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Covalent Binding of Oxidative Biotransformation Intermediates Is Associated with Halothane Hepatotoxicity in Guinea Pigs

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***In vivo* covalent binding of halothane biotransformation-reactive intermediates to hepatic protein and lipid was examined in association with the subsequent development of hepatic necrosis in the guinea pig. Oxidative halothane biotransformation was inhibited by the use of deuterated halothane, whereas reductive metabolism was enhanced by low inspired oxygen concentrations. Male outbred Hartley guinea pigs (n = 8) were exposed to either 1% (v/v) halothane or deuterated halothane—with a fractional inspired O_2 concentration (FI_{O_2}) of 0.40 or 0.10—for 4 h. Livers removed from half of the animals immediately after anesthesia were evaluated for organic fluorine bound to protein and lipid. The remaining animals were evaluated for a hepatotoxic response up to 96 h after exposure. Only guinea pigs that received 1% halothane at an FI_{O_2} of 0.40 had centrilobular necrosis develop with significantly increased plasma alanine aminotransferase activities. All other treatment conditions significantly reduced oxidative halothane biotransformation, as indicated by decreased plasma trifluoroacetic acid concentrations. These reductions were associated with a significant decrease in organic fluorine bound to hepatic proteins. An FI_{O_2} of 0.10 during halothane anesthesia significantly enhanced reductive biotransformation, as indicated by plasma fluoride ion concentrations. This was associated with a significant increase in organic fluoride bound to hepatic lipids. Centrilobular necrosis did not develop under these conditions. Thus, covalent binding to subcellular proteins by the trifluoroacetyl acid chloride intermediate generated by oxidative halothane biotransformation is implicated as a mechanism of centrilobular necrosis in guinea pigs. Binding to lipids by reductive pathway generated free radicals does not appear to be involved in production of the lesion. (Key words: Anesthetics, volatile: deuterated halothane; halothane. Animal: guinea pig. Binding: reactive intermediates. Biotransformation: halothane. Liver: hepatotoxicity.)**

HALOTHANE was first shown to cause hepatic necrosis in guinea pigs by Hughes and Lang in 1972.[1] However, the potential for use of this species in studying halothane-associated hepatotoxicity was not realized until the work of Lunam *et al.* in 1985.[2] In this study, it was demonstrated that extensive acute centrilobular necrosis consistently developed in naive guinea pigs after a 4-h anesthesia with 1% (v/v) halothane. The use of a wide range of fractional inspired oxygen tensions (FI_{O_2} = 0.14–0.80) had no effect on either the incidence or severity of the lesion.[2] Pathologic characteristics of the liver injury in guinea pigs have been found to closely resemble the spectrum of injury observed in nonfatal halothane hepatitis in humans.[3] Additional studies have demonstrated that a variety of factors have an influence on the susceptibility of guinea pigs to halothane-induced hepatic necrosis, including strain, gender, age, and heredity.[4–6]

More recently, the oxidative biotransformation of halothane by the hepatic cytochrome P-450 enzyme system has been implicated as a mechanism of cytotoxicity in the guinea pig.[7] With the use of deuterated halothane (d-halothane) to selectively inhibit oxidative metabolism, a significant reduction in centrilobular necrosis was observed.[7] This implies the trifluoroacetyl acid chloride reactive intermediate that is created during oxidative biotransformation is involved in the production of cell death, perhaps through its ability to covalently bind to free amino groups on subcellular proteins (fig. 1).[8] In addition, this intermediate can react with water within the hepatocyte to produce the metabolite trifluoroacetic acid.[9] Analysis for this metabolite in plasma can provide an indication of the amount of oxidative biotransformation that has occurred.[7] Although the vast majority (>98%) of halothane metabolism *in vivo* is along the oxidative route,[7] a lack of sufficient oxygen will lead to the insertion of electrons into the molecule by cytochrome P-450.[10] This second route of halothane biotransformation, known as the "reductive pathway," generates free radical intermediates that can react with the double bonds of subcellular lipids (fig. 1).[10] Additional breakdown of the free radicals releases fluoride ion (F^-), which can be used as a monitor of flux along this pathway.[9]

The presence of fluorine atoms on the halothane molecule allows for determination of the covalent binding of its reactive intermediates by analysis for these moieties.[11] By this analysis, it can be determined whether or not covalent binding of reactive halothane metabolic intermediates to cellular proteins or lipids is associated with halothane hepatotoxicity in guinea pigs. To alter flux along the two metabolic pathways, d-halothane (selectively inhibits oxidative metabolism)[7,9] or very low inspired oxygen concentrations (inhibit oxidative metabolism, promote reductive metabolism)[12] were used. By removal of the

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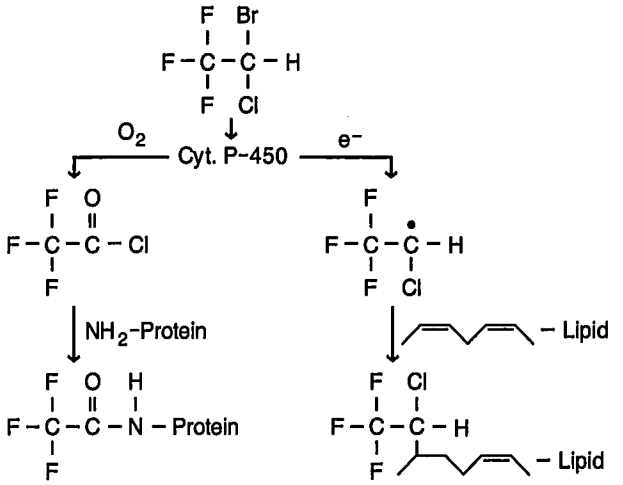

FIG. 1. The pathways (simplified) of halothane biotransformation, demonstrating the formation of reactive intermediates and their ability covalently to bind to protein and lipid.

livers of guinea pigs immediately after anesthesia, isolation of the protein and lipid, and determination of the amount of bound residues containing fluorine, a measure of covalent binding could be made. The amount of binding could then be compared with the hepatotoxic response in animals allowed to survive until 96 h after anesthesia.

Materials and Methods

ANIMALS

Male outbred Hartley guinea pigs (600–700 g) were obtained from Sasco, Inc., (Omaha, NE) and housed in stainless steel cages. The animals were kept on a 12-h light/dark cycle and fed food and water *ad libitum*. All treatments were made according to a protocol approved by the University of Arizona Animal Care Committee.

D-HALOTHANE SYNTHESIS

d-Halothane was synthesized by modification of the procedure of Hine *et al.*[13] Distilled halothane (350 ml; Abbott Laboratories, North Chicago, IL) was mixed vigorously with 800 ml 0.4 M sodium deuteroxide in deuterated water (40% sodium deuteroxide in deuterated water, 99 + atom percent deuterium, Aldrich Chemical Company, Inc., Milwaukee, WI; and deuterated water, 99.8 atom percent deuterium, ICN Biomedicals, Inc., Cambridge, MA) in the dark for 24 h. After removal of the aqueous layer, this procedure was repeated. The organic layer was then washed three times with a total volume of 400 ml deuterated water, dried over anhydrous sodium sulfate, and then distilled. The yield of d-halothane was 80% and proved to be more than 99% pure by gas chromatograph–mass spectrometer analysis.[7]

EXPOSURE CONDITIONS

Groups of guinea pigs (n = 8) were anesthetized with either 1% (v/v) halothane or d-halothane, $F_{I_{O_2}} = 0.40$ or 0.10 (balance N_2) and flow rate = 3 l/min, for 4 h in a 180-l Plexiglas acrylic plastic chamber. Anesthetic concentrations were measured at regular intervals by gas chromatography while oxygen concentrations were monitored with a polarographic oxygen electrode.[7] During anesthesia, four of the eight animals in each group were randomly selected to have body temperatures monitored by rectal probes attached to a telethermometer.[7] Temperatures were maintained at near normothermia by warming with two 20-W heaters attached to the underside of a raised metal plate that served as the floor of the chamber. There were no differences between groups in changes in body temperature during the 4-h exposure. Individual temperatures decreased by no more than 2° C.

SAMPLE COLLECTION

Some animals were killed immediately after anesthetic exposure by cervical dislocation, inferior vena cava blood was drawn, and their livers were removed. Liver tissue sections were fixed in buffered formalin and the remainder immediately frozen on dry ice for later bound organic fluorine analysis. Multiple blood samples were obtained from the remaining guinea pigs by toenail bleedings while they were restrained in Rodent Restraint Cones (Harvard Apparatus, South Natick, MA). Blood volumes removed (1–2 ml at 0 h, 0.3–0.5 ml at other sampling times) were kept small to avoid effects on blood volume. Animals were killed with lethal injections of pentobarbital, intraperitoneally. Inferior vena cava blood was drawn and liver tissue sections fixed in buffered formalin.

PLASMA ALT AND METABOLITE ANALYSIS

Plasma alanine aminotransferase (ALT) activities were measured spectrophotometrically (Procedure 59-UV®; Sigma Chemical Company, St. Louis, MO). Plasma concentrations of the halothane metabolites, F^-, and trifluoroacetic acid (TFA) were determined with specific ion electrodes[4] and by a gas chromatographic headspace technique,[14] respectively.

HISTOPATHOLOGIC EVALUATION

A randomly selected section of formalin-fixed hepatic tissue from each animal was processed and stained with hematoxylin and eosin. Slides were coded before submission to the pathologist (P. H.) for light microscopic evaluation. The presence of focal to confluent centrilobular necrosis in the sections collected at 96 h was used to classify an animal as being responsive to halothane.[7] Those with-

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out centrilobular necrosis or with scattered small foci of necrosis were considered nonresponsive.[7] Sections collected immediately after anesthesia were evaluated for the presence of hydropic changes (watery vacuolization), indicative of a severely hypoxic (anoxic) or ischemic event within the liver.[15]

Protein and Lipid Isolation

Protein and lipid were isolated from quadruplicate samples of liver tissue (100 mg). The tissue samples were sonicated in 1 ml of distilled water. Protein was precipitated and lipid extracted by addition of 5 ml ethanol (−20° C). After centrifugation (1,800 × *g* for 20 min), the protein pellets were washed twice with 1 ml 0.6 mM trichloroacetic acid and twice with 5 ml 2:1 chloroform–methanol (C/M). They were dried with nitrogen and then lyophilized to assure dryness. The ethanol and C/M extracts were combined as the lipid fraction and dried with nitrogen. The lipids were then dissolved in 5 ml C/M, transferred to another test tube, washed twice with 1 ml C/M-saturated water, dried under nitrogen, and then lyophilyzed. One tube from each set of quadruplicate samples was set aside for analysis of protein or lipid content. Proteins were dissolved in 1 N sodium hydroxide and content determined with the BCA Protein Assay® (Pierce, Rockford, IL). The average protein content was found to be 11.1 ± 0.86 mg protein/sample. The lipid fraction was analyzed for its phospholipid content by the method of Chen *et al.*[16] and was an average of 1.07 ± 0.05 mg phospholipid/sample. These averages were used to normalize bound organic fluorine values.

Bound Organic Fluorine Analysis

The amount of bound organic fluorine was determined by a modification of our sodium fusion technique.[11] Sodium metal (15–20 mg) was added to each of the remaining samples and the test tubes heated in a burner flame. After cooling, 1 ml water and 50 μl glacial acetic acid were added. Aliquots were mixed 50/50 with TISAB® (Orion Research, Boston, MA) and directly analyzed for F^- content with Orion® specific ion electrodes. The halothane metabolites (F^- and TFA) can potentially interfere with the results of the assay. Hepatic tissue concentrations of these metabolites were determined by sonicating 100 mg tissue in 0.9 ml distilled water, centrifuging the sample, and analyzing the supernatant fraction in the same manner as the plasma samples. Mean values ranged from 6 to 8 nmol F^- per 100 mg liver and 3 to 27 nmol TFA per 100 mg liver. To assure their removal from the protein and lipid samples by wash steps in the assay, large excesses (200 nmol F^- and 340 nmol TFA) were added to tissue samples from untreated controls at the outset of the assay. More than 98% of added F^- and all of the TFA were found to be removed after sodium fusion. Thus, tissue concentrations of these metabolites had no effect on bound organic fluorine results. After sodium fusion, background concentrations of F^- in control tissues were below the limits of detection in lipid samples and an average of 0.17 nmol F^-/mg protein in the protein fraction.

Experimental Protocol

There were four separate experimental groups with eight guinea pigs in each. One group was anesthetized with halothane at an FI_{O_2} of 0.40, another with halothane at an FI_{O_2} of 0.10. The other two groups were anesthetized with d-halothane again at an FI_{O_2} of 0.40 or 0.10. Half of the animals in each group were killed immediately after exposure to obtain hepatic tissue for bound organic fluorine analysis and histopathologic evaluation. Plasma ALT activities were measured in the remaining guinea pigs immediately after exposure (0 h) and at 24, 48, 72, and 96 h after exposure. These animals were killed at 96 h and liver tissue collected for histopathologic evaluation of abnormalities. Plasma ALT activities at 48 h were used for comparing degrees of hepatic injury.[2,7] The extent of halothane biotransformation was evaluated by measurement of halothane plasma metabolite concentrations in samples collected from all animals immediately after exposure.[7] A group of untreated control animals (n = 4) were killed and blood and liver tissue taken to provide baseline plasma levels of halothane metabolites and ALT, normal hepatic tissue for histopathologic comparison, and background values in the bound organic fluorine assay.

Statistical Analysis

All values are reported as mean ± SD. Analyses of ALT, halothane metabolites, and bound organic fluorine values were made with the use of analysis of variance (ANOVA) with a Newman-Keuls multiple comparison test. Because of increasing standard deviations with increasing means, a log transformation of ALT values was performed before analysis.[17] Chi-squared analysis was used to compare incidences of centrilobular necrosis. A P value less than 0.05 was considered significant.

Results

The use of d-halothane or low oxygen concentrations (FI_{O_2} = 0.10) during anesthesia produced significant decreases in oxidative biotransformation, as indicated by 0-h plasma TFA concentrations (table 1). The covalent binding of halothane to hepatic proteins, as indicated by bound organic fluorine released by the sodium fusion assay, was also significantly decreased by these conditions (table 1). These decreases were associated with an amelioration of halothane hepatotoxicity, as demonstrated by

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TABLE 1. Effect of Inspired Oxygen Concentration and D-Halothane on the Biotransformation and Covalent Binding of Reactive Intermediates of Halothane to Hepatic Proteins and Lipids

		Plasma Metabolite Concentration**		Bound Organic Fluorine	
FI_{O_2}	Treatment*	TFA (μM)	Fluoride Ion (μM)	Protein (nmoles F^- released per mg protein)	Lipid (nmoles F^- released per mg phospholipid)
0.40	Halothane	546 ± 73††	6.7 ± 1.9†	1.36 ± 0.26††	5.7 ± 1.8†
	D-Halothane	203 ± 40†	7.7 ± 2.7†	0.91 ± 0.09†††	6.3 ± 1.7†
0.10	Halothane	88 ± 27	10.8 ± 2.3	0.81 ± 0.05	33.7 ± 2.6
	D-Halothane	65 ± 27	15.5 ± 2.9	0.55 ± 0.19	31.4 ± 15.0

Values are means ± SD. N = 8 for plasma metabolites. N = 4 for bound organic fluorine.
* 1% for 4 h.
** Immediately after anesthesia.
† $P < 0.05$ *versus* $FI_{O_2} = 0.10$ values.
†† $P < 0.01$ *versus* all other values.
††† $P < 0.05$ *versus* $FI_{O_2} = 0.10$, D-halothane value.

significant decreases in plasma ALT and incidences of centrilobular necrosis (fig. 2).

At an FI_{O_2} of 0.40, d-halothane had no effect on reductive biotransformation, as indicated by 0-h plasma F^- concentrations (table 1). The covalent binding of free radical intermediates to hepatic lipids, as indicated by bound organic fluorine released, was also unaltered (table 1). Significant increases in reductive biotransformation and covalent binding to lipids occurred with low inspired oxygen concentrations ($FI_{O_2} = 0.10$) during exposure to halothane or d-halothane (table 1). Centrilobular necrosis did not develop under these conditions (fig. 2).

Immediately after anesthesia, plasma ALT levels were not increased above untreated control values (23 ± 3 units/ml, n = 4) in any of the treatment groups. Of the animals killed at this time, all of those exposed at an FI_{O_2} of 0.10 (n = 8) showed both hydropic change (watery vacuolization) and shrunken, deeply eosinophilic cells in centrilobular regions.[15,18] Of those killed immediately after anesthesia at an FI_{O_2} of 0.40, two of eight demonstrated only minor hydropic change, focally, whereas the remainder were essentially normal. At 24 h after anesthesia, there were no differences in ALT activity between the treatment groups. By 48 h, plasma ALT activities in the group that received halothane with an FI_{O_2} of 0.40 were significantly greater than in all other groups (fig. 2), remained so at 72 h (164 ± 122 units/ml), and were still significantly above control values at 96 h (71 ± 44 units/ml). ALT activities in the other treatment groups, although slightly increased (mean values = 25–69 units/ml), were not significantly different from untreated control values at the 48-, 72-, and 96-h time points. Evaluation of hepatic tissue taken at 96 h demonstrated that centrilobular necrosis[4,5] was only present in the group exposed to halothane at an FI_{O_2} of 0.40 (fig. 2). Guinea pigs anesthetized with d-halothane at an FI_{O_2} of 0.40 (n = 4) displayed essentially normal tissue morphologic characteristics. Of those anesthetized with either halothane or d-halothane at an FI_{O_2} of 0.10, five of eight exhibited scattered foci of necrosis, previously classified as a "mild" injury.[2,4] These animals were not considered "responders" to halothane because centrilobular necrosis was not present.[7] By 96 h, there was no longer any evidence of the hydropic changes that were seen in tissues taken at 0 h.

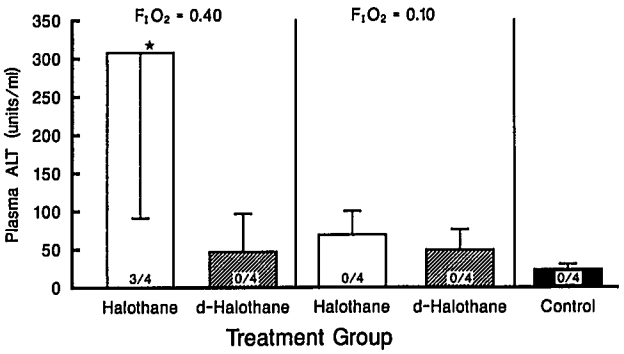

FIG. 2. Effect of inspired oxygen concentration and D-halothane on halothane hepatotoxicity in guinea pigs. Plasma ALT levels are shown at 48 h after anesthesia with either 1% halothane or D-halothane, each at an FI_{O_2} of either 0.40 or 0.10. Number of animals with centrilobular necrosis per n values in bars. Star indicates $P < 0.05$ *versus* all other groups. P (χ^2) < 0.01 for incidence of necrosis in the halothane + $FI_{O_2} = 0.40$ group *versus* all other groups. Values are mean ± SD.

Discussion

Halothane hepatotoxicity in guinea pigs is associated with the covalent binding to hepatic proteins by reactive intermediates generated during oxidative biotransformation. Binding to subcellular lipids by free radicals produced by reductive halothane biotransformation does not appear to be involved in producing the centrilobular lesion.

In this study, the difference in bound organic fluorine to protein between groups receiving either halothane or d-halothane at an FI_{O_2} of 0.40 is only 33%. However,

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there are large differences in hepatotoxicity. It would appear that there is a critical threshold in the amount of covalent binding of halothane intermediates to subcellular proteins that must be exceeded before the degree of damage to the hepatocyte is sufficient to cause cell death.[19] There may be critical target proteins or enzymes within the cell whose dysfunction resulting from covalent binding ultimately leads to cell death. Indeed, several hepatic proteins and enzymes that are targets for halothane reactive intermediates have now been identified.[20–22] Whether or not covalent binding to them is involved in the development of necrosis remains to be elucidated.

Changes in metabolite production with the different anesthesia conditions were not of equal proportion to changes in bound organic fluorine. The use of d-halothane at an $F_{I_{O_2}}$ of 0.40 decreased plasma TFA concentrations by more than half, whereas organic fluorine bound to hepatic protein was decreased by only one third. This may well result from differences between the trifluoroacetyl acid chloride reactive intermediate's preference for binding sites on proteins and its reaction rate with water to produce TFA.[8,9] In addition, reductive halothane biotransformation generates both free radicals and the metabolite 2-chloro-1,1-difluoroethene, both of which can bind to protein.[23,24] This would contribute to the total amount of organic fluorine bound to protein, preventing a decrease as great as that observed for the oxidative metabolite (TFA). Low inspired oxygen concentrations ($F_{I_{O_2}} = 0.10$), although decreasing oxidative biotransformation and consequently binding to protein, also increased reductive metabolism (F^-) and any contribution of free radicals and chlorodifluoroethene to total binding to protein. Thus, at an $F_{I_{O_2}}$ of 0.10, decreases in protein binding were not as great as decreases in TFA concentrations. In contrast to protein binding, organic fluorine bound to lipid increased fivefold to sixfold with hypoxia, whereas increases in the reductive metabolite (F^-) were only 1.5-fold to 2-fold. Again, this is probably because of differences in the preference of free radical intermediates to bind to lipid and their tendency to release F^- and form the chlorodifluoroethene metabolite.[9,23] Chlorodifluoroethene can be further metabolized, thus releasing more F^-.[24]

The free radicals generated by reductive biotransformation do seem to prefer to bind to lipid rather than protein. With hypoxia, organic fluorine bound to lipid is enhanced fivefold to sixfold, whereas binding to protein is decreased by 40%. These findings are in agreement with those of the only previous study concerning covalently bound halothane intermediates to hepatic proteins and lipids that was performed *in vivo* with animals exposed to anesthetic concentrations of halothane.[25] It also demonstrated that hypoxia greatly enhanced binding to lipid and decreased binding to protein.

Contrary to our evidence linking hepatotoxicity to oxidative halothane biotransformation, a recent study in guinea pigs attempted to link halothane-induced necrosis to microsomal lipid peroxidation produced by free radical generation.[26] The investigators observed changes in serum enzyme (transaminase) activities 6 h after exposure, along with the appearance of small necrotic foci within the liver. Beyond 12 h after anesthesia, serum enzyme activities were not different from control values. Thus, they were not producing the frank centrilobular necrosis that is the hallmark of our guinea pig model of halothane hepatotoxicity.[4,5] Undoubtedly their findings result from their use of young animals (<400 g) and a shorter period of halothane anesthesia (3 h). Younger guinea pigs have been found to be more refractory to the centrilobular lesion.[5] Anesthesia times exceeding 3 h appear to be required to consistently produce a centrilobular lesion (unpublished results). The early increases in serum liver enzyme activities and the development of small foci of necrosis may well result from lipid peroxidation. In this study, low inspired oxygen concentrations (0.10) during anesthesia significantly increased binding to lipid, and scattered small necrotic foci were observed to have developed in the animals.

An earlier study by Lunam *et al.* showed no correlation between inspired oxygen concentration during halothane anesthesia and the subsequent incidences of either frank centrilobular necrosis or small necrotic foci (mild injury).[2] However, the lowest inspired oxygen concentration used was an $F_{I_{O_2}}$ of 0.14 that did not affect the oxidative biotransformation of halothane as evidenced by a lack of change in urinary TFA excretion after anesthesia.[2] Thus, covalent binding to protein would have been unchanged and, according to the results of this study, so would development of centrilobular necrosis. Our use of an $F_{I_{O_2}}$ of 0.10 produced a state of hepatic hypoxia that significantly decreased oxidative biotransformation as indicated by plasma TFA concentrations. This would account for the observed differences in hepatic injury between the halothane treatment groups with $F_{I_{O_2}}$s of 0.40 and 0.10. In fact, the degree of hepatic hypoxia during anesthesia was so great with an $F_{I_{O_2}}$ of 0.10 that hydropic changes (watery vacuolization) of centrilobular hepatocytes, an indication of a severely hypoxic (anoxic) or ischemic event,[15,18] were evident in the tissue sections taken immediately after anesthesia. Because centrilobular necrosis did not develop under these conditions, the results provide evidence that decreases in hepatic oxygenation, which can occur during halothane anesthesia in guinea pigs,[27] are not a mechanism for development of the centrilobular lesion.

We have previously reported that anesthesia with d-halothane caused a decrease in the incidence and severity of centrilobular necrosis that was not as great in magni-

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tude as reported herein.[7] This most likely results from the use of smaller "n" values in this study and natural biologic variations in the hepatotoxic response of the guinea pigs. We have also reported in a small preliminary study that an FI_{O_2} of 0.10 during halothane anesthesia had no effect on the development of centrilobular necrosis in a mixed group of strain 13 male and female guinea pigs.[12] Although at odds with the results of this study that showed an amelioration of the lesion with these conditions, the discrepancy can best be explained by the extreme susceptibility of female strain 13 guinea pigs to halothane-associated hepatotoxicity,[5] allowing development of necrosis even though oxidative biotransformation was significantly decreased.[12]

Additional studies to identify the specific hepatic proteins (enzymes?) that show differential degrees of bound halothane intermediates under the conditions of this study could well elucidate the specific mechanism(s) by which oxidative halothane biotransformation can cause acute hepatic necrosis.[8,20-22]

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