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The Role of Oxidative Biotransformation of Halothane in the Guinea Pig Model of Halothane-associated Hepatotoxicity

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The role of the oxidative pathway of halothane biotransformation in mediating the hepatotoxicity of halothane in the guinea pig was examined by utilizing the deuterated form of halothane (*d*-halothane), which is resistant to oxidative metabolism. Male outbred Hartley guinea pigs were exposed to either 1% v/v halothane or *d*-halothane, FI_{O_2} = 0.21, for 4 h. Significant reductions in both oxidative and overall halothane biotransformation were observed with the use of *d*-halothane as indicated by decreased plasma levels of trifluoroacetic acid and bromide ion, respectively, immediately following exposure. Plasma fluoride ion, indicative of the reductive metabolism of halothane, was significantly increased with the use of *d*-halothane. These changes in metabolism were accompanied by a reduced hepatotoxic response as indicated by significantly decreased plasma ALT levels 24–96 h following exposure and a significantly lesser incidence of centrilobular necrosis. Thus, the oxidative biotransformation of halothane is implicated as a mechanism of injury in guinea pigs. (Key words: Anesthetics, volatile: halothane; deuterated halothane. Animal: guinea pig. Biotransformation: halothane. Liver: hepatotoxicity.)

BIOTRANSFORMATION of halothane by the cytochrome P-450 enzyme system, which proceeds along either oxidative or reductive pathways,[1] is considered to play a role in the development of halothane-associated hepatic necrosis in animals[1] and in humans.[2] Generation of free radical intermediates *via* reductive metabolism of halothane and their subsequent binding to subcellular macromolecules is a potential mechanism for halothane-associated hepatotoxicity in phenobarbital-pretreated, hypoxic rats.[1] Oxidative metabolism of halothane produces a trifluoroacetyl acid chloride intermediate, which hydrolyzes to produce the oxidative metabolite trifluoroacetic acid or binds to free amino groups on proteins.[2] Labeling of hepatocyte proteins with the trifluoroacetyl moiety is believed to create antigens that may be involved in the development of the rare, immunologically related, fulminant, halothane hepatitis in humans.[2] In addition, the oxidative pathway has been implicated as a factor in the generation of halothane-associated hepatic necrosis in normoxic rats pretreated with isoniazid.[3]

In the guinea pig model of halothane-induced liver injury,[4,5] it has been shown that inhibition of cytochrome P-450-mediated metabolism *via* pretreatment with SKF-525A attenuates the development of centrilobular necrosis.[4] Although this implies that the biotransformation of halothane is necessary for the production of hepatic necrosis in guinea pigs, it remains to be determined which metabolic pathway, oxidative or reductive, is responsible. Manipulation of the degree of reductive metabolism by utilizing different oxygen tensions during halothane exposure has not been found to alter lesion severity[4] and, thus, may not be critical in the development of hepatic necrosis. To elucidate the potential for involvement of the oxidative pathway as a mechanism of halothane-associated hepatotoxicity in guinea pigs, we utilized the deuterated form of halothane (*d*-halothane), which is resistant to oxidative biotransformation.[1]

Materials and Methods

ANIMALS

Male outbred Hartley guinea pigs (600–800 g) were obtained from Harlan/Sprague-Dawley (Indianapolis, Indiana). Preliminary studies in our laboratory have demonstrated an incidence of halothane-associated hepatic necrosis of approximately 50% in this strain. The animals were housed in stainless steel cages, kept on a 12-h light/dark cycle, and allowed food and water *ad libitum*. All animals were treated under a protocol approved by the University of Arizona Animal Care Committee.

MATERIALS

Halothane was obtained from Abbott Laboratories (King of Prussia, Pennsylvania). *d*-Halothane, synthesized for previous experiments in our laboratory,[1] had been stored under nitrogen in the dark at −20° C. Analysis on a Finnigan model 3300 gas chromatograph–mass spectrometer (Finnigan MAT, San Jose, California), equipped with a 4-ft × 2 mm column packed with 10% SP-1000 on Supelcoport, demonstrated that the compound was still >99% pure. All other materials were obtained as previously described.[5,6,§]

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§ Maiorino RM, Gandolfi AJ, Sipes IG: Gas chromatographic method for the halothane metabolites, trifluoroacetic acid and bromide in biological fluids. Journal of Analytical Toxicology 4:250–254, 1980

TABLE 1. Plasma Metabolite Levels in Guinea Pigs Immediately Following Exposure to Either Halothane or *d*-Halothane

Treatment†	Metabolite Levels (μM)*		
	Fluoride Ion	TFA	Bromide Ion
Halothane	5.8 ± 2.0	350 ± 78	578 ± 105
d-Halothane	8.1 ± 2.2‡	177 ± 44‡	382 ± 92‡

Values are mean ± SD (n = 11–15). TFA = trifluoroactetic acid.
* Plasma metabolite levels at 0 h.
† 1% halothane or *d*-halothane, FIO2 = 0.21, 4 h.
‡ $P < 0.01$ *versus* halothane treatment group.

Exposure Conditions

The animals were exposed in groups of nine or ten to either 1% (v/v) halothane or *d*-halothane for 4 h in a 180-l plexiglass chamber. The flow rate was 3 l/min with oxygen concentrations maintained at $F_{I_{O_2}} = 0.21–0.22$ (balance N_2). Anesthetic concentrations were measured at regular intervals on a Varian model 1420 gas chromatograph (Varian Instruments, Palo Alto, California) equipped with a thermal conductivity detector and a 5 ft × ⅛ in column packed with 5% SE-30 on Varaport. Oxygen concentrations were monitored with an Instrumentation Laboratories Model 408 polarographic oxygen electrode (Instrumentation Laboratories, Lexington, Massachusetts). Body temperatures were monitored using rectal probes attached to a YSI tele-thermometer (Yellow Springs Instruments, Yellow Springs, Ohio) and maintained to near normothermia by warming with two 20 W heaters attached to the underside of the metal plate that served as the floor of the chamber. During the 4-h exposures, body temperatures decreased only 0.5–2.0° C.

Sample Collection and Analysis

For the determination of blood and liver concentrations of the anesthetics, animals were killed during exposure by cervical dislocation during the final hour of anesthesia. Inferior vena cava blood was immediately drawn and duplicate 1-ml samples were mixed with 2 ml water-saturated heptane in 5-ml reaction vials equipped with Teflon®-lined septa. Duplicate samples of liver tissue (approximately 1 g) were weighed and placed in 2-ml water-saturated heptane in 5-ml reaction vials. The tissue samples were later disrupted with sonication while at a temperature of 4° C to minimize the loss of the anesthetics from the heptane. Analysis of anesthetic concentrations was performed on the Varian 1420 gas chromatograph using our method previously described for enflurane concentrations in blood.[6]

Multiple blood samples were obtained from all other animals *via* toenail bleedings. No additional anesthetics were required for sampling immediately after the exposure period. For other blood samples, the animals were lightly anesthetized with ketamine (10 mg/kg im). Only 1–2 ml of blood was taken immediately following anesthesia and 0.3–0.5 ml at other sampling times to avoid significant effects on blood volume. The guinea pigs were killed by cervical dislocation while anesthetized with ether, inferior vena cava blood was drawn, and liver tissue sections were fixed in buffered formalin.

Plasma alanine aminotransferase (ALT) levels were determined spectrophotometrically (Procedure 59-UV; Sigma Chemical Company, St. Louis, Missouri). Plasma concentrations of the halothane metabolites, inorganic fluoride ion, bromide ion, and trifluoroacetic acid were measured as previously described.[5,§]

A single, randomly selected section of hepatic tissue from each animal was processed, stained with hematoxylin and eosin, and coded prior to submission to the pathologist (P.H.) for light microscopic evaluation. Animals demonstrating focal to confluent centrilobular necrosis were considered responsive to halothane. Those without centrilobular necrosis or with scattered small foci of necrosis were considered nonresponsive.

Experimental Protocol

Two separate experiments were carried out with nine or ten animals in each treatment group. In each experiment, one randomly selected group of animals was anesthetized with halothane while the other breathed *d*-halothane. The anesthetics were administered at the same time of day to avoid diurnal variations in response. The first experiment examined the potential for deuteration of halothane to alter the time course of development and resolution of the hepatic lesion. This was carried out by measuring plasma ALT levels immediately after exposure (0 h) and at 24, 48, 72, and 96 h. At 96 h the animals were killed and hepatic tissue samples were also taken. In the second experiment, three guinea pigs from each group were killed after 3.0–3.5 h of the 4-h exposure for determination of blood and liver anesthetic concentrations. Plasma ALT levels were determined for the remaining animals in each group at 0, 24, and 48 h, which was the termination point of this experiment. Liver tissue samples were also taken at termination. In both experiments, 48-h plasma ALT levels were used for comparison of degrees of hepatic injury.[4,5] Plasma halothane metabolite concentrations were measured in the samples collected immediately following anesthetic exposure as volumes permitted. In addition, a group of untreated colony control guinea pigs (n = 10) were killed with samples taken to provide baseline levels of the metabolites and plasma ALT and normal hepatic tissue.

Statistical Analysis

All values are reported as mean ± SD. For comparison of ALT values, statistical analyses were made using ANOVA with a Newman-Keuls multiple comparison test.

Due to increasing standard deviations with increasing means, logarithmic transformation of ALT values was performed prior to statistical analysis.[7] Plasma metabolite levels were compared *via* a one-tailed Student's *t* test. Comparison of the incidences of centrilobular necrosis were made by chi-square analysis. A $P < 0.05$ was considered significant.

Results

There were no differences in the concentrations of either halothane or *d*-halothane in the blood or livers of the animals killed during the exposure period (combined halothane + *d*-halothane values for blood = 474 ± 115 μM, for liver = 1.85 ± 1.06 μmol/g wet wt, n = 6). *d*-Halothane underwent less oxidative metabolism during the exposure period as demonstrated by the significantly lower plasma levels of the oxidative metabolite, trifluoroacetic acid (−49%), and bromide ion (−33%), an indicator of overall halothane metabolism[1] (table 1). The use of *d*-halothane also generated greater flux along the reductive pathway of metabolism as indicated by significantly more plasma fluoride ion (+40%) than that produced during exposure to halothane (table 1). Compared with halothane, *d*-halothane exposure attenuated the hepatotoxic response following exposure as indicated by the combined data from both experiments, which showed significantly lower 48 h plasma ALT levels and a significantly lower incidence of centrilobular necrosis (fig. 1). Although ALT levels increased significantly above control and 0 h values in *d*-halothane exposed animals over the 96-h time course generated by experiment 1, the values were significantly less than those attained in the halothane group at the 24, 48, 72, and 96 h time points (fig. 2). As previously observed,[5] each treatment group had responders (animals with centrilobular necrosis) with large (10–20-fold) increases in ALT levels and nonresponders (animals without centrilobular necrosis) with small (less than twofold) increases in ALT levels, which caused the standard deviations to be as large as the means in some cases (figs. 1 and 2). In addition, several guinea pigs in each treatment group developed scattered foci of necrosis in their livers, which was accompanied by twofold to eightfold increases in ALT levels. These animals were considered nonresponders due to a lack of centrilobular necrosis. In spite of the large standard deviations, significant differences between means were observed upon analysis of the data.

Discussion

As previously shown,[1] deuteration of halothane inhibited its oxidative but not reductive *in vivo* biotransformation, which in the guinea pig was accompanied by a significant reduction in halothane-associated hepatotoxicity. This was not due to differences in either the bioavailability or the potency of halothane and *d*-halothane. Similar concentrations of the anesthetics were observed in the blood and liver of the guinea pigs during anesthesia. Deuterated analogs are considered equipotent.[8,¶] Deuteration of volatile anesthetics does not alter MAC, hemodynamic or respiratory responses,¶,** or waking times following anesthesia.[1] Thus, oxidative biotransformation of halothane is implicated as a mechanism of toxicity in the guinea pig model.

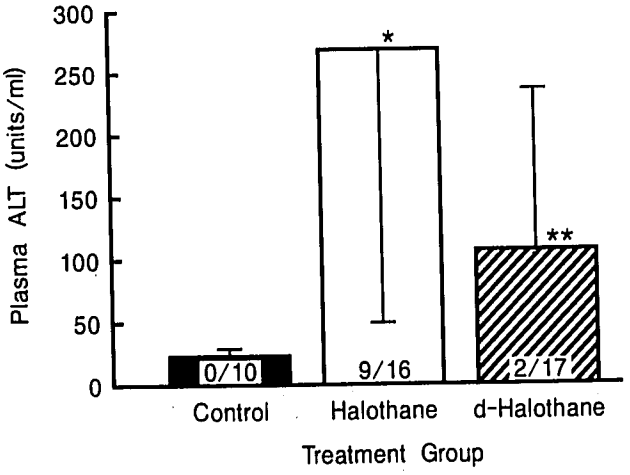

FIG. 1. Plasma ALT levels in guinea pigs 48 h after exposure to either halothane or *d*-halothane. Values are combined from experiments 1 and 2. Control; untreated animals. Halothane; 1% halothane, $F_{I_{O_2}} = 0.21$, 4 h. *d*-Halothane; 1% *d*-halothane, $F_{I_{O_2}} = 0.21$, 4 h. Number of animals with centrilobular necrosis/N values in bars. $*P < 0.01$ *versus* control ALT values. $**P < 0.01$ *versus* control and halothane treatment group ALT values. Incidence of centrilobular necrosis between groups differs by $P(\chi^2) < 0.02$ with greater than one-half contribution from the halothane treatment group. Values are mean ± SD.

Binding of cytochrome P-450-generated reactive metabolic intermediates to subcellular components is considered an initiating event in the development of hepatic necrosis following exposure to a variety of xenobiotics.[9] This mechanism is believed to be involved in the development of halothane-associated hepatotoxicity produced in mildly hypoxic ($F_{I_{O_2}} = 0.14$) rats.[1] In this animal model of hepatotoxicity, cytochrome P-450-mediated metabolism is enhanced *via* pretreatment with phenobarbital.[10] This, coupled with hypoxia during anesthetic exposure, promotes the free radical generating reductive pathway, increasing the binding of metabolic intermediates to subcellular macromolecules to a such an extent as to cause cell death.[11] Consistent with this theory, the use of *d*-halothane in phenobarbital-pretreated hypoxic rats had no

¶ Tinker JH, Van Dyke RA, McCarty LP: Deuterated halothane: Studies of potency and in vitro metabolism (abstract). International Anesthesiology Research Society, Miami, Florida, 1979

** Tinker JH, Milde JH, Noback CR: Deuterated enflurane: No MAC or cardiorespiratory isotope effect (abstract). ANESTHESIOLOGY 55:A3, 1981

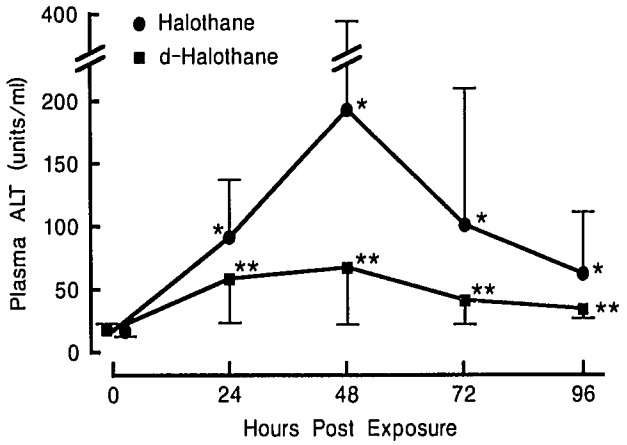

FIG. 2. Time course of plasma ALT levels in guinea pigs exposed to either 1% halothane (●) or 1% *d*-halothane (■), FI_{O_2} = 0.21, for 4 h. Values are from experiment 1 (n = nine or ten at each time point). *$P < 0.05$ *versus* 0 h and control values (ALT = 23 ± 6, n = 10). **$P < 0.05$ *versus* halothane, 0 h, and control values. Values are mean ± SD.

significant effect on either fluoride ion production (reductive pathway) or the severity of resulting hepatotoxicity.[1] However, in guinea pigs the reductive pathway does not appear to be a principal factor in the development of hepatotoxicity. The use of different oxygen tensions during halothane exposure causes variations in the degree of reductive metabolism but does not significantly alter the severity of the resulting lesion in the guinea pig.[4] In this study exposure to *d*-halothane produced significantly higher plasma levels of the reductive metabolite, fluoride ion, over those observed in halothane exposed animals; yet hepatotoxicity was attenuated.

Halothane-induced deprivation of liver oxygen supply, rather than halothane metabolism, has been proposed as being responsible for the development of hepatotoxicity in the guinea pig.[12] Although no evidence has ever been presented to indicate that deuterated analogs of the volatile anesthetics are other than equipotent, it is possible that the effect of *d*-halothane on hepatic oxygen supply is different from that of halothane. Plasma fluoride ion levels observed in guinea pigs exposed to *d*-halothane were greater than those obtained with halothane. This indicates that there was greater flux along the reductive (lack of oxygen) pathway during *d*-halothane exposure and possibly lower hepatic oxygen levels than in animals receiving halothane. Because a lesser degree of hepatotoxicity occurred with the use of *d*-halothane, it would appear that changes in hepatic oxygenation, as indicated by the degree of reductive metabolism, are not a principal factor in the development of the centrilobular lesion in guinea pigs. Yet another explanation for the increased reductive metabolism observed with *d*-halothane would be that deuterium substitution-induced inhibition of oxidative metabolism at the active site of cytochrome P-450 left more unmetabolized parent available for the insertion of electrons. Although slightly greater plasma fluoride ion levels (+25%) were previously observed with the use of *d*-halothane in phenobarbital-pretreated hypoxic rats, the values were not significantly different from those produced by halothane exposure.[1] Compared to guinea pigs, the much greater degree of reductive metabolism (>2×) that occurs in rats, due to the combination of phenobarbital pretreatment and hypoxic exposure conditions, most likely masked any potential differences between halothane and *d*-halothane in their tendency to undergo reductive biotransformation *in vivo*.

It has been recently reported that isoniazid pretreatment of rats coupled with exposure to halothane under normoxic conditions produced a hepatic lesion.[3] Under these conditions flux along the oxidative pathway of halothane metabolism was significantly increased, whereas low levels of reductive metabolism were not affected.[3] It was concluded that the oxidative biotransformation of halothane was responsible for the lesion.[3] Although it has been shown in mildly hypoxic rats that the total *in vivo* binding of halothane metabolites is less during normal to high oxygen exposure conditions than during hypoxia,[11] the oxidative pathway does generate a trifluoroacetyl acid–chloride intermediate that binds to the free amino groups of proteins.[2] The presence of bound trifluoroacetyl groups has been demonstrated by immunohistochemistry in the centrilobular hepatocytes of rats administered halothane while breathing normoxia.[2] The use of *d*-halothane greatly reduced the binding.[2] Because the centrilobular region of the liver is the area of lesion development,[4,5] binding of oxidative pathway-generated trifluoroacetyl moieties to critical subcellular proteins may be involved in the pathogenesis of hepatic injury in the guinea pig model of halothane-associated hepatic necrosis and in normoxic rats pretreated with isoniazid prior to halothane exposure.

The rat requires enhancement of cytochrome P-450-mediated metabolism *via* pretreatment with either phenobarbital[10] or isoniazid[3] to produce hepatic necrosis subsequent to halothane exposure, whereas the guinea pig does not.[4,5] This may be due to higher basal levels of microsomal cytochrome P-450 found in the guinea pig.[13,14] In addition, the few published reports comparing the species indicate that the guinea pig is more susceptible to the deleterious effects of hepatotoxins, such as carbon tetrachloride[15] and acetaminophen.[16] These inherent differences between the species could account for the lack of an enzyme induction requirement for producing halothane-associated hepatotoxicity in the guinea pig.

The oxidative pathway of halothane metabolism has been linked to fulminant halothane hepatitis in humans through its ability to trifluoroacetylate hepatic proteins,

transforming them into neoantigens, which subsequently provoke an immune response.[2] In regards to both halothane biotransformation and lesion morphology, the guinea pig closely resembles clinical observations of halothane metabolism and cases of halothane-associated acute hepatic dysfunction that can occur in over 20% of patients anesthetized with the compound.[4,5,17] Thus, if the oxidative pathway proves to be a source of hepatotoxicity, it would have a significant impact upon resolving the etiology of the acute disease as well.

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