

An Animal Model of Halothane Hepatotoxicity:

Roles of Enzyme Induction and Hypoxia

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Exposure of phenobarbital-pretreated male Sprague-Dawley rats to halothane, 1 per cent, for two hours under conditions of hypoxia (F_{I_0} , 0.14) resulted in extensive centrilobular necrosis within 24 hours. Accompanying the morphologic damage were an increase in serum glutamic pyruvic transaminase (SGPT) and a decrease in hepatic microsomal cytochrome P-450. Glutathione levels in the liver were unchanged. Phenobarbital-pretreated rats anesthetized with halothane, 1 per cent, at F_{I_0} , 0.21 had only minor morphologic changes at 24 hours. Hepatic injury was not apparent in any non-phenobarbital-induced rat or in any induced animal exposed to ether at F_{I_0} , 0.10 or to halothane at F_{I_0} , 0.99. There was a 2.6-fold increase in the 24-hour urinary excretion of fluoride in those rats in which extensive centrilobular necrosis developed. The *in-vivo* covalent binding to lipids of ^{14}C from ^{14}C -halothane also was increased markedly when ^{14}C -halothane was administered intraperitoneally to phenobarbital-induced rats maintained hypoxic (F_{I_0} , 0.14) for two hours. These results support the authors' hypothesis that halothane is metabolized to hepatotoxic intermediates by a reductive or non-oxygen-dependent cytochrome P-450-dependent pathway. This animal model of halothane-induced hepatotoxicity may be clinically relevant. A decrease in hepatic blood flow during halothane anesthesia may decrease the P_{O_2} available to hepatocytes and thus direct the metabolism of halothane along its reductive, hepatotoxic pathway. (Key words: Anesthetics, volatile: halothane. Bio-transformation: enzyme induction, microsomes. Hypoxia: liver. Induction: enzymes. Ions: fluoride. Liver: hepatotoxicity; microsomes.)

HALOTHANE has been widely used as an inhalational anesthetic since 1957. Perhaps a major drawback to more widespread application is the body of case reports implicating halothane as the causative agent in postoperative hepatitis.¹⁻³ Since the incidence of halothane-associated hepatic injury is slight, sporadic, and unpredictable, its mechanism and even existence have been widely debated. One reason for this debate is the lack of appropriate animal models of halothane-induced hepatotoxicity. Recently, Sipes and Brown⁴ were able to produce classic centrilobular hepatic necrosis in rats by pretreating the animals with Aroclor

1254 (a mixture of polychlorinated biphenyls) and anesthetizing them with halothane, 1 per cent, for two hours. They suggested that Aroclor 1254 may alter the qualitative biotransformation of halothane, particularly along a reductive or non-oxygen-dependent pathway. Reynolds and Moslen⁵ obtained similar results in Aroclor 1254-pretreated rats, and suggested that reductive biotransformation of halothane could lead to reactive intermediates. These reactive intermediates could ultimately initiate the events leading to hepatic injury. Abundant evidence in the medical literature supports the reductive metabolism of halothane. Uehleke *et al.*,⁶ Van Dyke and Gandolfi,⁷ and our group^{4,8} have demonstrated increased covalent binding of halothane metabolites *in vitro* when microsomal incubations are performed in nitrogen rather than oxygen. Similarly, Widger *et al.*⁹ demonstrated that hypoxia enhances the *in-vivo* release of fluoride and lipid binding of ^{14}C -halothane metabolites. These results suggest that hypoxia promotes the formation of reactive intermediates or toxic halothane metabolites. Therefore, we decided to investigate the role of hypoxia in halothane-induced hepatotoxicity. Since biotransformation of halothane appears to be prerequisite for this injury, the roles of microsomal enzyme induction and metabolism were also studied.

Methods

Adult male Sprague-Dawley rats weighing approximately 150–160 g were obtained from Hilltop Laboratories and maintained on a 12-hour dark–light cycle. The animals had free access to water and a diet of Wayne rodent food. To induce the hepatic microsomal drug-metabolizing enzymes, 110 animals were given phenobarbital (1 mg/ml) in their drinking water for ten days prior to any experimentation.

For exposure to halothane, control or phenobarbital-pretreated animals were placed into 26-l plexiglass cages in groups of six to eight per cage. These animals were then anesthetized for two hours with halothane, 1 per cent, at various concentrations of oxygen ($F_{I_{O_2}}$) according to the following schedule: Group B, control animals maintained at $F_{I_{O_2}}$ 0.99; Group C, control animals maintained at $F_{I_{O_2}}$ 0.14; Groups D, E, and F, phenobarbital-pretreated animals maintained at $F_{I_{O_2}}$ 0.99, 0.21, and 0.14, respectively. The anesthetic, nitrogen, and oxygen were delivered

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to the chamber by a standard Ohio Vernitrol® apparatus at a flow rate of 6 l/min. Chamber halothane concentrations were monitored with a calibrated Beckman® IL-2 infrared analyzer and the chamber oxygen concentration was monitored with an Ohio® 600 oxygen analyzer.

Sixty-two rats were included in Group A. These animals were not exposed to halothane, but were treated as follows: absolute controls, no phenobarbital pretreatment or exposure to hypoxia (n = 12); no phenobarbital pretreatment, but maintained at FI_{O_2} 0.10 for two hours (n = 12); phenobarbital pretreatment only (n = 12); phenobarbital pretreatment and maintained at FI_{O_2} 0.10 for two hours (n = 14); phenobarbital pretreatment and anesthesia with ether for two hours at FI_{O_2} 0.10 (n = 12). Primarily, the animals in this group served as controls for the effects of severe hypoxia and hypoxia coupled with a non-hepatotoxic general anesthetic. Since no histologic differences were observed among the various exposure conditions, they are grouped together to simplify presentation of data.

After anesthesia or the appropriate exposure, the animals were either placed in Econo® metabolism units or returned to their metal cages. They were sacrificed by decapitation 24 hours after anesthesia. Blood from the trunks was collected into heparinized

beakers, and the livers were rapidly removed and placed in chilled petri dishes. Sections 2 mm thick were removed from the right anterior lobe and placed in buffered formalin for fixation. The remaining liver was then processed for biochemical determinations.

Hepatic injury was assessed by measurement of plasma glutamic pyruvic transaminase, levels of microsomal cytochrome P-450 and reduced glutathione (GSH), and microscopic examination of sections of liver stained with hematoxylin-eosin. Plasma transaminase values were measured according to the procedure supplied with the Sigma® SGPT kits. Microsomes were obtained by differential centrifugation, as previously described.⁸ After adjustment of the resuspended microsomes to the desired protein concentration,¹⁰ cytochrome P-450 was measured spectrophotometrically.¹¹ Reduced GSH was determined by Ellman's procedure¹² in homogenates of liver rendered protein-free by meta-phosphoric acid precipitation and centrifugation. The slides of each section were graded according to the following scale: 0 = normal, no pathologic changes; 1 = slight cellular disruption; 2 = some staining variations and occasional appearance of vacuoles or balloon cells; 3 = occasional centrilobular necrosis; 4 = many centrilobular necrotic areas; 5 = confluent centrilobular necrosis. The investigators classified each slide. In addition, a third

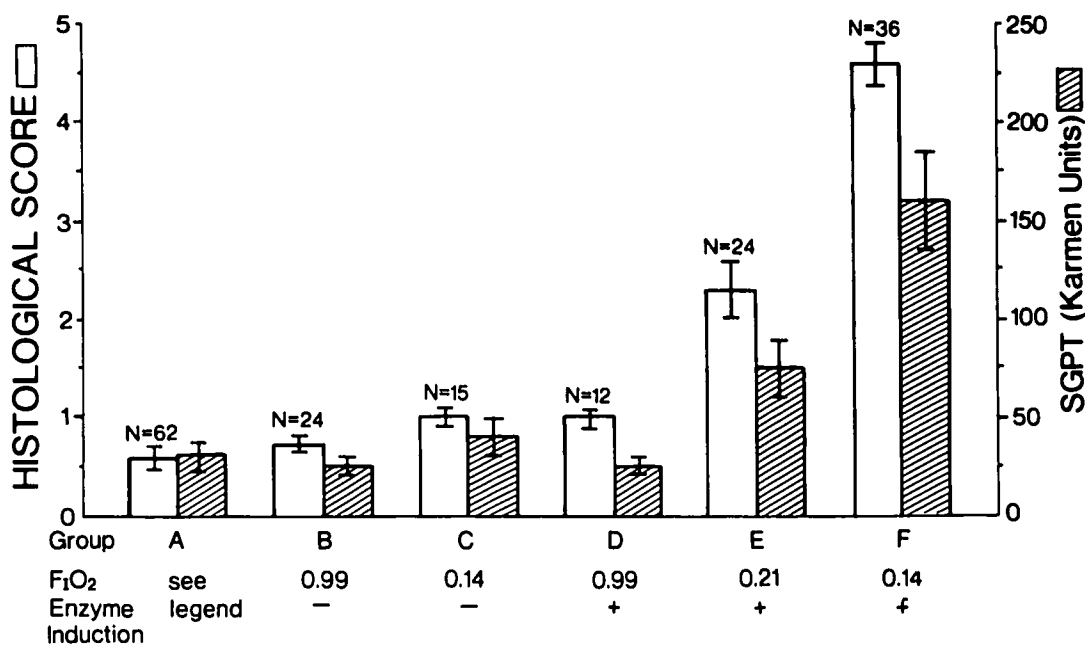


FIG. 1. Effects of microsomal enzyme induction with phenobarbital and hypoxia (FI_{O_2} 0.14) on halothane-induced hepatic injury. Graph represents means \pm SEM for hepatic histologic scores and SGPT units. Group A represents various pretreatments or exposure conditions, without halothane anesthesia, that serve as controls. These include absolute controls; phenobarbital pretreatment alone or coupled with two hours of hypoxia; phenobarbital pretreatment and two hours of anesthesia with diethyl ether; two hours of hypoxia without pretreatment. N = number of animals evaluated by histologic examination and SGPT determination.

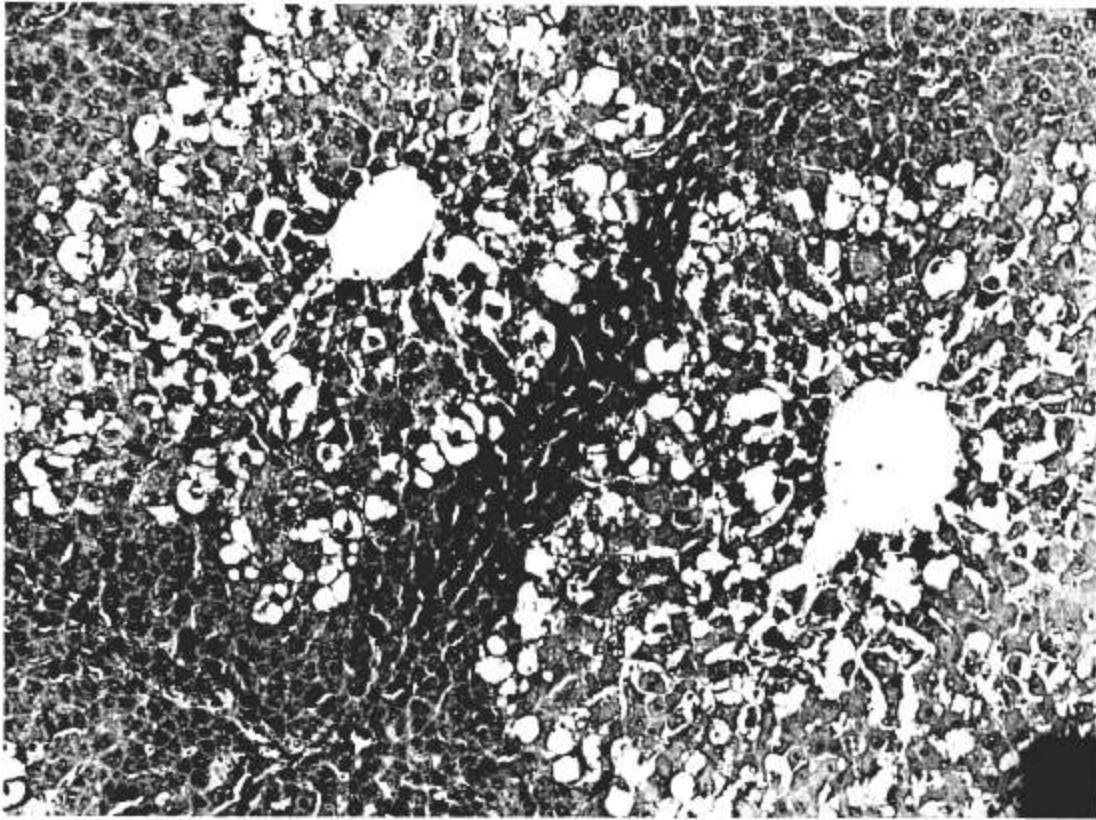


FIG. 2. Photomicrograph of a section of liver obtained from a phenobarbital-pretreated rat 24 hours after cessation of two hours of anesthesia with halothane, 1 per cent, under conditions of hypoxia ($F_{I_{O_2}}$ 0.14). The histologic score is 4. This illustrates the multiple, circumscribed lesions that radiate from the central veins. $\times 100$.

of the slides were scored double-blind by members of the Department of Anatomy and the Department of Pathology of the University of Arizona College of Medicine for corroboration purposes.

To assess the role of biotransformation of halothane in the production of hepatic lesions, serum bromide, urinary fluoride, and hepatic microsomal covalently bound ^{14}C -halothane metabolites were measured. Bromide was determined according to the tetra-bromosulfaniline technique reported by Goodwin¹³ and fluoride was determined with a conventional fluoride electrode (Orion® #94-09).

To determine the effects of hypoxia ($F_{I_{O_2}}$ 0.14) or phenobarbital induction on covalent binding of ^{14}C -halothane metabolites, eight rats were placed into the anesthesia chamber for exposure to room air or a hypoxic environment 60 min prior to intraperitoneal injection of ^{14}C -halothane (88 $\mu\text{Ci}/\text{kg}$, 3.7 mCi/mmol). After ^{14}C -halothane administration the rats were immediately returned to the proper environment for two hours. The animals were then sacrificed and the livers removed. Microsomes were obtained as described above, and proteins and lipids were separated

and exhaustively extracted to determine the extent of covalent binding as previously reported.^{4,8} The student *t* test or analysis of variance was used to determine statistical significance of differences among the various groups.

Results

Under conditions of hypoxia and induction of the microsomal drug-metabolizing enzymes with phenobarbital, halothane anesthesia produced extensive hepatic injury (fig. 1). Within 24 hours after exposure to halothane at $F_{I_{O_2}}$ 0.14, 31 of 36 animals had many areas of hepatic necrosis or confluent necrosis that radiated from the central veins. The mean histologic score for the animals in this group (F) was 4.6 ± 0.2 . SGPT values were also increased. Morphologic examination indicated normal hepatic architecture with sharply circumscribed zones of degeneration and coagulation, particularly in the centrilobular area (fig. 2). These necrotic areas were often infiltrated with clusters of lymphocytes, histocytes and neutrophils, and were often encircled by a layer of swollen hepatocytes containing single large vacuoles, strands

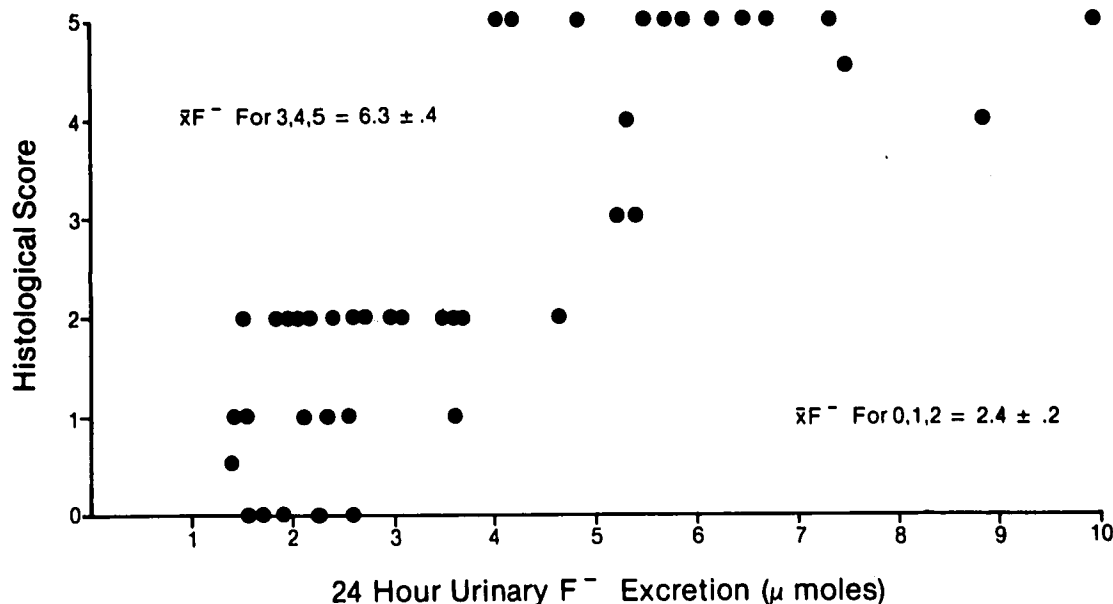


FIG. 3. Comparison of 24-hour urinary fluoride excretion and hepatic damage (histologic score). Rats that showed extensive hepatic damage (scores 3–5) were primarily in the hypoxic-phenobarbital-induced group (F), and had increased urinary fluoride ion excretion, 6.3 $\mu\text{mol}/24$ hr. The mean fluoride excretion for all other groups was 2.4 $\mu\text{mol}/24$ hr. Correlation coefficient, $r = 0.81$.

of degenerating cytoplasm, and eccentric intact or pyknotic nuclei. The marginally degenerating hepatocytes in less damaged sections of liver were pale, swollen and more eosinophilic than normal, but contained intact nuclei. Occasionally, midzonal cells contained small, sharply circumscribed vacuoles.

Halothane anesthesia at FI_{O_2} 0.21 also resulted in hepatic damage when the rats were pretreated with phenobarbital (Group E). Damage was apparent in nine of 24 animals, but the predominate histologic scores were 2 or 3, indicating slight cellular disruption and occasional focal necrosis.

TABLE 1. Levels of Cytochrome P-450 and Reduced Glutathione in the Livers of Phenobarbital-pretreated Rats 24 Hours after Halothane Anesthesia and Hypoxia*

Exposure	Cytochrome P-450 (Nanomoles/mg Microsomal Protein)	Reduced Glutathione ($\mu\text{mol}/\text{g}$ Liver)
None	1.5 ± 0.1	7.6 ± 0.7
Hypoxia (FI_{O_2} 0.14)	3 hr	1.5 ± 0.1
	24 hr	1.3 ± 0.1
Halothane anesthesia at FI_{O_2} 0.14	3 hr	6.6 ± 0.9
	24 hr	12.1 ± 1.6
Halothane anesthesia at FI_{O_2} 0.14	3 hr	6.6 ± 0.2
	24 hr	8.4 ± 0.6

* Values are means \pm SE for six to 12 animals per group; anesthesia with halothane, 1 per cent, for two hours.

† Significant difference, $P < 0.001$.

No statistically significant histologic change was found for the following variables (relative to controls): phenobarbital induction with no anesthesia; phenobarbital pretreatment with exposure to hypoxia, but no anesthesia; anesthesia of phenobarbital-induced rats with diethyl ether at FI_{O_2} 0.10; halothane anesthesia of non-phenobarbital-pretreated rats at FI_{O_2} 0.99 or FI_{O_2} 0.14; anesthesia of phenobarbital-induced rats at FI_{O_2} 0.99.

Halothane anesthesia of phenobarbital-treated rats at FI_{O_2} 0.14 decreased the level of cytochrome P-450 by 53 per cent within 24 hours (table 1). Hypoxia alone did not alter cytochrome P-450 levels. No significant change was found in the levels of reduced glutathione in the liver under conditions of hypoxia or hypoxia and halothane anesthesia (table 1). Twenty-four hours following anesthesia, urinary fluoride excretion had increased 2.6-fold in those animals that had histologic scores of 3–5 compared with those that had scores of 0–2 (fig. 3). The animals in the 0–2-score group had a mean fluoride excretion of $2.4 \pm 0.2 \mu\text{mol}/24$ hr. This group included mainly phenobarbital-pretreated rats anesthetized with halothane at FI_{O_2} 0.21 or 0.99. The other group, with a mean fluoride excretion of $6.3 \pm 0.4 \mu\text{mol}/24$ hr included mainly animals anesthetized at FI_{O_2} 0.14. Obviously, there was some overlap, particularly at a histologic score of 2.

Serum bromide levels were unchanged by hypoxia. Twenty-four hours after anesthesia at FI_{O_2} 0.14, the serum bromide level was $1.8 \pm 0.3 \mu\text{mol}/\text{ml}$, while

at FI_{O_2} 0.21 or 0.99, mean serum bromide was 1.4 ± 0.1 $\mu\text{mol/ml}$. Hypoxia markedly increased the covalent binding to lipids of radiolabel derived from ^{14}C -halothane (table 2), particularly in those animals pretreated with phenobarbital. Binding to protein was also increased, but only twofold instead of 14-fold as observed for lipids. Phenobarbital pretreatment significantly increased the covalent binding to proteins during breathing of air as well as hypoxia. However, for lipid binding hypoxia was the critical and determinant factor.

Discussion

The results of the present study confirm that halothane is hepatotoxic. Hypoxia forced the metabolism of halothane induced by phenobarbital pretreatment to a reductive or non-oxygen-dependent pathway, and severe hepatic damage occurred. Slight hepatic damage developed in a third of the phenobarbital-induced rats anesthetized with halothane in air. Phenobarbital-induced rats anesthetized at FI_{O_2} 0.99 did not show hepatic lesions. From this we conclude that as the oxygen concentration is decreased, hepatic damage from halothane is increased.

Hypoxia alone is not sufficient to trigger halothane-induced hepatic damage. Our findings confirm the findings of Leinweber *et al.*,¹⁴ who found that neither hypoxia (FI_{O_2} 0.12) nor hypoxia plus halothane anesthesia resulted in marked hepatic damage. Enzyme induction is also necessary, at least following single exposures to halothane. Apparently non-induced livers cannot metabolize halothane rapidly enough to overwhelm endogenous defense mechanisms, even under conditions of hypoxia.

The increase in urinary fluoride levels and the increased covalent binding provide strong evidence that hypoxia actually enhances the biotransformation of halothane. This is important because it could be argued that hypoxia is only sensitizing the liver to halothane. Hypoxia could indeed labilize certain subcellular organelles. However, it is apparent that toxic metabolites or reactive intermediates of halothane are formed at a faster rate under conditions of hypoxia. These metabolites then initiate the sequence of events that ultimately lead to hepatotoxicity.

Undoubtedly these reactive metabolites are produced by the hepatic microsomal cytochrome P-450 system.^{15,16} Being highly reactive, they can interact directly with constituents of the endoplasmic reticulum. This interaction probably explains the loss of cytochrome P-450 that was observed in the hypoxic model. Hepatic levels of glutathione, a cytosolic tripeptide, were not decreased by hypoxia or hypoxia

TABLE 2. Effects of Phenobarbital Induction and Hypoxia on the Covalent Binding of ^{14}C from ^{14}C -Halothane to Microsomal Proteins and Lipids

Atmosphere	Protein (Picomoles $^{14}\text{C}/\text{mg}$)	Lipid (Picomoles $^{14}\text{C}/\mu\text{mol Lipid P}$)
FI_{O_2} 0.21		
Control	18 ± 2	16 ± 5
Induced	$32 \pm 5^\dagger$	25 ± 4
FI_{O_2} 0.14		
Control	17 ± 6	40 ± 12
Induced	$68 \pm 16^*$	$364 \pm 32^*$

n = four animals per group.

* $P < 0.01$ for FI_{O_2} 0.14 vs. FI_{O_2} 0.21.

† $P < 0.05$ for induced vs. control.

and halothane anesthesia. However, this is not surprising, since glutathione is ineffective in preventing *in-vitro* covalent binding of ^{14}C -halothane metabolites under anaerobic conditions.¹⁰

Although it is difficult to extrapolate these data directly to man, it could be argued that since hepatic necrosis can be produced with halothane in animals, it certainly could occur in man. It can be speculated that a qualitative or quantitative change in halothane biotransformation to a reductive pathway could produce sufficient reactive intermediates to cause hepatic necrosis in man. Variables producing this change would include status of the microsomal enzymes (genetic alterations or environmental changes due to prior drug therapy or pollutants), total blood flow, and state of oxygenation of the liver. Halothane diminishes hepatic arterial blood flow in man,¹⁷⁻¹⁹ which may decrease available centrilobular hepatocellular P_{O_2} . Various surgical procedures may also decrease hepatic blood flow.¹⁹ It would be anticipated that these decreases in oxygenation of the liver may direct halothane to biotransformation along its reductive, hepatotoxic pathway. Another variable that has been considered a possible predisposing factor in halothane hepatotoxicity is obesity.^{3,20} Livers from obese patients showed moderate to severe fat infiltration, which could lead to increased anesthetic accumulation. More importantly, obese patients have an increased capacity for the reductive metabolism of halothane, as judged by the significantly increased serum fluoride levels.²¹

In summary, we have developed an animal model of halothane-induced hepatotoxicity that may be clinically relevant. Prerequisite for lesion development is induction of the microsomal drug-metabolizing enzymes. This induction, coupled with a diminished supply of available oxygen in the liver, favors halothane biotransformation along reductive, hepatotoxic pathways.

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