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THE DECISIVE pO_2-LEVELS IN HALOALKANE-MEDIATED LIVER CELL INJURY

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The model hepatotoxine carbon tetrachloride (CCl_4) was used to study haloalkane free radical-induced lipid peroxidation in isolated rat hepatocytes at steady state oxygen partial pressures (pO_2) between 0.2 and 100 mmHg. Equilibrium oxygen conditions were achieved by using an oxystat system.

Monitoring of hepatocellular oxygen uptake, malondialdehyde-formation and low-level chemiluminescence during incubations of CCl_4-supplemented hepatocytes indicated a drastic stimulation of lipid peroxidation at pO_2-levels between 1 and 10 mmHg. Above and below this pO_2-region the potency of CCl_4 to induce lipid peroxidation sharply decreased. The evaluation of cellular damages by determining trypan blue exclusion and lactate dehydrogenase leakage revealed that in the presence of CCl_4 hepatocellular injury was significantly increased at those pO_2-levels which were optimal for CCl_4-mediated lipid peroxidation.

The present results demonstrate that CCl_4 is a potent inducer of lipid peroxidation also in the intact hepatocyte, provided that the pO_2 is maintained at distinct low levels. The coincidence of lipid peroxidation and loss of cell viability at the same pO_2-range provides further evidence for the assumption that the haloalkane-mediated liver cell injury is due to a peroxidative process which primarily occurs at the hypoxic end of the physiological pO_2-levels (1–70 mmHg) in liver.

KEY WORDS: Carbon tetrachloride, liver cell injury, hypoxia, lipid peroxidation, low-level chemiluminescence.

INTRODUCTION

The crucial role of low steady state oxygen partial pressures (pO_2) in haloalkane-mediated lipid peroxidation and in the accompanying events, including loss of membrane barrier functions and inactivation of membrane-linked enzyme activities, has been established in the microsomal fraction[1] using an especially designed incubation system, the oxystat.[2] These observations led us to postulate a mechanism of haloalkane-mediated lipid peroxidation in which molecular oxygen plays contradictory roles:[1,3] on the one hand, it inhibits the reductive activation of haloalkanes such as CCl_4 to reactive free radicals at the haem moiety of cytochrome P-450 and, on the other hand, it is the key reagent in the propagation steps of the peroxidative breakdown of polyunsaturated fatty acids of membrane lipids.

The rationale of the present study was to validate our hypothesis in isolated hepatocytes in which CCl_4-induced lipid peroxidation and cell damage are inhibited at high pO_2-levels.[4] Equilibrium oxygen conditions at selected pO_2-levels in suspen-

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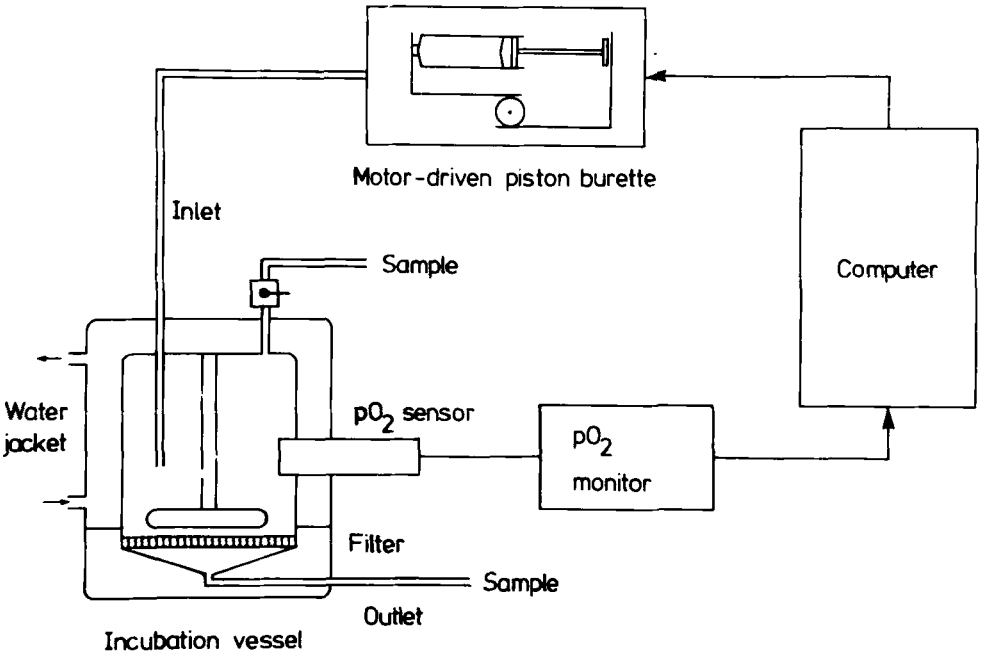

FIGURE 1 Computer-supported oxystat system.

sions of isolated hepatocytes and simultaneous monitoring of oxygen uptake were achieved by using a computer-supported oxystat which had been successfully employed in microsomes. The occurrence of low-level chemiluminescence was continuously monitored by adapting a single-photon counting instrument to the oxystat.

MATERIALS AND METHODS

Hepatocytes

Hepatocytes were isolated from male Wistar rats (180–200 g) following the procedure of Berry and Friend[5] as described by Sies *et al.*[6] They excluded trypan blue (80–85%). The animals were pretreated with phenobarbital (0.1 g/l potassium phenobarbital dissolved in drinking water for four days).

Incubations

Hepatocytes were incubated at 3–4 $\times$ 10^6 cells/ml in Krebs-Henseleit buffer, pH 7.4, supplemented with 10 mM glucose, 2.1 mM lactate, and 0.3 mM pyruvate. CCl_4 was added in 5 μl, ethanol to achieve a final concentration of 0.5 mM. Incubations were performed at 37°C.

Oxystat System

Equilibrium oxygen conditions and continuous monitoring of oxygen uptake at selected pO_2 were achieved by using a computer-supported oxystat system.[2] It is based

on the principle that the oxygen supply of respiring particles in an incubation vessel is maintained by injection of oxygen, dissolved in aqueous medium. To prevent dilution of the isolated hepatocytes during long-term incubations, the incubation vessel was equipped with a Millipore membrane filter of 8 μm pore size at the outlet (Fig. 1). A polarographic oxygen sensor was employed for continuous monitoring of the actual pO_2, a motor-driven piston burette for injection of oxygen-saturated medium, and a computer for process control. The computer reads the actual pO_2, compares it with the preselected value and activates the motor-driven piston burette to add appropriate amounts of oxygen-saturated medium (95% O_2/5% CO_2) until the set-point is reached. Furthermore, it calculates the oxygen uptake from the amounts of oxygen-saturated medium added.

Detection of Low-Level Chemiluminescence

Detection of electronically excited species under steady state oxygen conditions was performed by combining the oxystat system with a single-photon counting instrument as described by Cadenas and Sies.[7]

Assays

Thiobarbituric acid-reactive material was determined as follows: 0.2 ml hepatocyte suspension (about 6 mg hepatocellular protein/ml) was mixed with 1.0 ml of ice-cold trichloroacetic acid (1 M). After centrifuging at 10,000 × g for 10 min, 1.0 ml of the supernatant was heated with 0.5 ml of 2-thiobarbituric acid (70 mM) at 95°C for 10 min. The sample was read at 532 nm against sample blank. The amounts of thiobarbituric acid-reactive material formed were expressed as malondialdehyde equivalents using 1,1,3,3-tetramethoxypropane as standard.

The viability of the isolated hepatocytes was evaluated by determining trypan blue exclusion[8] and lactate dehydrogenase leakage.[9]

Protein was assayed with the method of Lowry *et al.*[10] using bovine serum albumin as standard.

Chemicals

All chemicals and biochemicals were purchased from Merck (Darmstadt, F.R.G.) and Boehringer (Mannheim, F.R.G.), respectively.

RESULTS AND DISCUSSION

Experiments with NADPH-reduced rat liver microsomes incubated at steady state pO_2-levels between 0.2 and 100 mmHg have revealed three basic features of haloalkane-induced peroxidation of polyunsaturated fatty acids of microsomal membrane lipids.[1,3,11–13]

(a) addition of halomethanes like CCl_4, $CBrCl_3$, $CHBr_3$ and haloethanes like $CF_3CHBrCl$ (Halothane) results in an immediate rise of microsomal lipid peroxidation as indicated by oxygen uptake, malondialdehyde-formation and diene conjugation;

(b) lipid peroxidation continues as long as the haloalkanes are metabolically

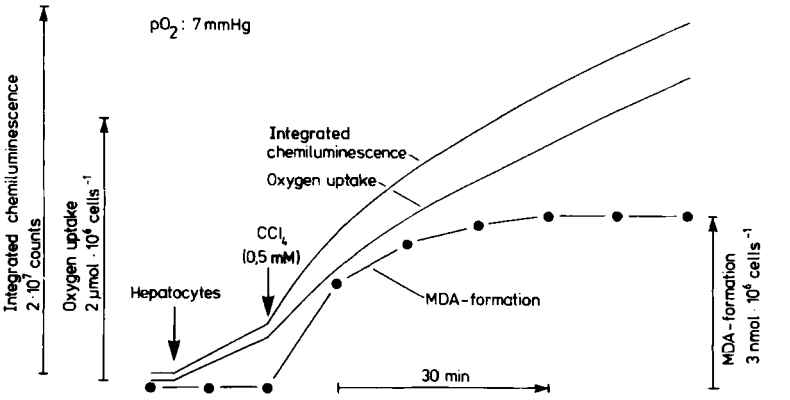

FIGURE 2 Plots of oxygen uptake, malondialdehyde (MDA)-formation and emission of low-level chemiluminescence during a typical incubation of isolated hepatocytes at a steady state pO_2-level of 7 mmHg.

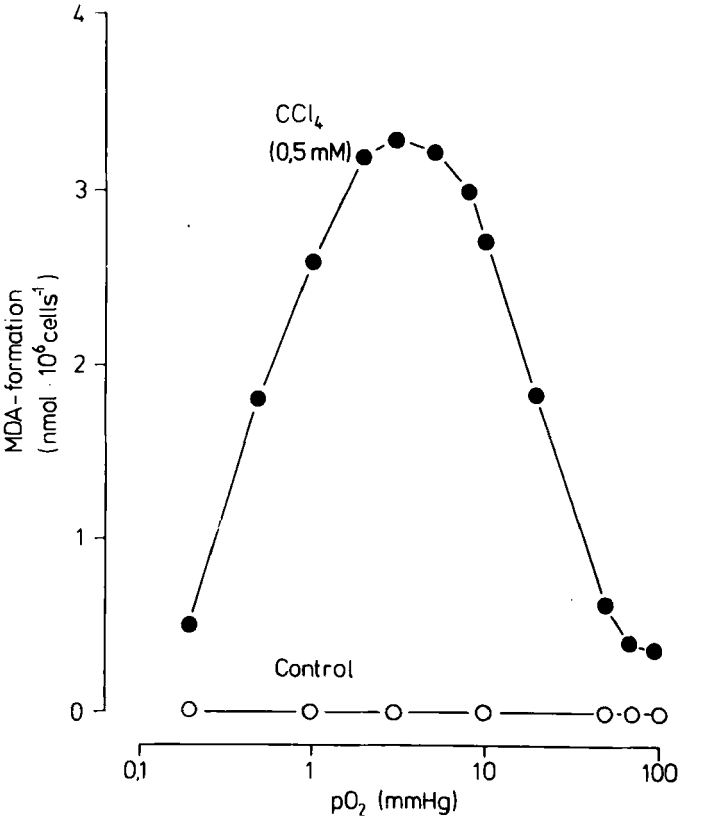

FIGURE 3 pO_2-dependence of CCl_4-induced lipid peroxidation in isolated rat hepatocytes. The cells were exposed to 0.5 mM CCl_4 for 1 h.

TABLE I

Effect of CCl_4 on malondialdehyde (MDA)-formation and cell viability of hepatocytes, expressed as lactate dehydrogenase (LDH) leakage and trypan blue exclusion, after 60 min of incubation at steady state oxygen partial pressures (pO_2). Means ± SD (n = 4)

Conditions	pO_2 (mmgH)	MDA-formation (nmol MDA × 10^6 cells^{-1})	Cell viability	
			LDH leakage	Trypan blue exclusion
Control	1	no	27 ± 3	68 ± 4
	7	no	18 ± 4	78 ± 6
	70	no	20 ± 3	75 ± 4
0.5 mM CCl_4	1	2.6 ± 0.2	59 ± 4	38 ± 5
	7	3.0 ± 0.3	43 ± 3	35 ± 3
	70	0.4 ± 0.1	23 ± 4	72 ± 3

activated by cytochrome P-450; when cytochrome P-450 activity decreases by haloalkane free-radical-mediated inactivation,[11,14–16] lipid peroxidation stops;

(c) haloalkane-induced lipid peroxidation is enhanced 10–20 times when the steady state pO_2-level is shifted from values around 70 mmHg, the upper level of the physiological pO_2 in liver, to values around 7 mmHg, the hypoxic level of the physiological pO_2 which is primarily present in areas adjacent to the central vein of the lobule.[1,3]

In line with these findings we observed a characteristic behaviour of lipid peroxidation during incubations of CCl_4-supplemented isolated hepatocytes. At a pO_2 of 7 mmHg addition of CCl_4 caused an immediate hepatocellular lipid peroxidation as indicated by the occurrence of malondialdehyde (Fig. 2). The concentration of malondialdehyde increased during an initial period of 10–20 min and approached a steady state level after about 30 min. Simultaneously with the occurrence of malondialdehyde there was a marked stimulation of the hepatocellular oxygen uptake and the emission of low-level chemiluminescence (Fig. 2). In this regard it is interesting to note that CCl_4-induced accumulation of malondialdehyde started without lag phase, which may point to the fact that under appropriate oxygen conditions, also in hepatocytes, the haloalkane free radicals liberated at the endoplasmic reticulum overcome the antioxidants defence system of native cellular membranes in a burst. Plotting the 60 min values of malondialdehyde accumulation versus pO_2 (Fig. 3) shows that CCl_4-induced lipid peroxidation was maximal between 1 and 10 mmHg; above and below this range the potency of CCl_4 to induce lipid peroxidation in this cellular system sharply decreased. Recently, a similar pO_2-range optimal for CCl_4-induced lipid peroxidation has been found in NADPH-reduced microsomes.[11] Because of this similarity it is concluded that the haloalkane-induced lipid peroxidation is not limited by oxygen supply in the intact hepatocyte.

To evaluate the effect of the haloalkane-induced lipid peroxidation on cell viability, we determined the ability of the cell membrane to exclude trypan blue and to prevent the release of lactate dehydrogenase during incubations at steady state pO_2-levels of 1, 7 and 70 mm Hg (Table I). Comparing the respective parameters of cell membrane integrity at pO_2 of 1 and 7 mmHg with those obtained at 70 mm Hg it was observed, that in the presence of CCl_4 a significant greater portion of hepatocytes was damaged at those pO_2-levels which were optimal for CCl_4-mediated lipid peroxidation.

In conclusion, incubations of isolated rat liver hepatocytes at physiologically low pO_2-levels between 0.2 and 100 mmHg demonstrate that CCl_4 is a potent inducer of

lipid peroxidation also in the intact hepatocyte, provided that the pO_2 is maintained at distinct low levels. The coincidence of lipid peroxidation and loss of cell viability at the same pO_2-range provides further evidence for the apparently paradoxical phenomenon that haloalkane-mediated liver cell injury is due to a peroxidative process which primarily occurs at the hypoxic end of the physiological pO_2-levels (1–70 mmHg)[17] in liver.

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