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Demonstration of Halothane-induced Hepatic Lipid Peroxidation in Rats by Quantification of F₂-Isoprostanes

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Background: Halothane can be reductively metabolized to free radical intermediates that may initiate lipid peroxidation. Hypoxia and phenobarbital pretreatment in Sprague-Dawley rats increases reductive metabolism of halothane. F₂-isoprostanes, a novel measure of lipid peroxidation *in vivo*, were used to quantify halothane-induced lipid peroxidation in rats.

Methods: Rats were exposed to 1% halothane at 21% or 14% O₂ for 2 h. Pretreatments included phenobarbital, isoniazid, or vehicle. Rats also were exposed to halothane, enflurane, and desflurane at 21% O₂. Lipid peroxidation was assessed by mass spectrometric quantification of F₂-isoprostanes.

Results: Exposure of phenobarbital-pretreated rats to 1% halothane at 21% O₂ for 2 h caused liver and plasma F₂-isoprostane concentrations to increase fivefold compared to nonhalothane control rats. This halothane-induced increase was enhanced by 14% O₂, but hypoxia alone had no significant effect. Alanine aminotransferase activity at 24 h was significantly increased only in the 1% halothane/14% O₂ group. The effect of cytochrome P450 enzyme induction on halothane-induced F₂-isoprostane production and liver injury was determined by comparing the effects of isoniazid and phenobarbital pretreatment with no pretreatment under hypoxic conditions. Halothane caused 4- and 11-fold increases in plasma and liver F₂-isoprostanes, respectively, in non-pretreated rats, whereas isoniazid pretreatment had no effect. Phenobarbital pretreatment potentiated halothane-induced lipid peroxidation with 9- and 20-fold increases in plasma and liver F₂-isoprostanes, respectively. Alanine aminotransferase activity was increased only in this group. At ambient oxygen concentrations, halothane but not enflurane or desflurane, caused F₂-isoprostanes to increase.

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Conclusions: Specific halothane-induced lipid peroxidation was demonstrated in Sprague-Dawley rats using quantification of F₂-isoprostanes and was increased by hypoxia and phenobarbital pretreatment, but not isoniazid pretreatment. (Key words: Anesthetics, volatile: halothane. Complications: hepatotoxicity. Peroxidation: lipid. Pharmacologic agents: isoniazid; phenobarbital. Prostanoids: F₂-isoprostanes.)

SHORTLY after the introduction of halothane, rare instances of severe liver injury attributed to the anesthetic were noted.¹ It has been suggested that mild liver injury caused by halothane is more common.^{2,3} Many investigations have been directed toward understanding potential mechanisms of halothane-induced hepatotoxicity. One experimental model of halothane-induced hepatotoxicity involves exposure of phenobarbital-pretreated rats to halothane under hypoxic conditions.⁴ This model has been referred to as the halothane-hypoxia model. Induction of phenobarbital-inducible cytochrome P450 enzymes and hypoxia in rats promotes increased reductive metabolism of halothane.⁵ This usually minor pathway of halothane metabolism *in vivo* can generate free radical intermediates capable of initiating lipid peroxidation.⁶ *In vivo* free radical generation after halothane exposure has been demonstrated by electron paramagnetic spectroscopy,⁷ however, the identification of lipid-derived radicals has been controversial.⁸ *In vitro* experiments demonstrate that halothane metabolism causes lipid peroxidation in microsomes under a variety of conditions.⁹ Nonetheless, difficulties in measuring lipid peroxidation *in vivo* have hampered efforts to implicate lipid peroxidation as a mechanism of injury in many pathologic processes including halothane-induced hepatotoxicity in whole animals or humans.¹⁰ Halothane-induced lipid peroxidation *in vivo* has been demonstrated by measuring exhaled ethane in rats, but only under conditions resulting in hepatic injury (*i.e.*, hypoxia and phenobarbital pretreatment).¹¹ In addition, the site of lipid peroxidation in these studies could not be directly determined.

The discovery of prostanoids produced catalyzed peroxidation is a important advance *in vivo*.¹² These biologically esterified to phospholipases. Qu plasma and lipid- other tissues has lipid peroxidation including CCl₄-induced

There are many hepatotoxicity and halothane-induced hydrocarbons can be generating free radical lipid peroxidation occur in the endocytosis showing an elle within 30 min biotic.¹⁵ The microscopic hepatic compound. The exposure to CCl₄ change, central serum alanine aminotransferase injury in the halothane model is mild similar to CCl₄ (<0.4 ml/kg)

Lipid peroxidation characterized using isoprostanes is a sensitive method for detecting CCl₄ hepatotoxicity. In these studies, a common finding was that F₂-isoprostanes were given CCl₄ with increasing within 30 min levels 100- to 200-fold. The increase in ethane occurs later compared to a lesser extent concentrations significantly 24 h after doses of CCl₄ dependent on CCl₄ dose with either phenobarbital or cytochrome P450

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The discovery of F₂-isoprostanes, a novel class of prostanoids produced by nonenzymatic free radical-catalyzed peroxidation of arachidonic acid, is an important advance in assessing lipid peroxidation *in vivo*.¹² These biologically active prostanoids are formed esterified to phospholipids and subsequently are released fully formed, presumably by the action of phospholipases.¹³ Quantification of free F₂-isoprostanes in plasma and lipid-esterified F₂-isoprostanes in liver and other tissues has proven to be an important index of lipid peroxidation in free radical-induced injury including CCl₄-induced liver injury.¹⁴

There are many similarities between CCl₄-induced hepatotoxicity and the halothane-hypoxia model of halothane-induced liver injury. Both halogenated hydrocarbons can be metabolized by a reductive pathway generating free radical intermediates that may induce lipid peroxidation. This metabolism is believed to occur in the endoplasmic reticulum with electron microscopy showing membrane disruption of this organelle within 30 min of exposure of rats to either xenobiotic.¹⁵ The histologic features and timing of microscopic hepatic injury also are similar with both compounds. This injury develops 12–48 h after exposure to CCl₄ or halothane and is typified by fatty change, centrilobular necrosis, and an increase in serum alanine aminotransferase (ALT) activity. Liver injury in the halothane-hypoxia model, however, usually is mild, similar to that seen with smaller doses of CCl₄ (≤ 0.1 ml/kg).

Lipid peroxidation in CCl₄ poisoning has been characterized using F₂-isoprostanes. Quantification of F₂-isoprostanes is approximately 30 times more sensitive for detecting CCl₄-induced lipid peroxidation *in vivo* than measurement of thiobarbituric acid reactive substances, a commonly used index of lipid peroxidation.¹⁶ F₂-isoprostanes increase primarily in the liver of rats given CCl₄ with hepatic lipid-esterified F₂-isoprostanes rising within 30 min of receiving CCl₄ and reaching levels 100- to 200-fold that of baseline values by 2 h.¹³ The increase in free F₂-isoprostanes in the plasma occurs later consistent with liberation of these compounds from the affected organs (liver primarily, but, to a lesser extent, kidney and lung).¹⁴ F₂-isoprostane concentrations in liver and plasma decrease significantly 24 h after the administration of low to moderate doses of CCl₄. Production of F₂-isoprostanes is dependent on CCl₄ dose and is enhanced by pretreatment with either phenobarbital or isoniazid to induce cytochrome P450 enzymes.¹⁴

The similarity between the halothane-hypoxia model of hepatotoxicity and CCl₄-induced liver injury led us to use F₂-isoprostanes quantification to confirm that halothane causes lipid peroxidation in rats *in vivo*. In addition, we determined the effects of hypoxia and phenobarbital or isoniazid pretreatment on this process. Finally, we determined that lipid peroxidation is specific to anesthesia with halothane, but not enflurane or desflurane.

Materials and Methods

Animal Care

Male Sprague-Dawley rats (weighing 150–200 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). The rats were housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care with alternating 12 h/12 h light/dark cycles. Experimental protocols were reviewed and approved by the Vanderbilt University Animal Care Committee in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences. Pretreatment with phenobarbital (Mallinckrodt, Paris, KY) consisted of administering 75 mg/kg intraperitoneally on the first day followed by adding 1 mg/ml phenobarbital to drinking water for the next 4 days. Pretreatment with isoniazid (Aldrich Chemical, Milwaukee, WI) consisted of adding it to the drinking water (1 mg/ml) for 10 days. Both drugs were withdrawn and rats were fasted 16 h before anesthetic exposure.

Administration of Halothane

Rats were placed in an 84-l methyl methacrylate polymer chamber preequilibrated with warmed and humidified gas flowing at 6 l/min. Halothane (Fluothane donated by Wyeth-Ayerst, Princeton, NJ) was delivered using a Fluotec 3 vaporizer (Ohmeda, West Yorkshire, England) precalibrated using gas chromatography. Enflurane (Ethrane, donated by Anaquest, Murray Hill, NJ) was delivered using an Enfluratec vaporizer (Ohio Medical Products, Madison, WI). Desflurane (Suprane, Ohmeda) was delivered using a Tec6 vaporizer (Ohmeda). The oxygen-nitrogen mixture was controlled using two rotameters and oxygen concentration in the chamber was continuously monitored using a precalibrated Ohmeda 5120 oxygen monitor. The chamber halothane concentration was periodically confirmed using gas chromatography. Enflurane and

desflurane concentrations were monitored using a Capnomac Airway Gas Monitor (Datex Instrumentarium, Helsinki, Finland). The body temperature of selected rats was monitored with a rectal probe and chamber temperature was adjusted to maintain normothermia.

Experimental Design

Determination of the Effect of Halothane and Hypoxia on F₂-isoprostane Generation. All animals were pretreated with phenobarbital and divided into four experimental groups, 0% halothane/21% O₂, 0% halothane/14% O₂, 1% halothane/21% O₂, and 1% halothane/14% O₂. In each group, ten rats were exposed to the assigned treatment for 2 h. Five rats were then killed within minutes after removal from the anesthetic chamber to assess lipid peroxidation. Rats were killed under pentobarbital anesthesia (60 mg/kg) and blood was withdrawn from the dorsal aorta. The liver was removed and frozen in liquid nitrogen before storage at -70°C. The remaining five animals in each group were fed and killed at 24 h to assess liver injury.

Determination of the Effect of Pretreatment on F₂-isoprostane Generation. Rats (15 per group) were divided into three pretreatment groups: no pretreatment, isoniazid pretreatment, or phenobarbital pretreatment. Ten animals from each group were exposed to 1% halothane/14% O₂ for 2 h. Five rats were killed immediately afterward and five were killed at 24 h as earlier. Five animals in each group were exposed to 0% halothane/14% O₂ for 2 h and killed immediately afterward to assess lipid peroxidation.

Determination of the Effect of Halothane, Enflurane, and Desflurane on F₂-isoprostane Generation. Three groups of ten rats were exposed to anesthetic concentrations of halothane (1.3%), enflurane (1.5%), or desflurane (5.2%) at 21% O₂ for 2 h. Five rats were killed immediately afterward and 5 were killed at 24 h as earlier. Five rats unexposed to anesthetic were used as controls for each experiment.

Biochemical Determinations

F₂-isoprostane concentrations were measured in freshly obtained plasma (1-3 ml) after purification and derivatization by gas chromatography/negative ion chemical ionization-mass spectrometry using selected ion monitoring as described previously.¹⁷ This is a stable isotope dilution assay using [²H₄]-Prostaglandin F_{2α} as the internal standard. Phospholipid-esterified F₂-isoprostanes in liver were quantified after extraction of

lipids by the method of Folch¹⁸ and base hydrolysis as described previously.¹³ Plasma alanine aminotransferase activities were measured using a commercial spectrophotometric kinetic assay kit (Sigma diagnostic procedures no. 59-10, St. Louis, MO).

Data Analysis

Data were analyzed by *t* test or analysis of variance with Fisher's (protected) least significant difference test using Statview software (Abacus Concepts, Berkeley, CA). When variances were found to be heterogeneous, log transformation of the data was performed first. Differences between group means were considered significant at *P* < 0.05. Data are presented as mean ± SEM.

Results

Effect of Halothane and Hypoxia on F₂-isoprostane Generation

The effect of hypoxia in the classic halothane-hypoxia model in which all animals were pretreated with phenobarbital was determined. Exposure of phenobarbital-pretreated rats to 1% halothane at atmospheric oxygen concentrations for 2 h increased plasma F₂-isoprostanes fivefold (fig. 1A). Exposure to 1% halothane under hypoxic conditions further increased plasma F₂-isoprostanes tenfold. However, hypoxia

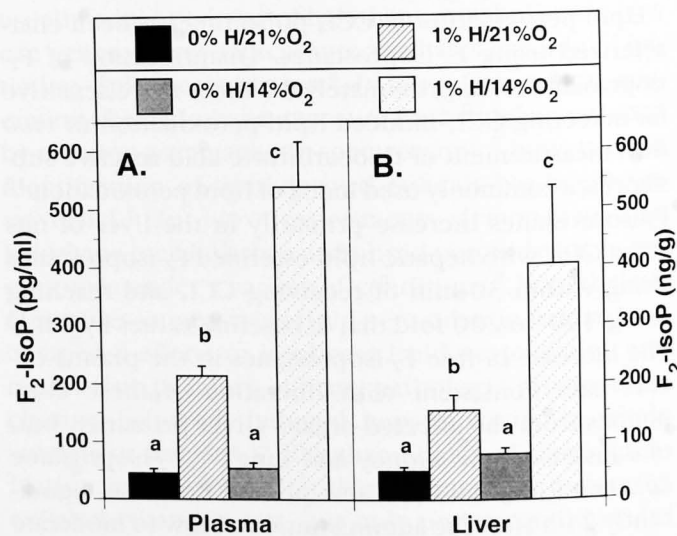


Fig. 1. Effect of various halothane and oxygen concentrations on plasma (A) and liver (B) F₂-isoprostane concentrations in phenobarbital pretreated rats. Data presented ± SEM (n = 5 per group). Bars not sharing a common superscript letter are significantly different (*P* < 0.05).

alone did not increase at the end of 2 h. Hepatic injury (fig. 1B). Half of the conditions were killed by measurement of plasma F₂-isoprostane was mild in this exposure to halothane and normoxia (4.0 ± 0.5 IU/l).

Effect of Phenobarbital on Halothane-Induced Liver Injury

The effect of phenobarbital on F₂-isoprostane generation induced by halothane was determined. Exposure to halothane under normoxia increased plasma F₂-isoprostanes (B) in the absence of phenobarbital. Phenobarbital pretreatment enhanced the production of F₂-isoprostanes caused by halothane. However, potentiation of F₂-isoprostane generation (9- and 20-fold, respectively) was assessed after anesthesia. Only plasma F₂-isoprostanes and aminotransferase activities were elevated for non-pretreated rats exposed to halothane.

Effect of Anesthesia on Liver Injury

To determine the effects of anesthesia on F₂-isoprostane generation, the effects of each anesthetic were evaluated. Plasma F₂-isoprostane concentrations were elevated (2.5-fold). Liver injury was elevated fivefold in rats exposed to desflurane. Elevations of liver injury were small. Plasma F₂-isoprostane concentrations for liver injury were elevated fivefold. Activities differed.

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alone did not increase plasma F₂-isoprostanes at the end of 2 h. Hepatic F₂-isoprostanes increased similarly (fig. 1B). Half of the rats exposed to the four anesthetic conditions were killed at 24 h to assess liver damage by measurement of plasma ALT activity. Liver injury was mild in this experiment. Only the group exposed to halothane and hypoxia showed a significant ALT elevation (77 ± 15 IU/l) when compared with no halothane/normoxemia (34 ± 4 IU/l), halothane/normoxia (49 ± 3 IU/l), or hypoxia alone (25 ± 2 IU/l).

Effect of Phenobarbital or Isoniazid Pretreatment on Halothane-Induced F₂-isoprostane Generation

The effect of pretreatment with phenobarbital or isoniazid on F₂-isoprostane production and liver injury induced by halothane and hypoxia or hypoxia alone was determined. As illustrated in figure 2, a 2-h exposure to halothane under hypoxic conditions caused an increase in F₂-isoprostanes in plasma (A) and liver (B) in the absence of pretreatment with isoniazid or phenobarbital. Pretreatment with isoniazid did not enhance the production of plasma or liver F₂-isoprostanes caused by halothane and hypoxia. Phenobarbital, however, potentiated the effect of halothane and hypoxia on F₂-isoprostane concentrations in plasma and liver (9- and 20-fold, respectively, *vs.* hypoxia alone). Liver injury was assessed in the animals killed 24 h after anesthesia. Only rats pretreated with phenobarbital had elevated plasma ALT activity, 235 ± 85 IU/l. Alanine aminotransferase activity was 41 ± 4 and 44 ± 3 IU/l for non-pretreated and isoniazid-pretreated rats exposed to halothane and hypoxia.

Effect of Anesthetic on F₂-isoprostane Generation

To determine whether F₂-isoprostane production was specific to metabolism of halothane, we determined the effects of halothane, enflurane, and desflurane on F₂-isoprostane generation at anesthetic concentrations of each anesthetic under normoxic conditions. As depicted in figure 3A, plasma F₂-isoprostane concentrations were elevated only in rats exposed to halothane (2.5-fold). Liver F₂-isoprostane concentrations were elevated fivefold in these animals. Although animals exposed to desflurane had statistically significant elevations of liver F₂-isoprostanes (60% increase), this increase was small and unaccompanied by an increase in plasma F₂-isoprostanes. None of the animals assessed for liver injury at 24 h in these experiments had ALT activities different from controls (data not shown).

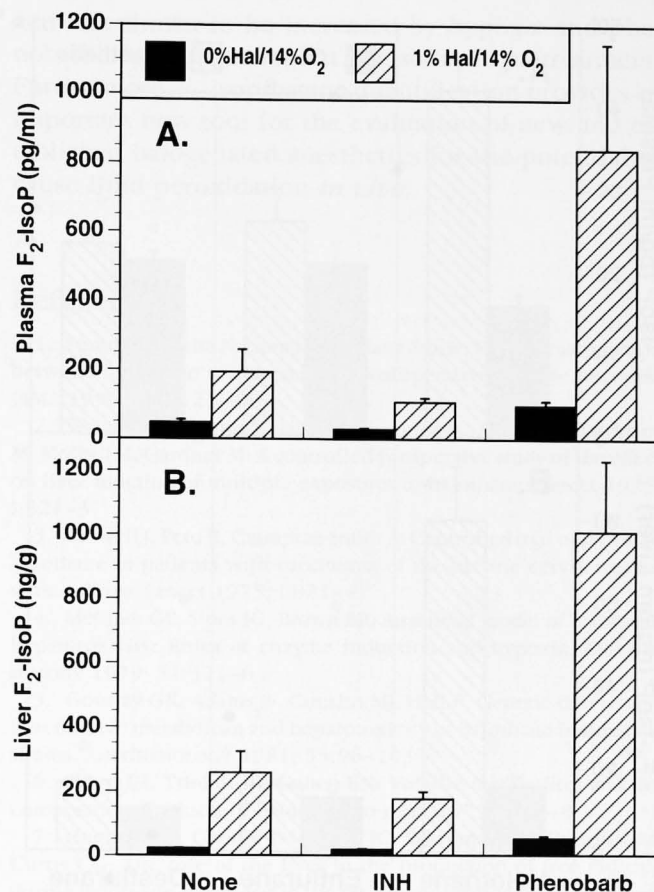


Fig. 2. Effect of drug pretreatment on halothane-induced plasma (A) and liver (B) F₂-isoprostane concentrations. Data presented \pm SEM (n = 5 per group).

Discussion

F₂-isoprostane formation is a specific result of free radical-initiated lipid peroxidation.¹⁹ Consequently, F₂-isoprostane quantification has been valuable in establishing the role of lipid peroxidation in a variety of free radical-induced hepatic and renal injury models in which existing techniques of measuring lipid *in vivo* peroxidation proved inadequate.^{14,20-22} Plasma and tissue F₂-isoprostane elevations in these models are not simply a nonspecific consequence of tissue necrosis. Tissue injury not induced by free radicals (*e.g.*, acetaminophen- or thioacetamide-induced liver necrosis) does not substantially increase F₂-isoprostanes.¹⁴ Thus, F₂-isoprostane quantification confirms that halothane exposure causes lipid peroxidation *in vivo* as previously demonstrated with measurement of exhaled ethane.¹¹ Moreover, F₂-isoprostane quantification provides important new information. Documentation of

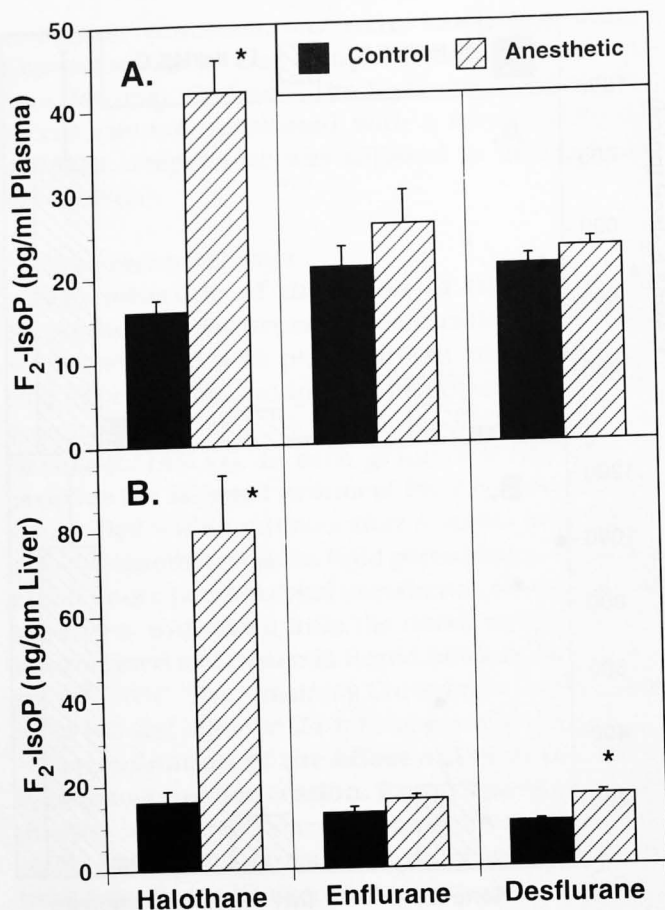


Fig. 3. Effect of anesthetic on plasma (A) and liver (B) F₂-isoprostane concentrations at normoxic, noninjurious conditions. Data presented \pm SEM (n = 5 per group). Bars denoted by * are different from control ($P < 0.01$).

increased hepatic F₂-isoprostane-containing lipids establishes that lipid peroxidation occurs in the organ injured by halothane. In addition, for the first time, halothane-induced lipid peroxidation *in vivo* has been demonstrated even in the absence of drug pretreatment and/or hypoxia. Thus, quantification of the increase in lipid peroxidation caused by hypoxia and phenobarbital pretreatment was possible.

These results correlate well with data regarding the reductive metabolism of halothane in Sprague-Dawley rats.⁵ Gourlay *et al.* measured reductive metabolites of halothane, 1-chloro-2,2,2-trifluoro ethane and 1-chloro-2,2-difluoro ethylene, during a 2-h exposure to 1% halothane. The quantity of these metabolites reflects the potential for halothane to initiate lipid peroxidation. They demonstrated that both 1-chloro-2,2,2-trifluoro ethane and 1-chloro-2,2-difluoro ethylene were exhaled by phenobarbital-pretreated rats exposed

to 1% halothane and 21% O₂. Exposure of rats to halothane at 14% O₂ approximately doubled the amount of these metabolites. This correlates well with the increase in halothane-induced F₂-isoprostane concentrations in phenobarbital-pretreated rats caused by 14% O₂ in our study (fig. 1). Gourlay *et al.* also reported that formation of reductive metabolites caused by exposure of rats to 1% halothane and 14% O₂ was increased twofold to threefold by phenobarbital pretreatment. This is similar to the effect of phenobarbital pretreatment on F₂-isoprostane concentrations that we observed under these conditions (fig. 2).

The relevance of these observations to human halothane hepatotoxicity is uncertain. Reductive metabolism of halothane is a minor, but established, metabolic pathway for halothane in humans. Specific reductive metabolites of halothane, 1-chloro-2,2,2-trifluoro ethane and 1-chloro-2,2-difluoro ethylene, have been measured in patients undergoing halothane anesthesia.²³ Thus, it is logical to postulate that some lipid peroxidation occurs in patients given halothane. Measurement of F₂-isoprostanes in patients exposed to halothane should allow determination of whether this occurs. Nevertheless, detection of lipid peroxidation does not mean that injury detrimental to the individual will invariably result. Our data demonstrate that halothane administration to Sprague-Dawley rats can induce a degree of lipid peroxidation that does not cause sufficient liver necrosis to increase plasma ALT activity. However, the data also suggest that under conditions that increase reductive metabolism and lipid peroxidation in rats (*i.e.*, hypoxia and phenobarbital pretreatment), liver injury becomes apparent. The F₂-isoprostane concentrations observed in the halothane/hypoxia model are similar to those observed after administering low doses of CCl₄ (0.01–0.1 ml/kg) causing only modest and variable amounts of injury.²⁴ Mild hepatic biochemical abnormalities are noted in some patients, even after a single exposure to halothane^{2,3} and it is possible that they are caused by reductive halothane metabolism. This would be more likely in patients in whom reductive metabolism of halothane is enhanced or antioxidant defenses are compromised. Much evidence, including a history of exposure to halothane in most patients, suggests that severe halothane hepatotoxicity in humans is immunologically mediated.²⁵ A variety of microsomal neoantigens containing a trifluoroacetyl group derived from oxidative metabolism of halothane and which react with antibodies from patients with halothane hepatotoxicity

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have been identified.^{26,27} Interestingly, the immunogenicity of membranes can be increased by alteration of the phase structure of phospholipids²⁸ leading to speculation that halothane-induced peroxidation of endoplasmic reticulum lipids may alter the phase of hepatocyte membranes sufficiently to enhance the immune response to halothane-induced neoantigens. This could occur after a halothane exposure that was insufficient to cause cellular necrosis and provide an indirect mechanism for halothane-induced lipid peroxidation to contribute to immunologically mediated halothane hepatotoxicity.

Isoniazid pretreatment has been shown to increase oxidative, but not reductive, metabolism of halothane in older male Fischer 344 rats.²⁹ Halothane causes liver injury in these animals in the absence of hypoxia but reductive metabolism of halothane is not believed to play a role in this model of halothane-induced liver injury. We found that isoniazid pretreatment did not increase halothane-induced lipid peroxidation (F₂-isoprostanes) or potentiate liver injury in Sprague-Dawley rats (fig. 2). We used the same isoniazid pretreatment protocol that we previously found to have increased CCl₄-induced F₂-isoprostane production sevenfold in Sprague-Dawley rats.¹⁴ In that study, CCl₄-induced F₂-isoprostane generation was also inhibited by the P4502E1 inhibitor 4-methylpyrazole. Isoniazid, like ethanol, induces cytochrome P4502E1³⁰ and this enzyme is largely responsible for reductive metabolism of CCl₄.³¹ However, the lack of an effect of isoniazid pretreatment on halothane-induced F₂-isoprostane generation suggests preliminarily that cytochrome P4502E1 is not involved in reductive metabolism of halothane in Sprague-Dawley rats *in vivo*.

Phenobarbital pretreatment potentiated the effect of halothane on F₂-isoprostane production. This is probably due to selective induction of cytochrome P450 enzymes, some of which are responsible for reductive metabolism of halothane. Interestingly, phenobarbital pretreatment alone caused an approximately twofold increase in baseline liver F₂-isoprostane concentrations, probably by increasing endoplasmic reticulum mass in hepatocytes. Phenobarbital pretreatment raises the yield of microsomal lipid and protein isolated from rat livers 1.5-fold.³² Thus, increased lipid content in livers from phenobarbital-pretreated animals would be expected to result in increased baseline hepatic F₂-isoprostane concentrations.

In summary, lipid peroxidation caused specifically by halothane was demonstrated in Sprague-Dawley rats

and was shown to be increased by hypoxia and phenobarbital pretreatment but not isoniazid pretreatment. Furthermore, F₂-isoprostane quantification provides an important new tool for the evaluation of new and established halogenated anesthetics for the potential to cause lipid peroxidation *in vivo*.

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Metabolism Phosphatase Diabetic

Effect of Insulin

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Background: Hypertension will exacerbate diabetes. Studies in a rat model of carotid occlusion plus acute respiration an insulin infusion, similar to normoglycemia study evaluated cerebral energy phosphate metabolism relates that might affect outcome.

Methods: Fifty-four Dawley rats were a perglycemic diabetic normoglycemic diabetic rats (ND; N = 18). groups of six rats each brain ischemia of 5 min in situ freezing, and automatic fluorometric analysis.

Results: Before ischemia of brain glucose (1.2 ± 1.33) or ND (3.20 ± 1.33) glycogen was similar in ID and 61% greater in intrischemic concentration (< 0.05). High-energy

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