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A Study of the Mechanism of Halothane-Induced Liver Necrosis. Role of Covalent Binding of Halothane Metabolites to Liver Proteins in the Rat[1]

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Various anesthetic and nonanesthetic doses of [1-^{14}C]halothane were administered separately to normal and phenobarbital-pretreated (PBP) rats by ip route. The rats were sacrificed at 0.5–24 h after dosing, and livers were removed and examined histopathogically for tissue necrosis. Only PBP rats that received anesthetic doses of halothane (11.5 or 23 mmol/kg) and sacrificed 24 h after dosing exhibited liver toxicity. Determination of the radioactivity distribution among various liver macromolecules revealed that the protein fraction contained the highest activity at all time points in all animals. The lipid fraction showed some radioactivity during the initial 1–6-h period which disappeared after 6–8 h, while the DNA fraction was devoid of radioactivity in all animals injected with [^{14}C] halothane. All the PBP rats that exhibited liver necrosis consistently attained higher covalent binding of halothane metabolites to liver proteins (2.13–2.20 nmol/mg of protein) when compared with the protein binding (1.12–1.41 nmol/mg of protein) observed among the rats that did not exhibit liver toxicity during the same time period. These results suggest a correlation between covalent binding of halothane metabolites to liver proteins and halothane-induced liver necrosis.

Halothane ($CF_3CHBrCl$), a widely used general anesthetic agent, is implicated in occasional fatal hepatic necrosis observed among exposed humans.[2-4] The toxic hazards appear to increase with repeated anesthetic exposures to halothane.[4] Although there were numerous studies in recent years,[5-20] the mechanism of halothane-induced liver necrosis remains poorly understood. The formation of chemically reactive metabolites of halothane by the liver microsomal cytochrome P-450 enzyme system and covalent binding of these reactive metabolites to hepatic lipids and proteins were reported by several investigators.[7-20] However, the covalent binding of halothane metabolites to liver macromolecules has not been correlated with liver toxicity. Recently, good correlation between tissue toxicity and covalent binding of reactive metabolites to target tissue macromolecules was observed with several halogenated hydrocarbons, e.g., chloroform, carbon tetrachloride, and bromobenzene.[21-23]

The present studies were initiated to evaluate possible relationship between covalent binding of halothane metabolites to liver macromolecules and halothane-induced liver necrosis. Rats were used in these experiments since earlier studies[24,25] showed that halothane toxicity can be induced in this species following phenobarbital pretreatment (PBP).

Experimental Section

Animals. Male Sprague–Dawley rats, weighing 100–120 g, were purchased from Camm Research Institute, Wayne, N.J., and maintained on Purina rat chow. The animals were fasted overnight prior to halothane administration and were fed thereafter. Drinking water was available ad libitum. The rats were divided into two major groups, normal and phenobarbital pretreated.

Phenobarbital Pretreatment (PBP). Rats were pretreated with phenobarbital sodium USP (80 mg/kg, in distilled water, ip) for three consecutive days and were used on the fourth day.[26,27]

Labeled Halothane Solutions. [1-^{14}C]Halothane (New England Nuclear, Boston, Mass.; specific activity 1.35 mCi/mmol) was dissolved in olive oil and made up to the requisite concentrations with unlabeled halothane (Ayerst Laboratories, Inc., New York, N.Y.). The halothane doses used were 4.6, 11.5, and 23.0 mmol/kg. Each animal received 50 μCi of ^{14}C/kg.

Halothane Administration. The protocol employed for in vivo studies with [^{14}C]halothane in normal and PBP rats is described in Table I. The requisite doses of halothane were administered by ip route. Six rats were used at each time point

Table I. Protocol for in Vivo Studies with [^{14}C]Halothane in Normal and Phenobarbital-Pretreated Rats[a]

Halothane dose[b] ip, mmol/kg	Duration of anesthesia[c]	Sacrifice time[d] after dosing, h	Liver necrosis[e]	
			Normal rats	Phenobarbital-pretreated rats
4.6	No anesthesia	0.5, 1, 2, 4, 8, 12, 18, 24	None	None
11.5	1 h 55 min-2 h 5 min	4, 8, 12, 18, 24	None	24-h group only
23.0	3 h 45 min-4 h	4, 8, 12, 18, 24	None	24-h group only

[a] Six rats were used at each time point in each group. [b] Each rat received 50 μCi of [1-^{14}C]halothane/kg. [c] The duration of anesthesia was not altered by phenobarbital pretreatment of rats. [d] There was no mortality due to halothane in any of the experimental groups. [e] See Table II for details on the extent of liver necrosis.

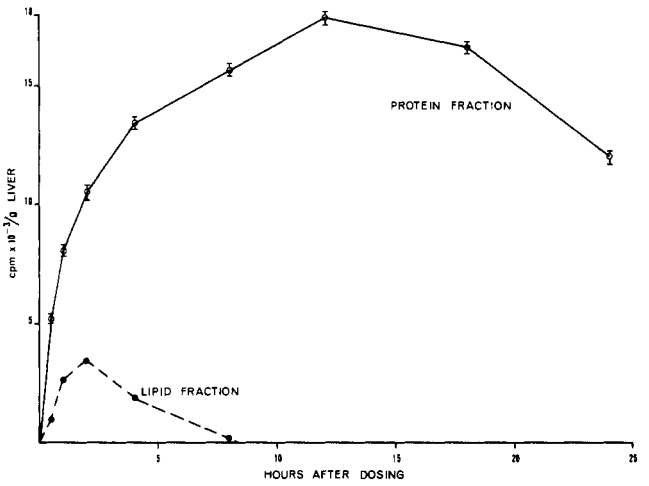

Figure 1. Distribution of radioactivity from [^{14}C]halothane (ip dose 4.6 mmol/kg; 50 μCi/kg) in protein and lipid fractions of livers from phenobarbital-pretreated rats.

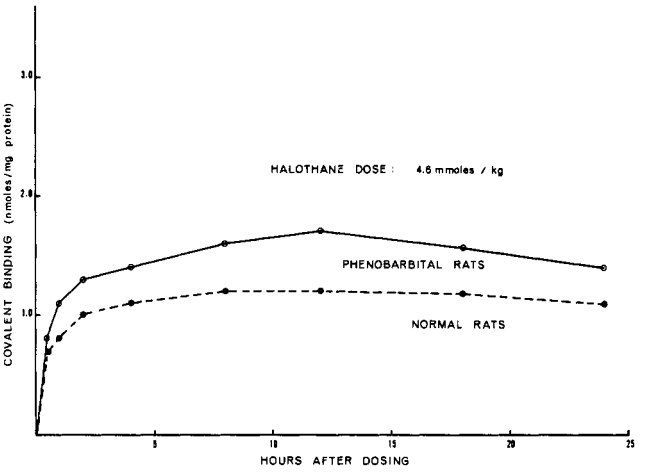

Figure 2. Covalent binding of [^{14}C]halothane (ip nonanesthetic dose 4.6 mmol/kg; 50 μCi/kg) metabolites to liver proteins from normal and phenobarbital-pretreated rats.

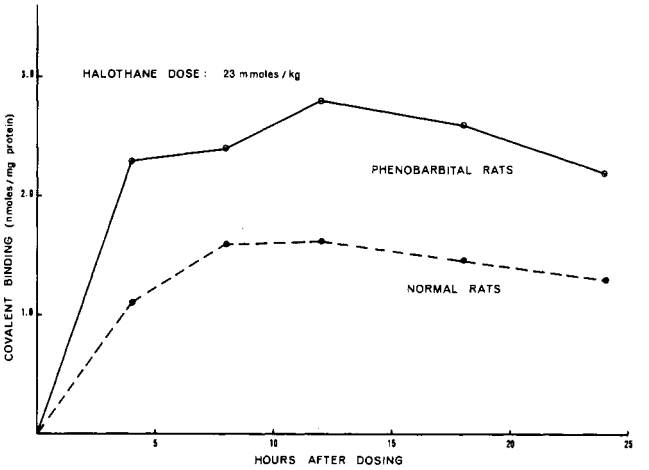

Figure 3. Covalent binding of [^{14}C]halothane (ip anesthetic dose 23 mmol/kg; 50 μCi/kg) metabolites to liver proteins from normal and phenobarbital-pretreated rats.

in each group. The control group of animals, both normal and PBP, received equivolumetric ip injection of the vehicle, olive oil.

Histopathological Studies. Rats were sacrificed by cervical dislocation at 0.5–24 h after dosing (Table I), and livers were removed and stored in dry ice. Liver slices of 1–2 mm thickness were fixed in 10% buffered formalin (pH 7.0), dehydrated in graded strengths of ethanol, cleared in xylol, and embedded in paraffin.[28] Sections of 6-μm thickness were stained with hematoxylin and eosin, and tissue necrosis was evaluated by light microscopy.[24,25]

Quantitative analysis of the liver necrosis produced by halothane was carried out by the procedure of Mitchell et al.[29] and the percent necrosis is reported as follows: 0 = absent, 1+ = necrosis of <6% of hepatocytes, 2+ = necrosis of 6–25% of hepatocytes, 3+ = necrosis of 26–50% of hepatocytes, and 4+ = necrosis of >50% of hepatocytes.

Serum Glutamic-Pyruvic Transaminase (SGPT) Assay. At the time of sacrifice, blood samples (5–8 ml) were collected from rats by cardiac puncture and serum was prepared by centrifugation. The SGPT assay was performed with a commercial assay kit (Sigma Chemical Co., St. Louis, Mo.) according to the procedure described in the accompanying technical bulletin (Sigma No. 55-UV, 1971). The enzyme levels are reported in international units (IU) per liter of serum at 25 °C.

Homogenization of Livers. Liver samples were homogenized (1 g/3 ml of distilled water) in a glass tissue grinder (Potter-Elvehjem type) fitted with motor driven Teflon pestle.[26] The entire procedure was carried out at 0 °C. A 100-μl aliquot of homogenate was mixed with 1 ml of tissue solubilizer (Soluene-100, Packard Instrument Co., Downers Grove, Ill.) and 10 ml of liquid scintillation cocktail (Insta-Gel, Packard) for radioactivity measurements.

Isolation of Lipid Fraction. A 1-g sample of liver was used for the isolation of total lipid fraction according to the procedure of Folch et al.[30] An aliquot of lipid fraction was used for measuring radioactivity. The values used in constructing the radioactivity distribution profile in Figure 1 represent the means of data from six animals (SE <0.09 × 10^3 cpm).

Isolation of DNA Fraction. The procedure of Irving and Veazey[31] was employed for the isolation of the DNA fraction from rat livers. An aliquot of DNA fraction was counted for radioactivity.

Isolation of Proteins and Covalent Binding Studies. To a 0.5-ml aliquot of liver homogenate (above), 9.5 ml of 10% trichloroacetic acid (TCA) was added at room temperature (25 °C) to precipitate the proteins. An aliquot of protein precipitate was counted for radioactivity. The values used for plotting the radioactivity distribution curve (Figure 1) represent the means of data from six rats and the vertical bars represent the standard errors of the means.

The covalent binding of [^{14}C]halothane metabolites to proteins was determined by the general procedure described previously for other halogenated hydrocarbons[21,32] and lipophilic drugs.[26,27,33] The procedure essentially involves ten consecutive washings of the protein precipitate with a hot (60 °C) methanol–ether (3:1) mixture to remove noncovalently bound radioactivity from [^{14}C]halothane. The protein precipitate was then digested in 1 ml of 1 N NaOH and an aliquot was used for measuring the

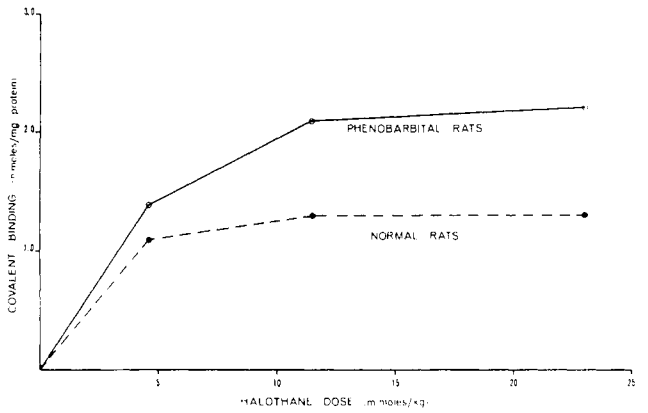

Figure 4. Relationship between [^{14}C]halothane dose and covalent binding of its metabolites to liver proteins from normal and phenobarbital-pretreated rats 24 h after dosing.

radioactivity. Proteins were estimated by the method of Lowry et al.[34] using bovine serum albumin as the standard. Covalent binding is expressed as nanomoles of halothane equivalents bound per milligram of liver protein. The values used for plotting graphs (Figures 2–4) represent the means of data from six rats (SE <0.03 nmol). Blank values, obtained by adding 0.5 μCi of [^{14}C]halothane to 0.5-ml aliquots of control rat (both normal and PBP) liver homogenates and processed as described above, were essentially negligible (0.002%) and the appropriate blank value was subtracted before calculating the covalent binding. Extraction with 8% phenol according to the procedure of Reid et al.[35] revealed that all the radioactivity in the TCA precipitate was bound to proteins rather than to any nucleic acid that might have coprecipitated with proteins.

Results and Discussion

In the present investigation, various anesthetic and nonanesthetic doses of [^{14}C]halothane were administered separately to normal and PBP rats by ip route (Table I) with the objective of evaluating the possible relationship between covalent binding of halothane metabolites to liver macromolecules and halothane-induced liver necrosis. The phenobarbital pretreatment of rats did not alter the duration of halothane anesthesia. No mortality was observed among the rats that received various doses of halothane in all the experiments outlined in Table I.

Histopathogical examination of liver tissues revealed that as reported earlier,[24,25] only PBP rats that received anesthetic doses of halothane (11.5 or 23.0 mmol/kg) and sacrificed 24 h after dosing exhibited multiple, discrete foci of liver parenchymal cell necrosis in the centrolobular region. On a scale of 0–4+, the extent of liver necrosis[29] in PBP rats at halothane doses 11.5 and 23.0 mmol/kg were 1+ and 2+, respectively. Similarly, two- to threefold increase in SGPT levels, which is an index of liver necrosis,[36] was observed in PBP rats that received the two anesthetic doses of halothane. These toxicological results are summarized in Table II.

Since phenobarbital pretreatment of rats had no effect on the duration of halothane anesthesia and liver necrosis was not observed in normal rats injected with identical anesthetic doses of halothane, any changes in cardiovascular dynamics produced by halothane anesthesia appear not to contribute significantly to the observed liver necrosis in PBP rats.

The distribution of radioactivity from administered [^{14}C]halothane in protein, lipid, and DNA fractions of liver was determined to evaluate which liver macromolecule was primarily associated with the uptake and retention of the radioactivity. The protein fraction contained the highest radioactivity at all time points in all animals, while the DNA fraction was devoid of radioactivity in all animals.

Table II. Extent of Liver Necrosis and Serum Glutamic-Pyruvic Transaminase (SGPT) Levels in Rats 24 h after Administration of Various Doses of Halothane

Rats	Halothane dose ip, mmol/kg	Extent of liver necrosis[a]	SGPT,[b] IU/l.
Normal	0	0	14 ± 2
	4.6	0	16 ± 2
	11.5[c]	0	18 ± 2
	23.0[c]	0	18 ± 2
Phenobarbital-pretreated	0	0	15 ± 2
	4.6	0	19 ± 3
	11.5[c]	1+	37 ± 5
	23.0[c]	2+	58 ± 7

[a] Based on a scale of 0–4+; see Experimental Section for details. [b] The results represent the means ± SE from six rats. [c] Anesthetic dose.

The lipid fraction showed some radioactivity during the initial 1–6-h period after dosing which disappeared after 6–8 h. In contrast, the liver proteins attained maximum radioactivity at about 12 h after dosing and maintained a substantial amount of the activity at 24 h in all animals treated with various doses of [^{14}C]halothane. Typical results are shown in Figure 1. A similar radioactivity distribution pattern was observed in normal and PBP rats.

Since the protein fraction contained the maximum radioactivity among the various liver macromolecules examined at all time points, it was studied further. Earlier studies showed that halothane binds covalently to liver microsomal proteins after undergoing metabolic activation by the drug-metabolizing cytochrome P-450 enzyme system.[7,20] In the present study, the covalent binding of radioactivity from [^{14}C]halothane to liver proteins was determined and the results are shown in Figures 2–4. These in vivo covalent binding values represent the binding of halothane metabolites since negligible binding of [^{14}C]halothane was observed when it was added to liver homogenates from control rats, both normal and PBP, that received olive oil only. In general, higher binding was observed with PBP rats which can be attributed to enhanced drug-metabolizing activity in the liver induced by phenobarbital.[26,27]

Both normal and PBP rats treated with a nonanesthetic dose of halothane (Figure 2) and normal rats treated with anesthetic doses (Figures 3 and 4) showed lower protein binding (0.8–1.7 nmol/mg of liver protein). These four groups of rats did not exhibit any definitive liver necrosis (Table I). In contrast, all the PBP rats treated with anesthetic doses of halothane consistently attained protein binding values higher than 2 nmol/mg of liver protein at all time points examined. Results obtained with the anesthetic dose of 23 mmol of halothane/kg are shown in Figure 3. A similar protein binding pattern was observed with the lower anesthetic dose of halothane (11.5 mmol/kg). All the PBP rats that exhibited definitive liver necrosis had covalent binding values of 2.13–2.20 nmol/mg of liver protein 24 h after the administration of anesthetic doses of [^{14}C]halothane, while considerably lower binding (1.12–1.41 nmol/mg liver protein) was observed in both normal and PBP rats that did not develop liver necrosis. The relationship between halothane dose and covalent binding of its metabolites to liver proteins is shown in Figure 4. The binding appeared to level off beyond the anesthetic dose of 11.5 mmol of halothane/kg in both normal and PBP rats.

These results appear to provide experimental support to the postulation that halothane is converted in vivo to reactive metabolites by the liver drug-metabolizing enzymes which then covalently bind to proteins with consequent development of liver necrosis. This mechanism

of liver toxicity is similar to that suggested for several other hepatotoxic halogenated hydrocarbons.[21-23] Studies are in progress to delineate the nature of chemically reactive metabolites of halothane.

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2-Cyano-1,3-dicarbonyl Compounds with Antiallergic Activity

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A number of 2-cyanoindan-1,3-diones and 3-cyano-4-hydroxycoumarins have been prepared and assessed for potential antiallergy activity as measured by their ability to inhibit passive cutaneous anaphylaxis in the rat, mediated by rat serum containing antigen specific IgE. The structural requirements for activity were similar not only for both series of compounds but also for the analogous 2-nitroindan-1,3-diones and 4-hydroxy-3-nitrocoumarins previously reported. The most active compounds were 2-cyano-5,6-diethylindan-1,3-dione (**4e**) and 3-cyano-6,7-diethyl-4-hydroxycoumarin (**11h**).

As part of a program relating to 2-nitro-1,3-dicarbonyl compounds, some of which were found to be capable of inhibiting rat passive cutaneous anaphylaxis (PCA),[1-4] we have studied the effects of replacing the nitro function in both the 2-nitroindan-1,3-diones **1** and the 4-hydroxy-3-nitrocoumarins **2**. The marked reduction or loss of activity observed on replacing the nitro group of **1** by such groups as hydrogen, alkyl, nitroso, acetyl, carboethoxy, halogen, phenyl, and sulfonic acid has been previously discussed,[5] and we have noticed a similar effect with such substitution in **2**.[6] It was of some interest, therefore, to find a high level of activity in the rat PCA test in both 2-cyanoindan-1,3-diones **4** and 3-cyano-4-hydroxycoumarins **11** (Belgian Patent 828690) in which the nitro moiety of **1** and **2**, respectively, had been replaced by nitrile. We now report our results obtained with these two series of compounds.

Chemistry. (a) 2-Cyanoindan-1,3-diones. Two routes for the synthesis of 2-cyanoindandiones **4** have been employed: the base-induced rearrangement of 3-cyanomethylenephthalides **3** (method A)[7] and the Claisen condensation of acetonitrile with phthalic esters **5** (method