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

Joseph A. Awad, M.D.,* Jean-Louis Horn, M.D.,† L. Jackson Roberts II, M.D.,‡ John J. Franks, M.D.§

Background: Halothane can be reductively metabolized to free radical intermediates that may initiate lipid peroxidation. 
Hexafluorophenobarbital 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 1/10-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. 

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 cytochrome P450 enzymes and lipid peroxidation 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 halothane 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.
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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, 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, is usually 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 100-200-fold that of baseline values by 2 h.15 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 (Ethane, donated by Anaquest, Murray Hill, NJ) was delivered using an Enflurane 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 F\textsubscript{2}-isoprostane Generation.** All animals were pretreated with phenobarbital and divided into four experimental groups, 0% halothane/21\% O\textsubscript{2}, 0% halothane/14\% O\textsubscript{2}, 1% halothane/21\% O\textsubscript{2}, and 1% halothane/14\% O\textsubscript{2}. 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 F\textsubscript{2}-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\% O\textsubscript{2} 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\% O\textsubscript{2} for 2 h and killed immediately afterward to assess lipid peroxidation.

**Determination of the Effect of Halothane, Enflurane, and Desflurane on F\textsubscript{2}-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\% O\textsubscript{2} for 2 h. Five rats were killed immediately afterward and five were killed at 24 h as earlier. Five rats unexposed to anesthetic were used as controls for each experiment.

**Biochemical Determinations**

F\textsubscript{2}-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. This is a stable isotope dilution assay using [\textsuperscript{13}C\textsubscript{18}]Prostaglandin F\textsubscript{2} as the internal standard. Phospholipid-esterified F\textsubscript{2}-isoprostanes in liver were quantified after extraction of lipids by the method of Folch and base hydrolysis as described previously. 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 F\textsubscript{2}-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 F\textsubscript{2}-isoprostanes concentrations fivefold (fig. 1A). Exposure to 1% halothane under hypoxic conditions further increased plasma F\textsubscript{2}-isoprostanes tenfold. However, hypoxia alone did not increase F\textsubscript{2}-isoprostanes (fig. 1B). Half of the effects of halothane were due to hypoxia elevated (77 ± 8 vs. halothane/hypoxia/normoxia (49 ± 8 IU/l).

**Effect of Phenobarbital and Isoniazid on F\textsubscript{2}-isoprostanes Induced by Halothane.**

The effect of pretreatment on F\textsubscript{2}-isoprostanes induced by halothane was determined. Exposure to halothane caused an increase in F\textsubscript{2}-isoprostanes (B) in the absence of phenobarbital. Pretreatment with phenobarbital mitigated the increase caused by halothane. However, F\textsubscript{2}-isoprostanes on F\textsubscript{2}-isoprostanes were elevated 5- to 20-fold, respectively, by phenobarbital or by hypoxia. Injury was assessed by plasma alanine aminotransferase activities. Only F\textsubscript{2}-isoprostanes were elevated in plasma of mice exposed to halothane or to hypoxia. In liver injury, alanine aminotransferase activities differed.

![Fig. 1. Effect of various halothane and oxygen concentrations on plasma (A) and liver (B) F\textsubscript{2}-isoprostanes 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).](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931825/)

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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, enfurane, 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).

Discussion

F2-isoprostane formation is a specific result of free radical-initiated lipid peroxidation. 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. 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. Thus, F2-isoprostane quantification confirms that halothane exposure causes lipid peroxidation in vivo as previously demonstrated with measurement of exhaled ethane. Moreover, F2-isoprostane quantification provides important new information. Documentation of
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. Gourlay et al. measured reductive metabolites of halothane, 1-chloro-2,2,2-trifluoro ethane and 1-chloro-2,2,2-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-2,2,2-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-2,2,2-difluoro ethylene, have been measured in patients undergoing halothane anesthesia. 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. Mild hepatic biochemical abnormalities are noted in some patients, even after a single exposure to halothane 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. A variety of microsomal neutonagines containing a trifluorocyclopentyl group derived from oxidative metabolism of halothane and which react with antibodies from patients with halothane hepatotoxicity have been identified as responsible for the phase structure of the hepatic microsomal enzyme. The speculation that halothane-induced hepatic microsomal enzyme dysfunction could occur after a latency period, consistent with the mechanism for halothane hepatotoxicity, is supported by our data.
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have been identified.\textsuperscript{26,27} Interestingly, the immuno-
genicity of membranes can be increased by alteration of the phase structure of phospholipids\textsuperscript{28} 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.

Isoxianzid pretreatment has been shown to increase oxidative, but not reductive, metabolism of halothane in older male Fischer 344 rats.\textsuperscript{59} 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 isoxianzid pretreatment did not increase halothane-induced lipid peroxidation (F2-isoprostanes) or potentiate liver injury in Sprague-Dawley rats (fig. 2). We used the same isoxianzid pretreatment protocol that we previously found to have increased CCL\textsubscript{1}-induced F2-isoprostane production sevenfold in Sprague-Dawley rats.\textsuperscript{14} In that study, CCL\textsubscript{1}-induced F2-isoprostane generation was also inhibited by the P4502E1 inhibitor 4-methylpyrazole. Isoxianzid, like ethanol, induces cytochrome P4502E1\textsuperscript{30} and this enzyme is largely responsible for reductive metabolism of CCL\textsubscript{1}.\textsuperscript{31} However, the lack of an effect of isoxianzid 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.\textsuperscript{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 isoxianzid 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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