreatment increases the sensitivity of rat liver to CCl$_4$ poisoning, presumably by increasing free radicals at any one dose. Castro \textit{et al.}\textsuperscript{24} indicated that SKF 523-A and antioxidants such as DPPD protect against CCl$_4$ hepatotoxicity. The observation that pretreatment of rats with phenobarbital increases the degree of morphologic damage in the liver of rats anesthetized with chloroform\textsuperscript{25} lends support to the thesis linking metabolism and toxicity of this halogenated anesthetic in a manner parallel to CCl$_4$.

Klaasen and Plaa\textsuperscript{5} disagreed with certain aspects of the lipoperoxidation hypothesis. These authors stated that while the temporal relationships of CCl$_4$ hepatotoxicity are compatible with the thesis, the dose relationship is weaker. No increase in diene conjugates was observed by these investigators after injection of chloroform ip, but this group did not use induced rats. Davis \textit{et al.}\textsuperscript{26} compared the actions of halothane and CCl$_4$ on components of the hepatic microsomal drug-metabolizing systems in normal and phenobarbital-pretreated rats. Rats were given phenobarbital, 50 mg/kg ip, for three days and then given halothane, 1 ml/kg, ip. These authors found no signifi-

\textbf{Fig. 5.} The mechanism of lipoperoxidation of a polyenoic fatty acid. Hydrogen abstraction and peroxo radical formation are associated with rearrangement of the double bonds to the diene configuration.

cant destructive effect by halothane in these experiments. There are several drawbacks to this study, however. The first is the short period of phenobarbital pretreatment. Scholler\textsuperscript{25} pretreated rat for 11 days before confirming enhanced toxicity with chloroform. Van Dyke and Wineman\textsuperscript{27} observed that optimal dechlorination of halothane requires an inducible factor found in the soluble fraction (non-microsomal) of the liver cell. The time course necessary for maximal induction of this factor is not known and may not be maximally increased in the rat with only three days of phenobarbital pretreatment. Another major drawback of the study by Davis \textit{et al.} and many other studies of this nature is the utilization of the ip route of administration for volatile anesthetics. Twenty-four-hour nonvolatile ether-extractable acid-halogenated urinary metabolites (measured by the Fujiiwa reaction) are less after trichloroethylene, methoxyflurane, and chloroform have been administered ip than following two-hour inhalation of these anesthetics at clinical concentrations.\textsuperscript{28}
The reason for this is obscure, but it may be due to diffusion through the bowel or to decreased extraction of anesthetics at high concentrations by the liver, in a manner similar to that reported by Sawyer et al.25

The in-vitro data presented suggest that neither halothane nor chloroform per se increases lipoperoxidation. In fact, these anesthetics tend to inhibit MA formation during both NADPH- and ascorbate-linked reactions in microsomal preparations. Since the anesthetics do not change the activity of NADPH cytochrome c reductase, alteration of this enzyme is not the mechanism of decreased MA formation with halothane and chloroform. This observation supports the viewpoint26 that this enzyme may not be necessary for NADPH-linked peroxidation.

The findings of these experiments suggest that halothane and chloroform may foster injury of the hepatic endoplasmic reticulum by initiating peroxidative breakdown of polyenoic lipids of these membranes. This is manifest only when the rate of metabolism of these agents is increased by means of enzyme induction with phenobarbital. We speculate that the act of metabolism (i.e., reactive intermediates formed during metabolism) of halothane and chloroform initiates this effect. The reason control animals anesthetized with these agents show no diene conjugate formation is that lipoperoxidation is an explosive reaction catalyzed only when a sufficient quantity of reactive intermediate accumulates. This speculation is reinforced by the findings that enhancement of MA formation is not produced by these anesthetics in vitro (during which time little anesthetic is metabolized) and by the observation that diene conjugate formation in vivo can be inhibited by SKF 525-A and DPPD. The lack of stimulation of diene conjugates and MA by trifluoroacetic acid implies that this end-product of halothane metabolism is not the primary offender. However, the lack of diene conjugate formation produced by ip injection of this polar compound in vivo does not rule out this possibility, since the acid may never reach the intracellular hepatic endoplasmic reticulum.

Chloroform anesthesia causes centriflobular necrosis in phenobarbital-pretreated rats,25 but halothane anesthesia produced only minor histologic changes for equivalent increases in diene conjugates. On this basis, it would appear that there are fundamental differences between the mechanisms leading to hepatic injury by the two halogenated anesthetics after initial lipoperoxidation.

The author acknowledges the technical assistance of Miss Ann Sagayla and Miss June Takeyama, Departments of Pharmacology and Anesthesia, Harvard Medical School.

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Metabolism

L-DOPA AND RBC ENZYMES Previous reports have indicated that after prolonged L-dopa therapy patients have a diminution in the activity of enzymes, mediating catecholamine synthesis (e.g., tyrosine hydroxylase, aromatic L-amino-acid decarboxylase), and, conversely, an increase in monoamine oxidase, one of the enzymes mediating catecholamine degradation. This paper reports diminished activity of COMT (catechol-O-methyltransferase), an enzyme important in the degradation of catecholamines, in the erythrocytes of patients chronically treated with L-dopa. A group of eight patients had a 40 per cent reduction in OMT activity compared with a control group of nine subjects. COMT activity began to be significantly reduced by the third week of treatment. The reduction seemed related to duration of therapy rather than total daily dose. The authors postulate three mechanisms: 1) diminished synthesis of COMT; 2) increased degradation; 3) inhibition of the enzyme. They feel that their data were most compatible with increased degradation of COMT. (Weiss, J. L., Coln, G. K., and Chase, T. N.: Reduction of Catechol-O-methyltransferase Activity by Chronic L-Dopa Therapy, Nature 234: 218–219, 1971.)