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Halothane-induced Lipid Peroxidation and Glucose-6-phosphatase Inactivation in Microsomes under Hypoxic Conditions

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Halothane-induced lipid peroxidation was studied in microsomes from phenobarbital-pretreated male rats at defined steady state oxygen partial pressures (P_{O_2}). At P_{O_2} less than 10 mmHg on addition of halothane to NADPH-reduced microsomes, significant increases in malondialdehyde (MDA) formation, oxygen uptake, and conjugated dienes were measured. At the maximum, near a P_{O_2} of 1 mmHg, halothane induced the formation of about 0.75 nmol MDA · mg microsomal protein^{-1} · min^{-1}; it also stimulated microsomal oxygen uptake twofold to threefold, and caused an almost threefold increase in conjugated diene absorption. Moreover, at this P_{O_2} microsomal glucose-6-phosphatase lost about 70% of its activity. At P_{O_2} greater than 10 mmHg, no significant effects of halothane on MDA formation, oxygen uptake, conjugated diene absorption, and glucose-6-phosphatase activity were observed; likewise under anaerobic conditions there was only a slight increase in conjugated dienes. The findings demonstrate that halothane induces microsomal lipid peroxidation at low P_{O_2} and in the presence of particular cytochrome P-450 isoenzymes, and that the halothane-induced lipid peroxidation leads to severe microsomal lesions, as indicated by the loss of glucose-6-phosphatase activity. (Key words: Anesthetics, volatile: halothane. Biotransformation (drug): fluorometabolites; microsomes. Hypoxia: hepatic. Liver: hepatotoxicity. Toxicity: hepatic.)

IT IS WELL RECOGNIZED that halothane-induced hepatocellular necrosis occurs when phenobarbital-pretreated male rats are anesthetized with halothane under hypoxic conditions.[1–3] According to this phenobarbital–hypoxia model of halothane hepatotoxicity, phenobarbital pretreatment is considered to be necessary to induce those particular isoenzymes of cytochrome P-450 metabolizing halothane, and hypoxia is thought to promote the reductive toxification of halothane to reactive metabolites, such as the $\cdot CF_3CHCl$ radical, rather than the oxidative detoxification to trifluoroacetic acid (cf, de Groot and Noll[4]).

Although the phenobarbital–hypoxia model provides strong evidence for the involvement of toxic-free radicals in halothane-induced liver cell necrosis, the way they do so is unknown. Since reductively formed halothane metabolites are known to bind covalently to microsomal proteins and lipids (cf, de Groot and Noll[4]), this covalent binding may contribute to liver cell damage. Indeed, experiments with rat liver microsomes under anaerobic conditions indicated that the observed suicidal inactivation of cytochrome P-450 may be the result of covalent binding of reactive halothane metabolites.[5,6]

On the other hand, it is known that interaction of free radicals with unsaturated fatty acids of membrane phospholipids may result in lipid peroxidation.[7–9] However, the formation of $\cdot CF_3CHCl$ radicals is inhibited strongly by oxygen, whereas lipid peroxidation necessarily depends on oxygen. Therefore, we speculated[4] that if halothane induces lipid peroxidation it should do so exclusively at a hypoxic oxygen partial pressure (P_{O_2}) that fulfills both requirements: 1) to be low enough to permit the reductive formation of $\cdot CF_3CHCl$ radicals but, 2) to be high enough to promote formation of lipid peroxides. Recently, we found preliminary evidence for this hypothesis.[10] In the present article we will give further support and demonstrate that the halothane-induced lipid peroxidation is accompanied by severe microsomal lesions, as indicated by an almost complete inactivation of microsomal glucose-6-phosphatase.

Methods

Male Wistar rats (160–200 g) fed on Altromin stock diet (Lage/Lippe, F. R. G.) were pretreated with phenobarbital (0.1% Na-phenobarbital, w/v, dissolved in drinking water) for 4 days. Microsomes were prepared as described previously.[11] The pellets were stored at 4° C and used within 1–4 h following preparation.

The incubation mixture contained $MgCl_2/KCl/$TRIS–HCl buffer (6 mM/104 mM/50 mM; *p*H 7.4), microsomes (1.5–3.0 mg microsomal protein · ml^{-1}), isocitrate (10 mM), isocitrate dehydrogenase (EC 1.1.1.42, 300 mU · ml^{-1}), and $NADP^+$ (1 mM). The NADPH regenerating system ensured a NADPH concentration of about 0.7 mM during the entire incubation period. Where indicated, halothane (final concentration 0.5 mM) was added as a solution of

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‡ Product information Fluothane®, ICI-Pharma, Plankstadt, F. R. G.

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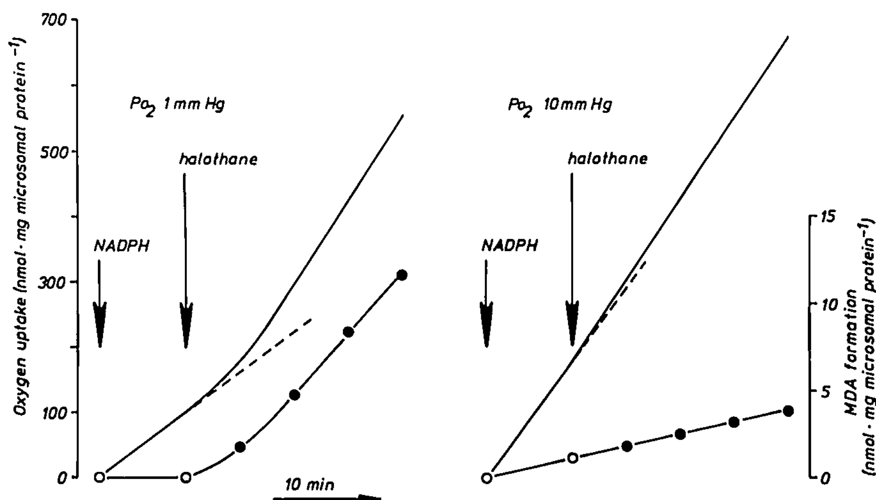

FIG. 1. Effects of halothane on oxygen uptake and MDA formation in NADPH-reduced rat liver microsomes at steady state P_{O_2} of 1 and 10 mmHg. The incubation system consisted of liver microsomes (1.5–3.0 mg microsomal protein·ml⁻¹) from pheno-barbital-pretreated male rats, $MgCl_2$/KCl/TRIS-HCl buffer (6 mM/104 mM/50 mM; *p*H 7.4), and NADPH (about 0.7 mM, regenerating system). Halothane (0.5 mM) was added where indicated. The incubations were performed at 37° C. Steady state P_{O_2} was maintained constant throughout the experimental period by the addition of oxygen-saturated buffer, using a feedback control system. (——) oxygen uptake; (● —— ●) MDA formation.

halothane-saturated (22.8 mM at 20° C‡) buffer. The incubations were performed at 37° C in a closed, thermostated incubation vessel equipped with inlet, outlet, and a Clark-type oxygen sensor (Eschweiler, Kiel, F. R. G.). A given P_{O_2} was maintained constant throughout the experimental period by addition of O_2-saturated buffer (1.2 mM oxygen at 22° C[12]) using a feedback control system comprising the oxygen sensor, an automatic control unit, and a motor-driven piston burette (Radiometer, Copenhagen, Denmark). The automatic control unit was allowed to increase the P_{O_2} setpoint continuously, a characteristic used to compensate the drift of the oxygen sensor, which is caused by halothane.

Glucose-6-phosphatase activity was measured under the conditions described by Nordlie and Arion.[13] P_i liberated was determined with an enzymatic method using inosine-5′-diphosphate (0.4 mM) and the enzymes, nucleoside phosphorylase (EC 2.4.2.1), and xanthine oxidase (EC 1.2.3.2). Uric acid formed by these coupled enzyme reactions was measured at 302 nm. Protein was determined with the method of Lowry *et al.*[14] using bovine serum albumin as a standard and malondialdehyde (MDA) as described by Reiner *et al.*,[15] using 1,1,3,3-tetramethoxypropane as a standard.

Diene conjugation was assayed as follows: 1.0 ml microsomal suspension was mixed thoroughly with 5.0 ml chloroform/methanol (2:1, v/v). After centrifugation, 1.0 ml of the lower phase was taken to dryness under a stream of nitrogen. The lipid residue was dissolved in 1.0 ml chloroform/methanol (2:1, v/v),

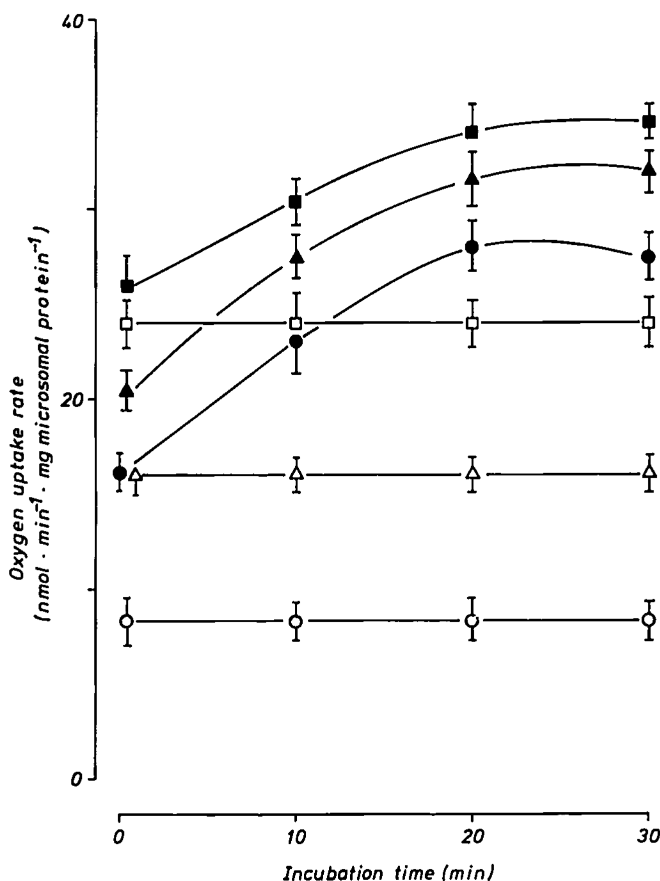

FIG. 2. Rates of microsomal oxygen uptake in presence of NADPH (open symbols) and NADPH/halothane (filled symbols) at steady state P_{O_2} of 1 (○, ●), 5 (△, ▲), and 10 mmHg (□, ■). Vertical bars denote SE of the mean for at least three separate incubations. Further experimental details are as in figure 1.

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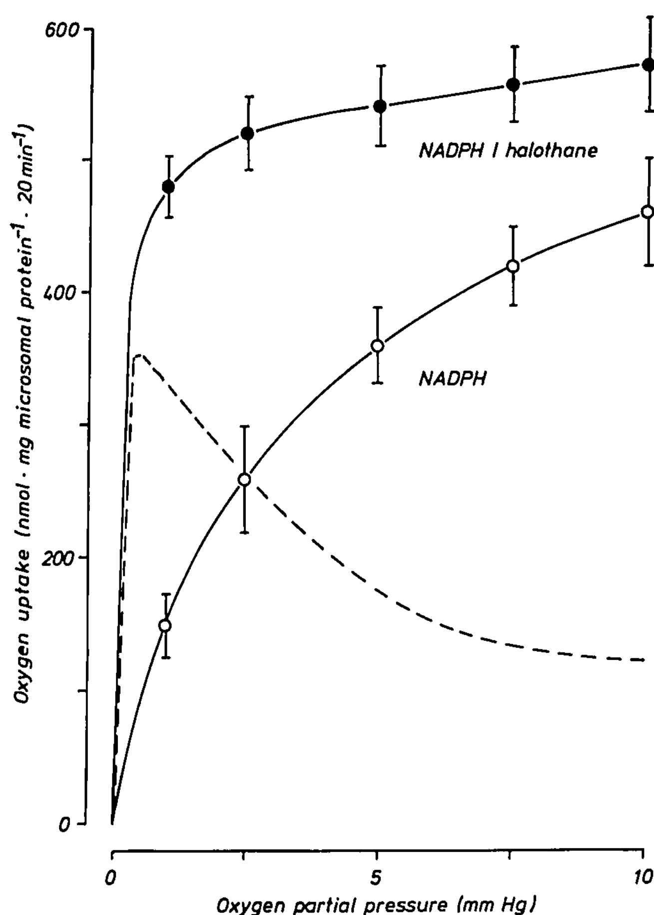

FIG. 3. Effect of increasing P_{O_2} on the microsomal oxygen uptake in presence of NADPH and NADPH/halothane. The amounts of oxygen taken up during 20 min of incubation at various P_{O_2} are given. The dashed line denotes the difference between NADPH/halothane and NADPH-mediated oxygen uptake. Further experimental details are as in figures 1 and 2.

and the absorbance of the diene conjugation peak at approximately 238 nm was determined against a chloroform/methanol (2 : 1, v/v) blank.

The Student's t test was used to determine statistical significance. Differences were designated as significant when $P \leq 0.05$.

Results

In figure 1, typical recordings of microsomal oxygen uptake at P_{O_2} of 1 and 10 mmHg are depicted. At a P_{O_2} of 1 mmHg addition of halothane to NADPH-reduced microsomes markedly stimulated microsomal oxygen uptake, while at a P_{O_2} of 10 mmHg, only a slight stimulating effect of halothane was observed. For comparison, the related malondialdehyde (MDA) measurements are included. In line with previous results,[10] a significant stimulating effect of halothane on MDA formation occurred at a P_{O_2} of 1 mmHg but not at a P_{O_2} of 10 mmHg.

In the presence of NADPH alone, rates of microsomal oxygen uptake increased with increasing P_{O_2} but remained constant at a given P_{O_2} during the entire incubation period (fig. 2). In presence of both NADPH and halothane microsomal oxygen uptake rates increased with increasing incubation time, reaching a plateau after about 20 min of incubation (fig. 2). At that time, microsomal oxygen uptake rates were stimulated significantly by the additional presence of halothane at all P_{O_2} studied, whereas the initial rates of microsomal oxygen uptake were stimulated significantly only at P_{O_2} of 1 and 5 mmHg but not at a P_{O_2} of 10 mmHg. Without NADPH, even in the presence of halothane, neither oxygen uptake nor MDA formation was observed (data not shown).

The oxygen dependence of the stimulating effect of halothane on microsomal oxygen uptake is shown more clearly in figure 3 where the amounts of oxygen taken up following 20 min of incubation are plotted against the chosen steady state P_{O_2}. Subtracting the oxygen uptake in the presence of NADPH alone from that in the presence of NADPH/halothane revealed that the particular effect of halothane on oxygen uptake was maximal near a P_{O_2} of 1 mmHg and that it significantly decreased with increasing P_{O_2}.

Marked increases in conjugated diene absorption were found after incubation in the presence of NADPH/halothane at a P_{O_2} of 5 mmHg and even more so at a P_{O_2} of 1 mmHg (table 1), while only small increases in conjugated diene absorption were observed after incubation at a P_{O_2} of 10 mmHg and under anaerobic conditions. The latter is in accordance with findings of Wood *et al.*,[16] who also reported a halo-

TABLE 1. Conjugated Diene Absorption in Microsomal Lipids after 20 Min of Incubation of NADPH-reduced Microsomes with Halothane at Various P_{O_2}

P_{O_2} (mmHg)	Conjugated Dienes	
	$A_{238} \cdot$ mg Microsomal Protein^{-1}	Per Cent of Control
Anaerobic (control)	0.18 ± 0.01	100
Anaerobic	0.24 ± 0.02	135
1	0.65 ± 0.10	362
5	0.50 ± 0.08	280
10	0.30 ± 0.06	168

The control value was obtained under anaerobic conditions in the absence of halothane. SE of the mean for at least four separate incubations are given. Experimental details are as in figure 1.

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thane-induced increase in conjugated dienes under anaerobic conditions. Control incubations in the presence of NADPH alone showed no significant effects on conjugated diene absorption at the P_{O_2} studied (data not shown).

Microsomal glucose-6-phosphatase activity remained almost unaffected during incubation in the sole presence of NADPH at P_{O_2} up to 10 mmHg (fig. 4). On incubation in the presence of both NADPH and halothane under anaerobic conditions and at a P_{O_2} of 10 mmHg, no significant alterations in glucose-6-phosphatase activity were observed as well. However, on incubation in the presence of NADPH/halothane at a P_{O_2} of 1 mmHg following a lag phase of about 10 min, a pronounced decrease in glucose-6-phosphatase activity occurred (fig. 4).

Discussion

Recently, we reported the formation of MDA on incubation of NADPH-reduced microsomes with halothane at a P_{O_2} between 0.5 and 10 mmHg[10] (also see fig. 1). In line with those results, microsomal oxygen uptake and conjugated diene absorption also reflect significant lipid peroxidation following addition of halothane to NADPH-reduced microsomes at low P_{O_2} (figs. 1–3, table 1). Like the formation of MDA, maximal increases of halothane-induced oxygen uptake and conjugated diene formation occurred at a P_{O_2} of 1 mmHg.

The positive results with all three indicators of lipid peroxidation used here leave little doubt that halothane does induce microsomal lipid peroxidation at a low P_{O_2} and in the presence of halothane-metabolizing cytochrome P-450 isoenzymes, as postulated previously.[4] To evaluate the significance of halothane-induced lipid peroxidation for microsomal damage, the effect on microsomal glucose-6-phosphatase activity was studied and revealed a close relationship between glucose-6-phosphatase inactivation and microsomal lipid peroxidation (fig. 4). The decline of glucose-6-phosphatase activity was maximal at that P_{O_2} where halothane-induced lipid peroxidation proceeded at maximal rate. No loss of glucose-6-phosphatase activity was observed under anaerobic conditions, excluding a direct damaging effect of reductively formed halothane metabolites on microsomal glucose-6-phosphatase. The almost complete inactivation of glucose-6-phosphatase mediated by halothane-induced lipid peroxidation indicates a severe microsomal lesion.

At normoxia the P_{O_2} in the liver varies between 1–56 mmHg, with its lowest values centrolobular.[17]

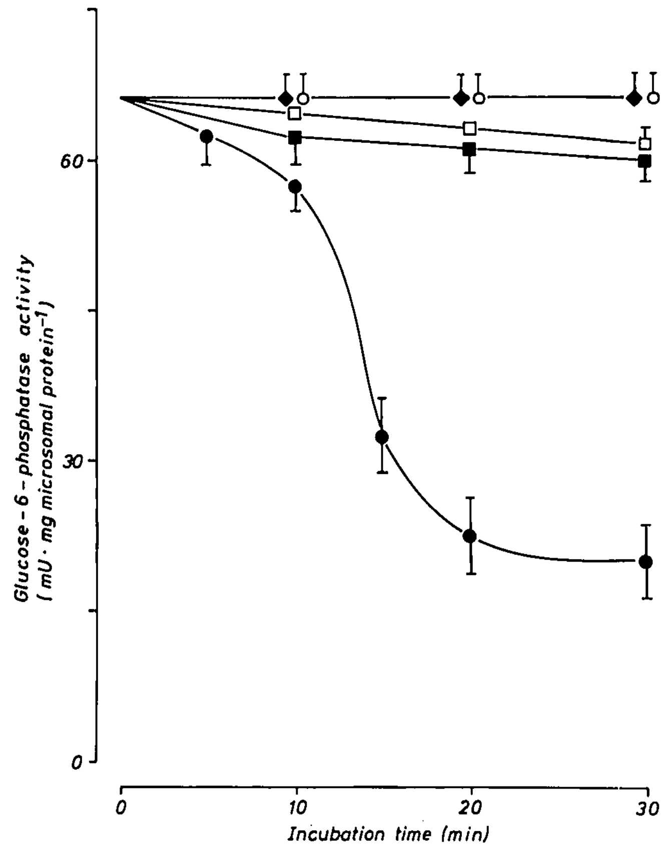

FIG. 4. Microsomal glucose-6-phosphatase activity on incubation of microsomes in presence of NADPH (open symbols) and NADPH/halothane (closed symbols) at steady state P_{O_2} of 1 (○, ●) and 10 mmHg (□, ■) and under anaerobic conditions (◆). Vertical bars denote SE of the mean for at least four separate incubations. Further experimental details are as in figure 1.

Therefore, even under normoxic conditions in a small pericentral region of the liver lobule a P_{O_2} should exist critical for halothane-induced lipid peroxidation. Even slight hypoxia considerably should increase this area. Thus, our assumption of halothane-induced lipid peroxidation as the underlying mechanism of halothane hepatotoxicity is in good agreement with the distribution of P_{O_2} in the liver and the effect of hypoxia in the phenobarbital–hypoxia model of halothane hepatotoxicity.[1-3]

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