O_2-INDEPENDENT DAMAGE OF CYTOCHROME P450 BY CCl_4-METABOLITES IN HEPATIC MICROSOMES

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1. Introduction

The metabolism of CCl_4 by the hepatic endoplasmic monooxygenase system results in the peroxidation of microsomal lipids and the covalent binding of CCl_4-metabolites to microsomal proteins and lipids (reviewed [1,2]). The inactivation of cytochrome P450, the terminus of the endoplasmic monooxygenase system, is one of the earliest signs of damage following the administration of CCl_4, a hepatotoxic compound, in vivo [3–6]. Similarly, the addition of CCl_4 in vitro to liver microsomes in the presence of NADPH also results in an early inactivation of cytochrome P450 [7–9]. It is unclear [10] whether cytochrome P450 is inactivated by the attack of reactive CCl_4-metabolites directly [11] or whether these metabolites act indirectly by initiating O_2-dependent lipid peroxidation [8]. We present here evidence that cytochrome P450 can be damaged by CCl_4-metabolites under conditions which prevent the production of detectable quantities of lipid peroxides.

2. Methods

2.1. *Isolation of microsomes*

Female Wistar rats (150–200 g) fed on Altromin stock diet were starved over night. Under ether anaesthesia livers from 4 rats were perfused in situ with ice-cold KCl (0.15 M), then excised and homogenized in 4 vol. KCl (0.15 M) with a Potter-Elvehjem homogenizer. Unless specified all manipulations were at 0°C. Mitochondrial supernatant was obtained by centrifuging the homogenate at 755 × *g* for 10 min and subsequently at 14 500 × *g* for 20 min. The supernatant was spun at 105 000 × *g* for 60 min. The microsomal pellets were resuspended at ~7 mg protein/ml in incubation buffer containing: NaCl (340 mM), KCl (104 mM), Tris · HCl (50 mM) (pH 7.4). The microsomes, maintained in suspension by continuous stirring, were deoxygenated by blowing argon (O_2 content $\leqslant$0.1 ppm) on the surface for $\geqslant$1 h. Under these conditions the oxygen content of the suspension was reduced from 0.26 mM to <0.025 μM.

2.2. *Assays*

Protein, lipid peroxidation, heme and cytochromes P450 and b_5 were determined according to [7,12–14]. O_2 at 1 nM–3 μM was measured by the O_2-dependent bioluminescence of the bacteria *Vibrio fischeri* (German Collection of Microorganisms, Göttingen). The luminescence was recorded with a fluorimeter (Baird Atomic SF-100), the incubation vessel being placed directly in front of the photomultiplier. Calibration of the O_2-concentration was performed by addition of defined volumes of air-saturated water (O_2 content = 0.27 mM, 22°C) under reaction conditions (see fig.1).

2.3. *Incubations*

The incubation vessel was a 45 ml glass cylinder equipped with water jacket, side arm, gas inlet and outlet and a magnetic stirrer.

The anaerobic incubations were performed with an incubation mixture of the following final composition: $MgCl_2$ (6 mM), glucose-6-phosphate (8 mM), glucose-6-phosphate dehydrogenase (400 U/l) and bacteria *Vibrio fischeri* (1 g wet wt/l) in incubation buffer previously deoxygenated by bubbling argon for $\geqslant$20 min. EDTA (1 mM) was included where indicated. After the addition of the deoxygenated micro-

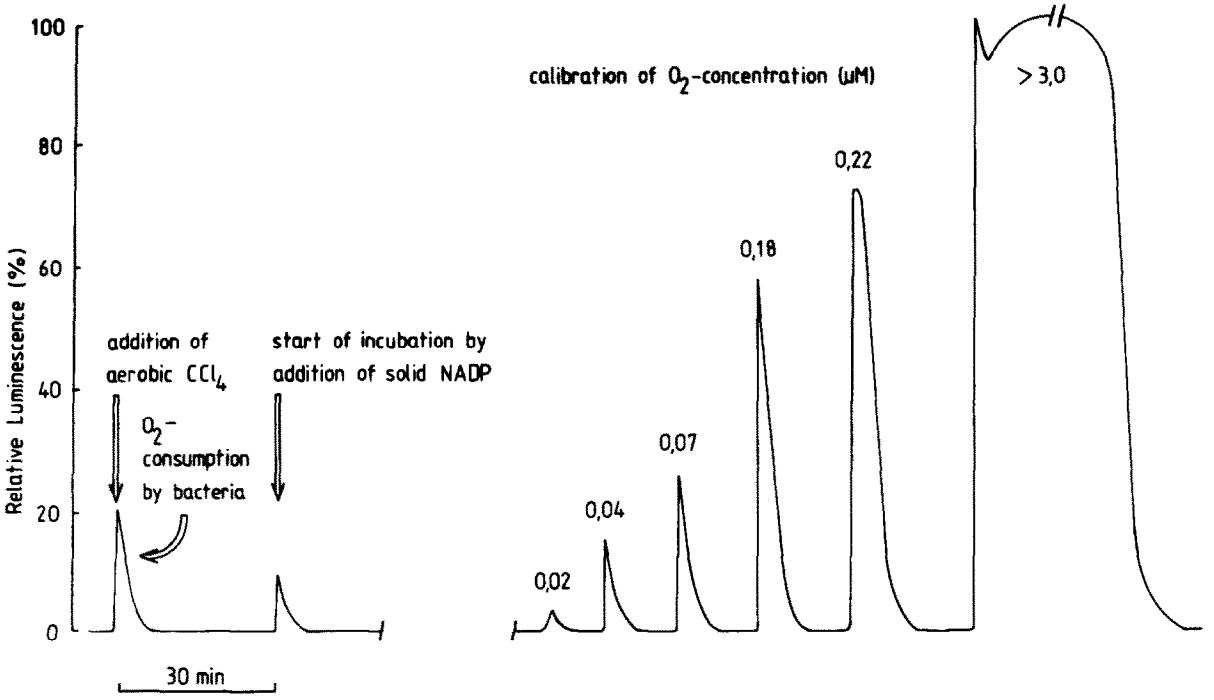

Fig.1. Determination and calibration of O_2 concentration during incubation. O_2 concentration was measured by the bioluminescence of photobacteria. Experimental details are in section 2.

somal suspension (1.5 g protein/l) and the equilibration to 22°C, sufficient ethanolic CCl_4 was injected to produce final conc. 5 mM EtOH and 1 mM CCl_4 (fig.1). Controls received EtOH but no CCl_4. The preincubation was continued to obtain, due to oxygen consumption by bacteria, <1 nM oxygen during 20 min. Then, the reaction was begun by addition of 12 mg solid NADP · Na_2 from the side arm. The NADP served to maintain NADPH ~0.15 mM during the entire incubation period of maximal 1 h. For aerobic incubations, solutions were not deoxygenated and the bacteria were omitted. At the beginning of the reaction oxygen was 0.26 mM.

3. Results

3.1. *Damage of cytochrome P450*

Under anaerobic conditions, in the presence of NADPH but absence of CCl_4, the microsomal cytochrome P450 concentration remained constant (fig.2). The addition of CCl_4 to this anaerobic system resulted in a pronounced decrease in the cytochrome P450 concentration, even in the presence of EDTA. The rate of this decline was similar to that observed under aerobic conditions. The damage of the cytochrome P450 paralleled the disappearance of microsomal heme (fig.3). In contrast, the content of the second microsomal hemoprotein, cytochrome b_5, remained essentially unaltered under these conditions (not shown).

3.2. *Lipid peroxidation*

Lipid peroxidation, measured by the thiobarbituric acid reaction, was only apparent under aerobic conditions (table 1). Under anaerobic conditions no lipid peroxidation was detectable.

4. Discussion

The purpose of this study was to examine the mechanism by which CCl_4-metabolites damage cytochrome P450. Specifically we wished to determine whether inactivation resulted from the direct interaction between cytochrome P450 and metabolites of CCl_4 or whether inactivation proceeded indirectly via the activation of lipid peroxidation. Since lipid

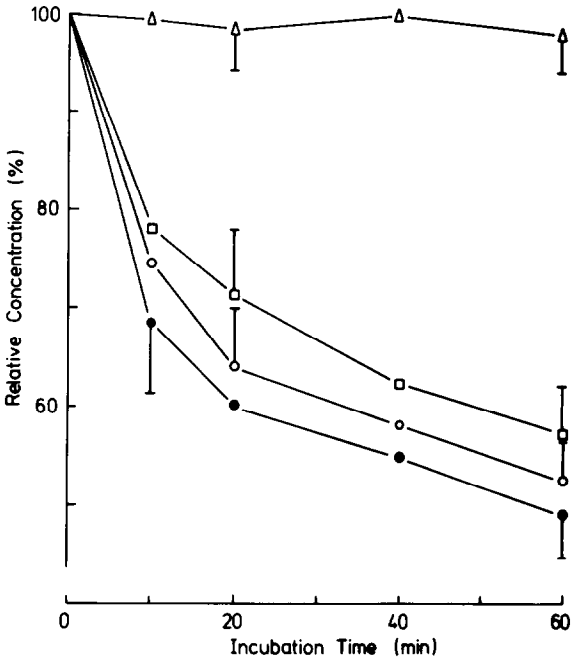

Fig.2. Decrease of cytochrome P450 concentration under various conditions. The starting concentration of cytochrome P450 determined in the absence of CCl_4 was 1.05 μM. Each value represents a mean of 3 incubations. The values were corrected for spectral interference of CCl_4 by multiplying the measured concentration by a factor of 1.4. The conditions were; (•) aerobic, 1 mM CCl_4; (○) anaerobic, 1 mM CCl_4; (□) anaerobic, 1 mM CCl_4, 1 mM EDTA; (△) anaerobic, no CCl_4. Further experimental details are in section 2.

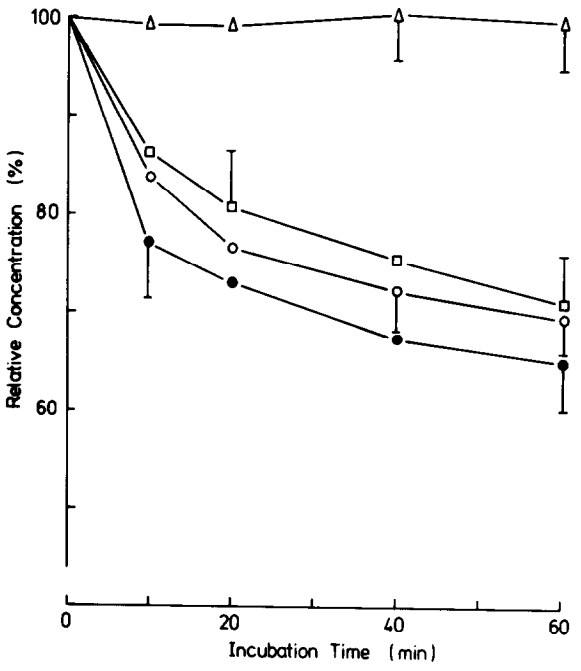

Fig.3. Decrease of heme concentration under various conditions. The starting concentration of heme was 1.56 μM. Each value represents a mean of 3 incubations. The conditions were; (•) aerobic, 1 mM CCl_4; (○) anaerobic, 1 mM CCl_4; (□) anaerobic, 1 mM CCl_4, 1 mM EDTA; (△) anaerobic, no CCl_4. Further experimental details are given in methods.

peroxidation is O_2-dependent we were interested in determining whether CCl_4 could inactivate cytochrome P450 under anaerobic conditions (fig.1,2). Under these conditions no lipid peroxidation could be detected by the thiobarbituric acid (TBA) assay (table 1). Unfortunately this assay is fairly inefficient; only 4% of the lipid peroxides are measured as TBA-positive material [15]. Thus, the standard deviation of the TBA-assay corresponds to abount ± 4.5 μM peroxides formed. Since cytochrome P450 is only 1 μM an accurate determination of residual oxygen content is crucial to the estimation of the maximal peroxide levels.

Table 1
Decrease of cytochrome P450 concentration and lipid peroxidation under various conditions

O_2	NADPH	CCL_4	EDTA	Malonaldehyde	Residual	Cyt P450
				μM	μM	%
(an) Aerobic	–	+	–	0.7 ± 0.2	1.05 ± 0.05	100 (control)
Aerobic	+	+	–	10.7 ± 0.6	0.51 ± 0.06	49
Anaerobic	+	–	–	0.7 ± 0.1	1.04 ± 0.05	99
Anaerobic	+	+	–	0.7 ± 0.2	0.56 ± 0.06	53
Anaerobic	+	+	+	0.7 ± 0.2	0.61 ± 0.07	58

The lipid peroxidation is expressed as μmol malonaldehyde/l. Each value represents a mean of 3 incubations for 1 h. Experimental details are given in section 2 and fig.2

The use of photobacteria permits the measurement of oxygen levels in the nM range [16]. Furthermore the bacteria efficiently consume oxygen. Fig.1 illustrates this point; e.g., in <5 min the photobacteria have reduced the oxygen level from 50 nM–<1 nM. In our standard assay oxygen was ≤25 nM. Under these conditions one would expect a maximal 25 nM peroxide, well below the lower limit for the TBA-assay. This level of peroxide formation is clearly an overestimate since bacteria consume oxygen. Even so, this level of peroxides is too low to account for the rapid rate and the amount at which cytochrome P450 is damaged (fig.2, table 1). The damage of cytochrome P450 also persists in the presence of 1 mM EDTA, a compound known to inhibit CCl_4-initiated lipid peroxidation [9]. These results are in marked contrast to those in [8]. Furthermore a similar rate of cytochrome P450 inactivation is observed under aerobic condition (fig.2) even though lipid peroxidation increases tremendously (table 1). The lack of correlation between the rate of cytochrome P450 inactivation and the rate of lipid peroxidation is strongly suggesting that the two mechanisms proceed independently.

Although lipid peroxidation can be efficiently suppressed by the exclusion of oxygen, the lipid peroxidation mechanism can not be entirely ruled out as the mechanism by which CCl_4 damages cytochrome P450. The main limitation is that the oxygen measured by the bacteria is only free oxygen; local concentrations, particularly in the microsomal versicles, may be considerably higher. One way of minimizing this problem is to pre-incubate the microsomes with the bacteria. Within 5 min this procedure reduces oxygen to <1 nM (fig.1). In our standard assays we used a pre-incubation time of 20 min, but varying this time from 5–45 min had no effect on the rate of inactivation of cytochrome P450 (data not shown). Taken together, these results indicate that cytochrome P450 can be inactivated by CCl_4-metabolites directly without requiring lipid peroxidation. This conclusion is supported by the results of in vitro and in vivo experiments that free-radical scavengers such as promethazine and phenyl-*t*-butylnitrone can suppress lipid peroxidation, measured by the TBA-assay, but not cytochrome P450 inactivation [17–20]. Furthermore, in mice, CCl_4-metabolism and cytochrome P450 inactivation occur in absence of lipid peroxidation [11].

The concentration of heme also decreases during the incubations with CCl_4 (fig.3). This must reflect a fairly specific inactivation process for cytochrome P450 since the level of cytochrome b_5, the second microsomal hemoprotein, is not significantly affected (not shown). This suggests that the damaging process proceeds by the attack of shortlived CCl_4-metabolites on the heme moiety of cytochrome P450, which is reported to be the locus of the metabolism of CCl_4 [2].

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