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THE CRUCIAL ROLE OF LOW STEADY STATE OXYGEN PARTIAL PRESSURES IN HALOALKANE FREE-RADICAL-MEDIATED LIPID PEROXIDATION

POSSIBLE IMPLICATIONS IN HALOALKANE LIVER INJURY

HERBERT DE GROOT and THOMAS NOLL

Institut für Physiologische Chemie I der Universität Düsseldorf, Moorenstrasse 5, D-4000 Düsseldorf, Federal Republic of Germany

It is well established that the hepatotoxicity of the completely halogenated alkanes CCl_4 and $CBrCl_3$ is closely linked to their reductive activation to free-radicals catalyzed by cytochrome P-450, the terminal oxidase of the hepatic microsomal mixed function oxidase system. Damage to cellular components may result either from a direct attack of the haloalkane free-radicals or from lipid peroxidation initiated by interaction of the free-radicals with unsaturated fatty acids of membrane phospholipids (see [1–4] for recent reviews). The inactivation of cytochrome P-450 itself, possibly due to covalent binding of haloalkane radicals to the haem moiety of the cytochrome, is an example of direct damage [5–8] while the inactivation of the microsomal enzymes glucose-6-phosphatase and UDPglucuronyl-transferase, as well as the loss of latent activity of microsomal nucleoside diphosphatase [9–13] exemplify damage arising from lipid peroxidation.

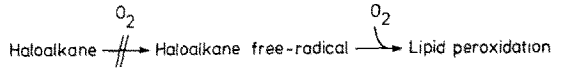
The completely halogenated compounds CCl_4 and $CBrCl_3$ are metabolized by cytochrome P-450 solely by reduction. In contrast, those haloalkanes containing a hydrogen atom in their molecule are also oxidatively metabolized by cytochrome P-450 by hydroxylation of the carbon–hydrogen bond. From haloforms such as $CHBr_3$ and $CHCl_3$ the respective dihalocarbonyl compounds are formed by elimination of a proton and a halide ion, and it is assumed that their formation is responsible for the hepatotoxicity of these halomethanes [14–18]. In the case of the anaesthetic $CF_3CHBrCl$ (halothane) the oxidative degradation to the stable end product trifluoroacetic acid [19, 20] is generally believed to be part of its detoxication pathway [2, 21]; even though there is evidence that it may also be involved in an immunological mechanism of halothane liver injury [22].

The subject of the present paper is the haloalkane free-radical-mediated lipid peroxidation and, in particular, the crucial role of low steady state O_2 partial pressures (P_{O_2}) in this potent destructive process. First, the theoretical background is given which led us to postulate that low steady state P_{O_2} are decisive for haloalkane free-radical-mediated lipid peroxidation. Subsequently, the experimental set-up is described which was utilized to validate this postulate and experiments with liver microsomes and isolated liver cells are shown which demonstrate that the postulate is generally applicable to polyhalogenated alkanes. Finally, possible implications in haloalkane liver injury are discussed.

DUAL ROLE OF MOLECULAR OXYGEN IN HALOALKANE FREE-RADICAL-MEDIATED LIPID PEROXIDATION

The reductive activation of haloalkanes to free radicals is catalyzed preferentially by those isoenzymes of cytochrome P-450 which are induced by pretreatment of experimental animals with phenobarbital [6, 23, 24]. The formation of the haloalkane free-radicals has been demonstrated by spin trapping [25–27] and, indirectly, by identification of products derivable from the respective free-radical intermediate, e.g. hexachloroethane [28] and chloroform [29–32] in the case of CCl_4-activation. As pointed out by V. Ullrich and his colleagues [32–34] the haloalkane first interacts with the active site of cytochrome P-450. Following one-electron reduction and elimination of a halide ion, a haloalkane radical ferric cytochrome P-450 complex is formed from which the radical either is released to yield the haloalkane free-radical or undergoes further one-electron reduction. The physiological electron donator for the first one-electron reduction is NADPH and the electron is transferred via NADPH cytochrome P-450 reductase to the cytochrome. The reductive cytochrome P-450-mediated activation of haloalkanes is somewhat analogous to the mechanism of O_2 activation by cytochrome P-450-dependent monooxygenation [35]. For example, the radical ferric cytochrome P-450 complex is formally similar to the oxycomplex involved in oxidative metabolism. Further, the electron transfer to a carbon–hydrogen bond requires the direct interaction of the haloalkanes with the reduced haem iron of the cytochrome. Thus, the reductive activation of haloalkanes should be inhibited by O_2 and therefore proceed preferentially under anaerobic conditions.

Once released from the ferric cytochrome P-450 complex the haloalkane free-radical as such or its O_2 adduct, the respective haloalkane peroxy radical [36], can react with cytochrome P-450 itself to inactivate the cytochrome (see above) or with components in the close vicinity of the membrane-bound enzyme. Reaction with polyunsaturated fatty acids of membrane phospholipids yields fatty acid radicals



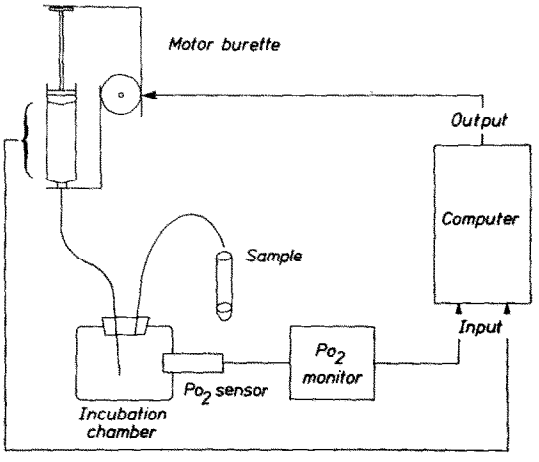
Scheme 1. The dual role of oxygen in haloalkane free-radical-mediated lipid peroxidation.

and in the presence of O_2 the process of lipid peroxidation is initiated [1–4, 37–40]. By addition of O_2 to the fatty acid radicals lipid peroxy radicals are formed which following rearrangements decompose via several pathways to a number of reactive products, some of which serve to propagate the free-radical chain reaction sequence. Measurable parameters of lipid peroxidation include O_2 uptake, formation of conjugated dienes, and decay products such as malondialdehyde, 4-hydroxynonenal, and hydrocarbons.

It is evident that the second part of the free-radical-mediated lipid peroxidation, namely the propagation reactions, depends on the presence of O_2. Thus, while the initial step, the formation of haloalkane free-radicals, is inhibited by O_2, the rate of the subsequent steps should increase with increasing P_{O_2}. Because of this dual role of O_2 (Scheme 1) it was tempting to speculate that haloalkane free-radical-mediated lipid peroxidation should proceed with preference at a P_{O_2} which accounts for both parts of the overall process: It must be low enough to allow the formation of haloalkane free-radicals but high enough to insure lipid peroxidation to proceed at sufficient rate [2].

THE OXYSTAT SYSTEM

To get experimental access to the haloalkane free-radical-mediated lipid peroxidation and the concomitant O_2 flux under steady state O_2 conditions in NADPH-reduced microsomes and isolated liver cells an incubation system has to meet the requirements to maintain a given P_{O_2} constant during a prolonged incubation period while allowing the determination of the O_2 uptake of the respiring particles continuously. To satisfy these requirements we developed a special incubation technique, the oxystat system, which makes use of the principle that the



Scheme 2. The oxystat system.

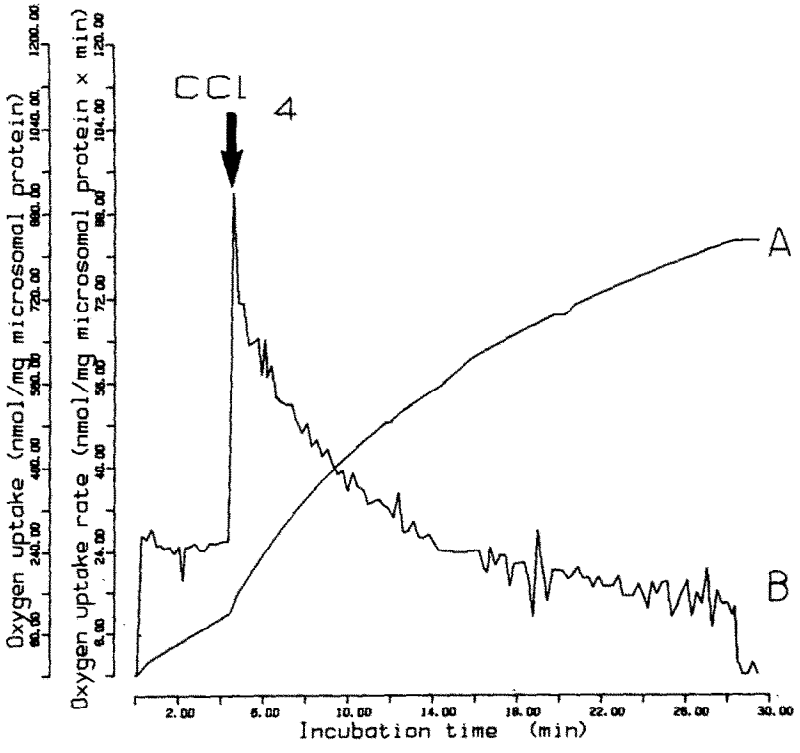


Fig. 1. Effect of CCl_4 on O_2 uptake of NADPH-reduced liver microsomes. Computer-generated plot of a typical experiment performed at 37° and at a steady state P_{O_2} of 10 mmHg using the oxystat system (Scheme 1). The incubation mixture consisted of liver microsomes (2.5 mg microsomal protein/ml at start) from phenobarbital-pretreated male rats, 6 mM-$MgCl_2$/104 mM-KCl/50 mM-Tris/HCl buffer, pH 7.4, and 0.3 mM-NADPH (regenerating system). CCl_4 (0.5 mM) was added as indicated. A, O_2 uptake; B, O_2 uptake rate.

O_2 supply in suspensions of respiring particles is maintained by injecting O_2 dissolved in aqueous medium. The oxystat system (Scheme 2) employs (i) a polarographic O_2 sensor ($P_{O_2} \geq 0.2$ mm Hg) or bacterial luminescence ($(P_{O_2} < 0.2$ mm Hg) [5, 41] for continuous P_{O_2}-monitoring, (ii) a motor burette for the injection of O_2-saturated medium, and (iii) a computer for process control. The latter reads the actual P_{O_2} in the incubation chamber, compares this value with a preselected P_{O_2} and activates the motor burette until the set point is approached. Further, the computer accounts for the dilution of the respiring particles and calculates the O_2 uptake from the amounts of O_2-saturated medium added.

EFFECT OF P_{O_2} ON HALOALKANE FREE-RADICAL-MEDIATED LIPID PEROXIDATION IN LIVER MICROSOMES AND ISOLATED HEPATOCYTES

Microsomes

In NADPH-reduced rat liver microsomes optimum P_{O_2} for the induction of lipid peroxidation by haloalkanes were between 1 and 20 mmHg as indicated by increases in oxygen uptake, conjugated diene absorption and formation of malondialdehyde [8, 12, 42, 43].

In Fig. 1 the computer-generated plot of microsomal O_2 uptake of a typical experiment with CCl_4 at a steady state P_{O_2} of 10 mmHg is depicted. Following addition of CCl_4 to the NADPH-reduced microsomes an immediate and dramatic rise in the O_2 uptake rate occurred. The O_2 uptake rate declined in the further incubation period, presumably as a consequence of the progressive inactivation of cytochrome P-450 [8]. CCl_4-induced microsomal lipid peroxidation proceeded at maximal rate at P_{O_2} of 1 to 10 mmHg [8] (see also Fig. 2). At P_{O_2} below and above these values there was a sharp decrease in the rate of CCl_4-mediated lipid peroxidation. At a P_{O_2} of 70 mmHg only a slight stimulation of lipid peroxidation by CCl_4 was observed.

A similar P_{O_2} dependence as described for CCl_4 was observed for $CBrCl_3$-induced lipid peroxidation in NADPH-reduced liver microsomes with the exception that the optimum P_{O_2} shifted to somewhat higher values (unpublished results).

Those haloalkanes containing a hydrogen atom in their molecule such as $CHBr_3$ and $CF_3CHBrCl$

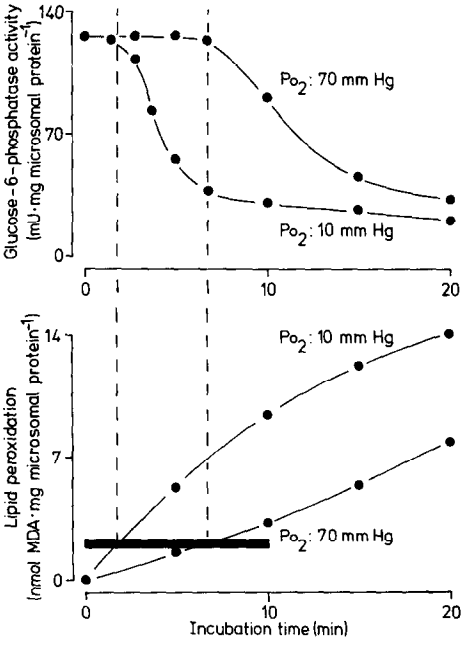


Fig. 2. Time courses of glucose-6-phosphatase activity and lipid peroxidation in NADPH-reduced microsomes supplemented with CCl_4 at steady state P_{O_2} of 10 and 70 mmHg. Glucose-6-phosphatase activity was determined as previously described [54]. Lipid peroxidation was estimated by the amounts of malondialdehyde (MDA) formed [55]. The bar indicates the threshold value of lipid peroxidation where the loss of glucose-6-phosphatase activity starts. Further experimental details are as indicated in the legend to Fig. 1.

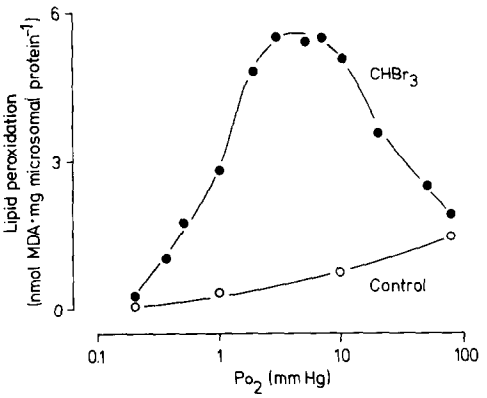


Fig. 3. $CHBr_3$-induced lipid peroxidation of NADPH-reduced liver microsomes as a function of P_{O_2}. The incubation time was 20 min and the $CHBr_3$ concentration 0.5 mM. The control experiments were performed in the presence of NADPH but absence of the haloalkane. Further experimental details are as indicated in the legends to Figs. 1 and 2.

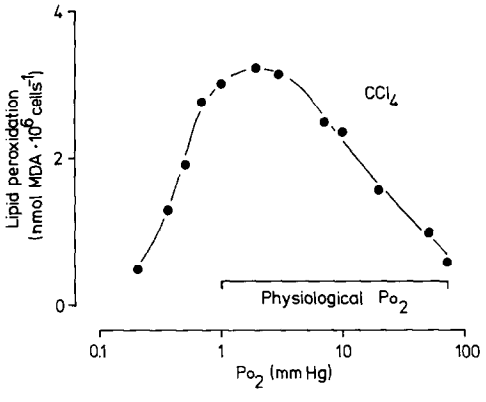
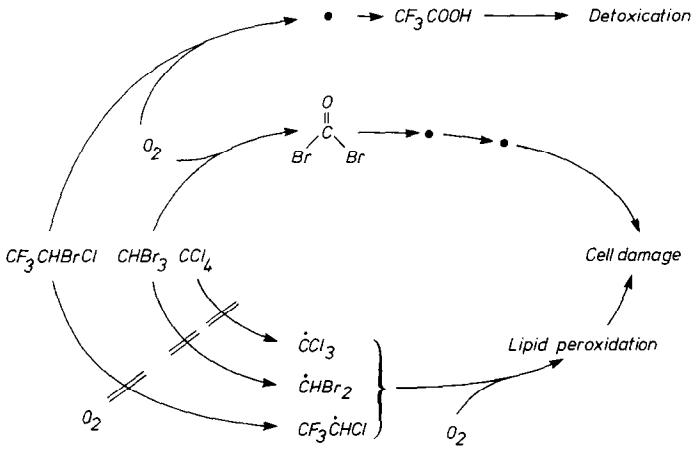


Fig. 4. CCl_4-induced lipid peroxidation in isolated rat hepatocytes. Hepatocytes ($3 \cdot 10^6$ cells/m at start) isolated from phenobarbital-pretreated male rats were incubated in Krebs–Henseleit saline supplemented with 10 mM-glucose, 2.1 mM-lactate, 0.3 mM-pyruvate, and 0.5 mM CCl_4 for 20 min. Further experimental details are as indicated in the legends to Figs 1 and 2. In the absence of CCl_4 no significant formation of malondialdehyde was detected. For comparison, the range of physiological P_{O_2} in liver [50] is indicated.



Scheme 3. Promotive and inhibitory effects of oxygen on haloalkane metabolism and haloalkane liver injury.

(halothane) stimulated lipid peroxidation to a significant extent exclusively at P_{O_2} below 40 mmHg. This is exemplified for $CHBr_3$ in Fig. 3. In this example the optimum P_{O_2} for the induction of lipid peroxidation was about 5 mmHg. The optimum P_{O_2} for $CF_3CHBrCl$-induced lipid peroxidation was even lower. Maximal stimulation of lipid peroxidation occurred at P_{O_2} around 2 mmHg [12, 42]. At P_{O_2} above 10 mmHg$CF_3CHBrCl$ was without effect on microsomal lipid peroxidation.

The potency of a series of halomethanes to induce lipid peroxidation in NADPH-reduced liver microsomes at a given low steady state P_{O_2} followed the order $CHBr_3 > CHBr_2Cl > CHBrCl_2 > CHCl_3$ revealing a close correlation to the relative rates of their reductive metabolism [43].

The marked increases of haloalkane free-radical-mediated microsomal lipid peroxidation by decreasing the P_{O_2} to values between 1 and 40 mmHg were accompanied by correspondingly marked increases of peroxidatively mediated microsomal damages. As an example in Fig. 2 the inactivation of microsomal glucose-6-phosphatase by CCl_4-induced lipid peroxidation is shown. The threshold value of microsomal peroxidation of about 2 nmol malondialdehyde/mg microsomal protein where glucose-6-phosphatase started to loose its activity was passed at a markedly earlier point of time at a P_{O_2} of 10 mmHg than it was at a P_{O_2} of 70 mmHg. Interestingly, other microsomal damages evoked by lipid peroxidation are also characterized by distinct threshold values. Examples are loss of the latent activity of nucleoside diphosphatase with a threshold value of about 12 nmol malondialdehyde/mg microsomal protein [13] and expression of morphologic alterations of the microsomal vesicles with a threshold value of about 25 nmol malondialdehyde/mg microsomal protein [44]. It is evident, that to reach the latter high values by haloalkane free-radical-mediated lipid peroxidation optimum low steady state P_{O_2} are a basic requirement.

Isolated hepatocytes

A similar dependence for P_{O_2} of haloalkane free-radical-mediated lipid peroxidation as described for microsomes were also found in isolated hepatocytes. For example, in Fig. 4 CCl_4-induced lipid peroxidation in isolated hepatocytes is plotted as a function of P_{O_2}. Maximum lipid peroxidation was observed at P_{O_2} between 0.5 and 10 mmHg while almost no lipid peroxidation was induced at P_{O_2} above 70 mmHg.

POSSIBLE IMPLICATIONS IN HALOALKANE LIVER INJURY

The experiments with liver microsomes and isolated hepatocytes indicate that low steady state P_{O_2} are crucial for haloalkane free-radical-mediated lipid peroxidation in liver. Because of the antagonistic behaviour of O_2 in this process (Schemes 1 and 3) low steady state P_{O_2} dramatically augmented the ability of the completely halogenated alkanes CCl_4 and $CBrCl_3$ to induce lipid peroxidation and in the cases of those haloalkanes containing a hydrogen atom in their molecule exclusively low steady state P_{O_2} permitted the induction of lipid peroxidation.

Characteristic of haloalkane liver injury is the centrolobular necrosis [45–48]. Within the liver lobule those isoenzymes of cytochrome P-450 inducible by phenobarbital are predominantly located in the centrolobular area [49]. From *in situ* determinations it is established that P_{O_2} in liver normally varies between 1–60 mmHg with its lowest values around the central vein of the lobule [50]. Thus, there is a striking accord between the site of liver injury, the site of haloalkane activation, the P_{O_2} distribution in liver, and the optimum P_{O_2} for haloalkane free-radical-mediated lipid peroxidation (see also Fig. 4).

The P_{O_2} distribution in liver together with the P_{O_2} dependence of haloalkane-induced lipid peroxidation may also explain why hypoxia increases the hepatotoxicity of CCl_4 [46, 47]; hypoxia enlarges the portion of the liver with P_{O_2} optimal for CCl_4-induced lipid peroxidation (Scheme 3). However, other factors such as an increased covalent binding of CCl_4-metabolites to cellular macromolecules may also contribute to the promoting effect of hypoxia.

The hepatotoxicity of haloforms such as $CHBr_3$ has been attributed to their oxidative metabolism to dihalocarbonyl compounds [14–18] (Scheme 3). However, the present results suggest that at least in the centrolobular regions haloalkane free-radical-mediated lipid peroxidation makes a significant contribution to haloform liver injury. This applies especially to those haloforms containing bromine or iodine atoms in their molecule.

The P_{O_2} required for halothane-induced lipid peroxidation (Scheme 3) is only found at the very low part of the physiological P_{O_2} scale of the liver. This may be one reason why liver injury is rare following halothane anaesthesia [2]. It can also account for the fact that halothane liver injury occurs in phenobarbital-pretreated experimental animals only when the O_2 content of the inspired air is decreased to 14% [51–53].

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