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Inhalation Anesthetics and Cytochrome P-450-dependent Reactions in Rat Liver Microsomes

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The activities of liver microsomal enzymes were studied in preparations from unanesthetized rats and rats anesthetized for one hour with nitrous oxide, diethyl ether, halothane or chloroform. Most of the enzymes studied were cytochrome P-450-dependent oxygenases that hydroxylate endogenous substrates. The other microsomal enzymes, assayed for comparison, included the cytochrome P-450-dependent aminopyrine demethylase, glucose-6-phosphatase, a dehydrogenase, and NADPH-cytochrome P-450 reductase. No anesthetic was associated with a significant change in activity of any enzyme studied. In rats pretreated with phenobarbital no anesthetic except chloroform changed enzymic activity. All hydroxylations were inhibited markedly by chloroform, as were a microsomal dehydrogenation, hydrolysis of glucose-6-phosphate, and NADPH-cytochrome P-450 reductase activity. Administration of α-tocopherol did not prevent the inhibition associated with chloroform in phenobarbital-induced animals.

It is concluded that cytochrome P-450-dependent hydroxylations involved in metabolic processes normally proceeding in the endoplasmic reticulum of the liver are not permanently affected by the anesthetics used in this study. The inhibitory effect of chloroform after pretreatment with phenobarbital is unspecific and affects a large number of different microsomal enzymes. Evidence that mechanisms other than lipid peroxidation may be responsible for the toxic effects of chloroform in the liver is presented. (Key words: Metabolism, hepatic microsomal; Liver, microsomes; Metabolism, cholesterol; Anesthetics, volatile, diethyl ether; Anesthetics, volatile, halothane; Anesthetics, volatile, chloroform; Anesthetics, gases, nitrous oxide.)

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MOST INHALATION ANESTHETICS are metabolized by the microsomal drug-metabolizing enzyme system(s) in the liver.[1,2] This enzyme system contains cytochrome P-450 and NADPH-cytochrome P-450 reductase and requires NADPH and molecular oxygen. The system catalyzes hydroxylations of a large number of exogenous and endogenous, mainly lipid-soluble, compounds.[1,2] The metabolism of anesthetics in the microsomal fraction in some cases yields toxic metabolites. The formation of toxic metabolites has been suggested to explain the hepatotoxicity of chloroform and possibly halothane (*cf.* ref. 1). Prior administration of certain substrates increases the catalytic activity of the cytochrome P-450-dependent system. A potent inducing agent is phenobarbital. Variable but significant induction has also been shown for many inhalation anesthetics.[1,2]

There is at present no information concerning the effect of inhalation anesthetics on cytochrome P-450-catalyzed hydroxylations of endogenous substrates in the liver. The conversion of cholesterol into bile acids and the metabolism of bile acids involve a number of cytochrome P-450-dependent hydroxylations.[3] These reactions have been studied in considerable detail and are well suited to studies of the possible effects of inhalation anesthetics on metabolic processes in the microsomal fraction of liver. The present report describes the effect of short-term inhalation of several inhalation anesthetics in the rat on these cytochrome P-450-dependent hydroxylations. To provide a comparison with the drug-metabolizing enzyme system(s), the oxidative demethylation of aminopyrine was also studied. For comparison with cytochrome P-450-independent reactions a microsomal dehydrogenation and the hydrolysis of glucose-6-phosphate were

TABLE 1. Summary of Enzymes and Reactions Studied

Enzyme System	Catalyzed Reaction	Reference
Cholesterol 7α-hydroxylase	Cholesterol → 5-cholestene-3β,7α-diol	4–6
5β-Cholestane-3α,7α-diol 12α-hydroxylase	5β-Cholestane-3α,7α-diol → 5β-cholestane-3α,7α,12α-triol	7, 8
5β-Cholestane-3α,7α-diol 26-hydroxylase	5β-Cholestane-3α,7α-diol → 5β-cholestane-3α,7α,26-triol	9
Lithocholate 6β-hydroxylase	Lithocholate → 3α,6β-dihydroxy-5β-cholanoate	10, 11
Taurodeoxycholate 7α-hydroxylase	Taurodeoxycholate → taurocholate	11, 12
5-Cholestene-3β,7α-diol 3β-dehydrogenase	5-Cholestene-3β,7α-diol → 7α-hydroxy-4-cholesten-3-one	13
Aminopyrine demethylase	Oxidative N-demethylation of aminopyrine	14
Glucose-6-phosphatase	Glucose-6-phosphate → glucose	15
NADPH-cytochrome P-450 reductase	(Cytochrome P-450 complex)ox → (cytochrome P-450 complex)red	16

assayed. Table 1 summarizes the reactions studied.

Methods

MATERIALS

[4-^{14}C] Cholesterol (56 μCi/μmole, Radiochemical Centre, Amersham, England) was purified prior to use by chromatography on a column of aluminum oxide, grade III. The column was eluted with increasing concentrations of benzene in hexane. 5-[7β-^{3}H] Cholestene-3β,7α-diol (8 μCi/μmole) was prepared according to the method of Danielsson and Einarsson.[17] 5β-[7β-^{3}H] Cholestane-3α,7α-diol (8 μCi/μmole) was prepared as described by Björkhem and Gustafsson.[9] [24-^{14}C] Deoxycholic acid (0.3 μCi/μmole) was obtained from ICN (Irvine, Calif.), and tritium-labeled lithocholic acid (12 μCi/μmole) from NEN Chemicals (Dreieichenhain, Germany). Taurodeoxycholic acid was prepared from deoxycholic acid according to the method of Norman.[18] NAD, NADPH and α-tocopherol were supplied by Sigma Chemical Co., St. Louis, Mo.

ANIMAL EXPERIMENTS

Male Sprague-Dawley rats weighing about 150 g were used. They were fed a commercial pellet food (Anticimex, Stockholm, Sweden) and tap water *ad libitum*. Conditions were optimized with respect to diurnal variations.[19] In experiments with phenobarbital-pretreated animals, phenobarbital (100 mg/kg body weight) in 1 ml saline solution was administered daily for 5 days. The animals were killed 24 hours after the last injection. α-Tocopherol (70 mg/kg body weight) in 1 ml saline solution containing 16 per cent Tween 80 was given intraperitoneally 14 hours prior to sacrifice.

ANESTHETIC PROCEDURES

The rats were anesthetized for one hour in groups of four in 8-l desiccators containing sodium hydroxide. Gas mixtures were delivered via a conventional anesthesia machine (AGA, Stockholm, Sweden) equipped with a calibrated Sorsil vaporizer,[20] and passed through the desiccator at a flow rate of 2.5 l/min for one hour. The rats were kept at a depth of anesthesia corresponding to stage 3, plane 1, by appropriate adjustment of the vapor concentration according to the vital signs of the rats.

The following gas mixtures were used: air, oxygen, nitrous oxide/oxygen 3:1, diethyl ether/air, halothane/oxygen and chloroform/oxygen.

Preparation of Rat Liver Microsomes

Each animal was killed with a blow on the head and the liver was removed immediately. Liver homogenates, 20 per cent (w/v), were prepared in 0.25 M sucrose containing 0.001 M EDTA. The homogenate was centrifuged at 20.000 × *g* for 20 min. The microsomal fraction was obtained by centrifuging the 20.000 × *g* supernatant fluid at 100,000 × *g* for 1 hour. Microsomal suspension was prepared in 0.1 M potassium phosphate buffer, *p*H 7.0, containing 0.028 M nicotinamide. The volume of buffer added corresponded to the volume of the 20,000 × *g* supernatant fluid from which the microsomal fraction had been isolated.

Assays

Cholesterol 7α-Hydroxylase Activity. [4-^{14}C] Cholesterol, 0.03 μmole in 50 μl of acetone, 3 ml microsomal suspension, and 3 μmoles NADPH in 2 ml buffer were incubated for 15 min at 37 C. Incubation was terminated by the addition of 20 volumes of chloroform/methanol (2:1, v/v). After filtration, 0.2 volumes of saline solution were added. The chloroform phase was collected and the solvent evaporated. The residue was subjected to thin-layer chromatography with benzene/ethyl acetate (8:3, v/v) as solvent. Extent of 7α-hydroxylation was calculated from scans of the chromatoplates with a radio scanner (Berthold, Karlsruhe, Germany).

5-Cholestene-3β,7α-diol 3β-Dehydrogenase Activity. 5-[7β-^{3}H] Cholestene-3β,7α-diol, 0.1 μmole in 50 μl of acetone, 1 ml microsomal suspension, and 1.3 μmoles of NAD in 2 ml buffer were incubated for 20 min at 37 C. Incubation was terminated and analyzed as described above, but with benzene/ethyl acetate (1:1, v/v) as solvent in thin-layer chromatography.

5β-Cholestane-3α,7α-diol 12α-Hydroxylase and 5β-Cholestane-3α, 7α-diol 26-Hydroxylase Activities. 5β-[7β-^{3}H] Cholestane-3α,7α-diol, 0.6 μmole in 50 μl of acetone, 1.5 ml microsomal suspension, and 3 μmoles of NADPH in 1.5 ml buffer were incubated for 20 min at 37 C. Incubation was terminated and analyzed as described above with ethyl acetate as solvent in thin-layer chromatography. The ratio between the two products, 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α,26-triol, was determined by radio-gas chromatography with a Barber-Colman 5000 instrument equipped with a column of 3 per cent QF-1. Prior to radio-gas chromatography, the mixture was converted into the trimethylsilyl ether.

Lithocholate 6β-Hydroxylase Activity. Tritium-labeled lithocholic acid, 0.2 μmole in 50 μl of acetone, 2 ml microsomal suspension, and 3 μmoles of NADPH were diluted with buffer to a final volume of 3 ml. In experiments with phenobarbital-treated rats, only 0.5 ml microsomal suspension was used. Incubation was carried out for 20 min at 37 C and terminated by the addition of 5 ml of 95 per cent (v/v) aqueous ethanol. After dilution with water and acidification, the mixture was extracted twice with ether. The combined ether extracts were washed with water until neutral and the solvent was evaporated. The residue was subjected to thin-layer chromatography in solvent system S 11.[21] The amount of product was calculated from the chromatoplates as described above.

Taurodeoxycholate 7α-Hydroxylase Activity. [24-^{14}C] Taurodeoxycholic acid, 0.2 μmole in 0.1 ml buffer, 0.5 ml microsomal suspension, and 3 μmoles of NADPH in 2.5 ml buffer were incubated for 20 min at 37 C. Incubation was terminated by the addition of 5 ml of 95 per cent (v/v) aqueous ethanol. The mixture was hydrolyzed with 1 M potassium hydroxide for 12 hours at 110 C. The hydrolyzed mixture was diluted with water, acidified, and extracted twice with ether. The combined ether extract was analyzed as described for incubations with lithocholic acid.

Aminopyrine Demethylase Activity. Aminopyrine, 15 μmoles, and NADPH, 3 μmoles in 2.5 ml of buffer, were incubated with 0.5 ml microsomal suspension for 20 min at 37 C. Incubation was terminated by the addition of 0.38 ml of a 25 per cent (w/v) solution of zinc sulfate and 0.38 ml of a saturated solution of barium hydroxide. The amount of formaldehyde formed was measured according to the method of Nash.[22]

Glucose-6-phosphatase Activity. D-Glucose-6-phosphate, 8 μmoles in 0.75 ml

TABLE 2. Effects of Inhalation Anesthetics on Enzymic Activity in Rat Liver Microsomes (Means ± SEM)

Reaction	Air	Oxygen	Nitrous oxide	Diethyl ether	Halothane	Chloroform	Chloroform–Tocopherol*
	nmoles of Product/mg Microsomal Protein						
Cholesterol 7α-hydroxylase							
Control	0.45 ± 0.04	0.54 ± 0.03	0.48 ± 0.10	0.55 ± 0.03	0.55 ± 0.06	0.42 ± 0.07	—
Phenobarbital-treated	0.12 ± 0.03	0.09 ± 0.01	—	0.09 ± 0.01	0.12 ± 0.03	0.02 ± 0.01†	0.03 ± 0.01†
5-Cholestene-3β,7α-diol 3β-dehydrogenase							
Control	5.0 ± 0.6	5.2 ± 0.3	3.2 ± 0.5	5.7 ± 0.6	3.1 ± 0.4	—	—
Phenobarbital-treated	4.8 ± 0.3	4.1 ± 0.4	6.6 ± 0.9	4.2 ± 0.4	6.0 ± 1.0	1.1 ± 0.1†	1.3 ± 0.1†
5β-Cholestane-3α,7α-diol 12α-hydroxylase							
Control	1.7 ± 0.3	1.8 ± 0.2	1.1 ± 0.2	1.4 ± 0.3	1.4 ± 0.3	1.6 ± 0.1	—
Phenobarbital-treated	0.6 ± 0.2	0.4 ± 0.1	1.1 ± 0.3	0.4 ± 0.1	(1.3 ± 0.1†)	(0.3 ± 0.1††)	(0.5 ± 0.1††)
5β-Cholestane-3α,7α-diol 26-hydroxylase							
Control	1.6 ± 0.2	1.4 ± 0.3	1.4 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	1.7 ± 0.1	—
Phenobarbital-treated	1.1 ± 0.2	0.7 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	(1.3 ± 0.1†)	(0.3 ± 0.1††)	(0.5 ± 0.1††)
Lithocholate 6β-hydroxylase							
Control	5.6 ± 0.3	6.7 ± 0.5	4.0 ± 0.4	7.1 ± 0.7	3.6 ± 0.6	5.2 ± 0.4	—
Phenobarbital-treated	11.4 ± 0.5	10.4 ± 0.3	9.9 ± 0.8	11.1 ± 0.7	9.3 ± 0.5	2.2 ± 0.1†	2.4 ± 0.2†
Taurodeoxycholate 7α-hydroxylase							
Control	10.1 ± 1.5	8.1 ± 1.0	9.4 ± 2.1	7.8 ± 0.8	6.5 ± 1.2	10.0 ± 0.3	—
Phenobarbital-treated	33.2 ± 3.4	31.6 ± 3.7	33.6 ± 4.4	24.3 ± 1.8	42.2 ± 6.1	9.4 ± 1.3†	8.3 ± 0.5†
	nmoles of Formaldehyde/mg Microsomal Protein						
Aminopyrine demethylase							
Control	54 ± 5	51 ± 2	56 ± 3	53 ± 4	44 ± 4	49 ± 5	—
Phenobarbital-treated	172 ± 11	170 ± 6	142 ± 14	138 ± 4	153 ± 3	59 ± 4†	53 ± 2†
	nmoles of Phosphate/mg Protein of the 20,000 × g Supernatant Fluid						
Glucose-6-phosphatase							
Phenobarbital-treated	461 ± 17	—	—	—	—	385 ± 16†	219 ± 56†
	nmoles reduced Cytochrome c/min × mg Microsomal Protein						
NADPH–cytochrome P-450 reductase							
Phenobarbital-treated	51 ± 2	—	—	—	—	29 ± 3†	23 ± 2†

* Rats pretreated with α-tocopherol.
† $P < 0.01$ in comparison with rats kept in air.
‡ The values given include the total amounts of 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α,26-triol that were formed.

0.032 M potassium citrate solution, pH 6.5, was incubated with 0.25 ml 20,000 × g supernatant fluid for 30 min at 30 C. Incubation was terminated with 4 ml of 0.35 M perchloric acid. The amount of phosphate formed was measured according to the method of Gomori.[23]

NADPH-Cytochrome P-450 Reductase Activity. This activity was determined as described by Masters *et al.*[24]

Protein and Cholesterol Concentrations. Protein was determined according to the method of Lowry *et al.*[25] and cholesterol according to the method of Hanel and Dam.[26]

Results

Table 2 summarizes the effects of one hour of anesthesia with nitrous oxide, diethyl ether, halothane, or chloroform on hydroxylations in the biosynthesis and metabolism of bile acids in untreated and phenobarbital-treated rats. For comparison, the oxidative demethylation of aminopyrine, the oxidation of a Δ^5-3β-hydroxysteroid, and the hydrolysis of glucose-6-phosphate were also assayed. In rats not pretreated with phenobarbital, no significant change in any of the reactions studied was observed. In rats pretreated with phenobarbital, nitrous oxide, diethyl ether and halothane had no significant effect, whereas with chloroform marked inhibition of all reactions was observed. Treatment with α-tocopherol did not prevent the effect of chloroform.

Discussion

The microsomal fraction of liver contains an enzyme system that catalyzes the hydroxylation of a large number of lipid-soluble compounds. The enzyme system consists of an electron-carrier chain containing NADPH-cytochrome P-450 reductase and cytochrome P-450. It requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen. In addition to drugs, including many anesthetics, hydrocarbons, fatty acids, steroid hormones and bile acids are hydroxylated by this system.[1-3] The microsomal fraction of liver also contains enzyme systems with a similar electron-carrier chain and requirement of NADPH and oxygen, which are involved in the biosynthesis of bile acids.[3] The differences between these enzymes and the drug-metabolizing enzyme system consist of differing responses to treatment *in vivo* with phenobarbital, to biliary drainage, to treatment with cholestyramine, and to the presence of an isotope effect in the hydroxylation.[3,27] The participation of a cytochrome P-450 is well established, and it has been suggested that the specific properties of these enzyme systems are due to differences in cytochrome P-450.[3-9]

Since there is a common requirement for a number of factors in the metabolism of anesthetics, as well as in the formation and metabolism of bile acids, it is conceivable that anesthetics during anesthesia may inhibit a number of the cytochrome P-450-dependent reactions normally occurring in the liver. The present study does not answer the question whether such inhibition occurs *in vivo* during anesthesia, since much of the anesthetic is removed during preparation of the microsomal fraction. The activity of the enzyme systems catalyzing the hydroxylations in biosynthesis and metabolism of bile acids varies under normal conditions. For instance, the activity of the rate-limiting enzyme, cholesterol 7α-hydroxylase, shows large diurnal variations.[19] Thus, the inhibition, if any, of these enzyme systems during anesthesia for a few hours might be of minor biologic significance provided it is easily reversible at the end of anesthesia. The present results obtained in livers removed from rats immediately after acute exposure for one hour to anesthetic concentrations of nitrous oxide, diethyl ether, halothane, and chloroform show that a number of cytochrome P-450 hydroxylations involved in the formation and metabolism of bile acids were unaffected. The activity of NADPH-cytochrome P-450 reductase and the drug-metabolizing enzyme system measured by the oxidative N-demethylation of aminopyrine were also unchanged. It has been reported that anesthetics might alter the amount of cytochrome P-450 following long-term exposure.[28-31] There is no information concerning the effect of acute exposure. If acute exposure led to decrease of cytochrome

P-450, the activities of the cytochrome P-450-dependent enzyme systems assayed in this study probably would have been affected. In rats pretreated with phenobarbital, acute administration of carbon tetrachloride leads to a rapid breakdown of cytochrome P-450 within an hour.[32] Chloroform and possibly halothane, both of which are chemically closely related to carbon tetrachloride, have been reported to affect animals pretreated with phenobarbital differently from untreated animals.[29,30,33-35] In the present study, when the same hydroxylations were assayed in phenobarbital-treated rats exposed to the same inhalation anesthetics, no significant effects were observed except with chloroform. With chloroform marked inhibition of all reactions assayed was observed. Not only hydroxylations but also a microsomal dehydrogenation and the microsomal hydrolysis of glucose-6-phosphate were inhibited. This suggests that anesthesia with chloroform in rats pretreated with phenobarbital yields a general unspecific destruction of microsomal enzymes.

The hepatotoxicity of chloroform is well recognized, but the mechanisms are not fully understood. There is evidence that a major effect of chloroform is stimulation of endogenous lipid peroxidation.[36,37] The mechanism of this effect is probably formation of radicals from chloroform by the drug-metabolizing enzyme system. Since the activity of this system is stimulated by pretreatment with phenobarbital, the formation of chloroform radicals is greater in phenobarbital-treated than in untreated animals. Some of the results of the present studies appear relevant to the question of the mechanism by which chloroform exerts its toxic effect. Cholesterol is very sensitive to lipid peroxides. Under conditions of lipid peroxidation in microsomes, endogenous cholesterol is oxidized nonenzymatically and the enzymatic 7α-hydroxylation of cholesterol is inhibited.[5] In the present experiments, the 7α-hydroxylation of cholesterol was unaffected by chloroform in untreated rats and inhibited strongly in phenobarbital-treated rats. Even if the rate of lipid peroxide formation during exposure to chloroform is higher in phenobarbital-treated rats than in untreated rats, it appears unlikely that this is the entire explanation for the differing effects, since lipid peroxides have short half-lives and would probably have decomposed to a considerable extent during isolation of the microsomal fraction. These considerations indicate that the toxic effects of chloroform primarily occurred during the exposure. If these effects consisted of an increased production of lipid peroxides, one might expect an effect of α-tocopherol.[38] However, no protective effect was obtained by pretreatment with α-tocopherol. The conclusion that not only increased lipid peroxidation but also other mechanisms mediate chloroform toxicity has also been drawn by Brown, from a study in which the effects of halothane and chloroform on lipid peroxidation in phenobarbital-treated rats were compared.[36] Both anesthetics stimulated lipid peroxidation, to about the same extent, yet morphologic lesions of the liver were seen with chloroform only.

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