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SELF-CATALYSED, O_2-INDEPENDENT INACTIVATION OF NADPH- OR DITHIONITE-REDUCED MICROSOMAL CYTOCHROME P-450 BY CARBON TETRACHLORIDE

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Abstract—Rat liver microsomes were incubated under anaerobic conditions in the presence of NADPH, dithionite and CCl_4. It was found: (1) that in the presence of NADPH/CCl_4 or dithionite/CCl_4 the microsomal cytochrome P-450 (P-450) content rapidly declined and low amounts of CO were produced; (2) that the decrease of P-450 was always accompanied by an almost equimolar loss of microsomal heme; (3) that both the loss of P-450 and the formation of CO were inhibited by metyrapone and O_2; the decrease of P-450 was also inhibited by high concentrations of CO; and (4) that the pretreatment of rats with phenobarbital enhanced markedly both the loss of P-450 and the formation of CO, whereas the pretreatment with 3-methylcholanthrene significantly stimulated only the formation of CO.

These results suggest: (1) that mainly the phenobarbital-induced species of P-450 are damaged by a direct, O_2-independent attack of CCl_4 metabolites on its heme moiety; (2) that these reactive CCl_4 metabolites, probably $\dot{C}Cl_3$ or $|CCl_2$, are intermediates of the metabolism of CCl_4 to CO; and (3) that this metabolism is catalysed in a suicidal manner by the reduced form of P-450 itself.

INTRODUCTION

There is a close connection between the microsomal cytochrome P-450 (P-450) of the liver parenchymal cell and the hepatotoxicity of CCl_4. This happens not only because the inactivation of P-450 is one of the earliest feasible sites of damage by CCl_4, but also because P-450 is reputed to be involved in the metabolism of CCl_4 to reactive products, a step prerequisite to its toxicity [1–3].

One of the reactive CCl_4 metabolites is the $\dot{C}Cl_3$ radical [4, 5], a species already proposed by Butler [6]. A further, reactive CCl_4 metabolite, the $|CCl_2$ carbene, was suggested by Wolf *et al.* [7]. They also presented evidence [7, 8] for a reaction pathway of CCl_4 to CO which is catalysed by P-450. In this pathway first the $\dot{C}Cl_3$ radical, and subsequently the $|CCl_2$ carbene is formed reductively via chloride elimination. The carbene can ultimately hydrolyse to CO.

A second possible site of the CCl_4 toxification is the P-450 reductase, another constituent of the microsomal monooxygenase system. This is indicated because of the failure of CO, an otherwise potent inhibitor of P-450-dependent enzymatic reactions [9], to inhibit the CCl_4-dependent lipid peroxidation [10, 11], a process which is supposed to be initiated by free $\dot{C}Cl_3$ [1].

At issue is the way in which the reactive CCl_4 metabolites produce the CCl_4 toxicity. Often it is assumed that they damage indirectly by stimulating lipid peroxidation and this O_2-dependent process leads not only to P-450 inactivation but also ultimately to cell death [12]. Recently, however, we have shown that lipid peroxidation is not necessary for the inactivation of P-450 and that a direct, O_2-independent damage of P-450 by reactive CCl_4 metabolites is likely [13].

We present here further evidence for this mechanism. In detail we have attempted to determine: (1) whether dithionite-reduced microsomes also metabolise CCl_4 to P-450-damaging products; (2) at which site the P-450-inactivating CCl_4 metabolites are formed; (3) which of the multiple forms of P-450 are mainly involved in the inactivation by CCl_4; and (4) whether a connection can be established between the metabolism of CCl_4 to CO and the damage of P-450 by CCl_4.

MATERIALS AND METHODS

Treatment of animals. Female Wistar rats (150–200 g) were fed on Altromin stock diet (Lage-Lippe, West Germany). Phenobarbital, dissolved in 0.9 per cent NaCl, was administered at a dose of 80 mg/kg for 3 days. 3-Methylcholanthrene, dissolved in olive oil, was administered at a dose of 20 mg/kg once 48 hr before killing the animals. Both drugs were injected intraperitoneally. Food was withdrawn 12 hr before death.

Isolation of microsomes. Under ether anaesthesia livers were retrogradely perfused *in situ* through the right atrium with ice-cold KCl (0.15 M), excised, minced with scissors and homogenised in 4 vol. of KCl (0.15 M) with a glass Potter-Elvehjem homogeniser. The homogenate was centrifuged at 4° and 755 × *g* for 10 min to sediment the nuclei and at 14,500 × *g* for 20 min to sediment the mitochondria. The supernatant was spun at 105,000 × *g* for 60 min. The microsomal pellet was resuspended in ice-cold KCl/Tris–HCl buffer (104 mM/50 mM, pH 7.4). The

microsomes were deoxygenated by blowing argon (O_2 content < 0.1 ppm) on the surface of the stirred suspensions for 1 hr.

Assays. P-450 was determined as ferrous CO complex [14]. Heme was measured by the pyridine hemochromogen method [15]. To prevent spectral interference by CCl_4 and CO in the determination of the content of P-450 and of heme, the reference cuvettes were filled before CCl_4 was added to the incubation mixture. CO was detected by its CO hemoglobin spectrum [7]. Calibration was performed by the addition of microliter quantities of CO-saturated water (1.0 mM CO at 20°). Protein was determined with the method of Lowry [16] using bovine serum albumin as a standard.

Incubations. The incubations were performed anaerobically in the absence of light at 37° in a 10 ml glass cylinder equipped with water jacket, oxygen electrode, gas inlet and outlet and a magnetic stirrer. The incubation mixture was of the following final composition: (a) in the case of reduction with dithionite, KCl (104 mM), Tris–HCl (50 mM, pH 7.4), Na-dithionite (1 mM) and microsomes (1.0–1.5 mg protein/ml); and (b) in the case of reduction with NADPH, KCl (104 mM), Tris–HCl (50 mM, pH 7.4), $MgCl_2$ (6 mM), glucose-6-phosphate (8 mM), glucose-6-phosphate dehydrogenase (400 U/l) and microsomes (1.0–1.5 mg protein/ml). The NADPH-regenerating system ensured a NADPH concentration of about 0.14 mM during the entire incubation period. Where indicated, CCl_4/EtOH (1 mM/5 mM), EtOH (5 mM), CO (10 μM or saturated by bubbling CO for 1 min, about 1 mM), metyrapone (2-methyl-1,2-bis(3-pyridyl)-1-propanone, 5 mM) and DPPD (*N,N'*-diphenyl-*p*-phenylenediamine, 1 μM) were included. To achieve and to maintain anaerobic conditions, the incubation mixtures were composed in the following order: first metyrapone, DPPD or the components of the NADPH-regenerating system were placed into the glass cylinder. This solution was deoxygenated by bubbling argon for at least 30 min. Then, where indicated, Na-dithionite, dissolved in deoxygenated KCl/Tris–HCl buffer (104 mM, 50 mM, pH 7.4), was added. Subsequently the anaerobic microsomal suspension was injected. After equilibration to 37°, and in some cases after addition of CO, the incubation was begun by the injection of CCl_4/EtOH. Controls contained EtOH but no CCl_4. During the anaerobic incubations the O_2 concentration never exceeds a value of 0.2 μM, the lower sensitivity of the oxygen electrode.

RESULTS

Loss of P-450 *and formation of* CO *by* NADPH/CCl_4 *and dithionite*/CCl_4

Under anaerobic conditions the P-450 content of microsomes from phenobarbital-pretreated rats remained unaltered in the presence of CCl_4 or NADPH, and only a small decline of about 10 per cent was observed in the presence of dithionite (Fig. 1). Under none of these conditions was the formation of CO detectable (not shown). In contrast, the simultaneous addition of NADPH/CCl_4 or dithionite/CCl_4 resulted in a rapid decrease in the P-450 content (Fig. 1), an almost equimolar loss of microsomal heme, and the evolution of approximately 2.5 mol CO per mol P-450 lost (Table 1). After incubation with NADPH/CCl_4 (Fig. 2) as well as after incubation with dithionite/CCl_4 (not shown), no further changes were observed in the CO difference spectra of the anaerobic reduced microsomes (spectral bandwidth, 2 nm), except from marked decreases in the maxima.

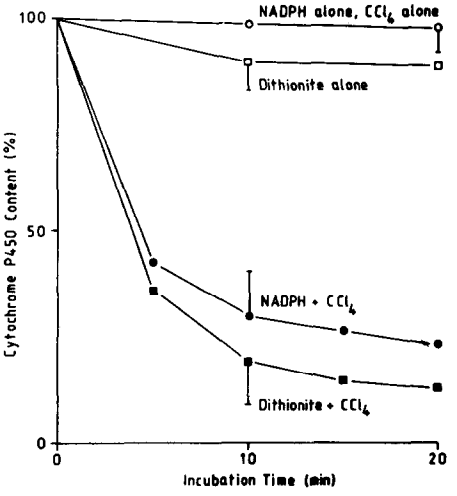

Fig. 1. Decrease of the cytochrome P-450 content of liver microsomes from phenobarbital-pretreated rats under anaerobic conditions. Each value represents a mean ± S.E.M. of at least 3 incubations at 37°. The starting content of cytochrome P-450 was 1.50 ± 0.16 nmol/mg microsomal protein. The substances were included in the following final concentrations: CCl_4 (1 mM), dithionite (1 mM), NADPH (about 0.14 mM, regenerating system). Further experimental details are given in methods.

Effects of inhibitors

The NADPH/CCl_4-dependent losses of P-450 and of heme were markedly inhibited by CO concentrations in the millimolar range (Fig. 2); 10 μM CO had no effect (Table 1). Metyrapone and O_2/DPPD were less effective as inhibitors. The inhibitory effects were also reflected in a decrease in CO formation (Table 1). The dithionite/CCl_4-dependent loss of P-450 was also markedly inhibited by high concentrations of CO (Table 1).

Effects of pretreatment

In microsomes from phenobarbital-pretreated rats the increased level of P-450 was decreased by NADPH/CCl_4 to a greater initial rate and to an absolutely and relatively higher extent compared to microsomes from untreated rats (Fig. 3). In contrast, the loss of P-450 (448) by NADPH/CCl_4 in microsomes from 3-methylcholanthrene-pretreated rats was, at an unaltered initial rate, absolutely only slightly higher and relatively even lower, with respect to microsomes from untreated rats. Both, pretreatment with phenobarbital and with 3-methylcholanthrene enhanced the anaerobic NADPH/CCl_4-dependent formation of CO, though in the latter case to a lesser degree (Fig. 4). Independent of

Table 1. Formation of CO and loss of microsomal cytochrome P-450 and heme by NADPH/CCl_4 and dithionite/CCl_4 under anaerobic conditions, effects of inhibitors

1 mM CCl_4		Loss of cytochrome P-450	Loss of total heme	Formation of CO
Reductant	Inhibitor	nmol/mg protein	nmol/mg protein	nmol/mg protein
None (control)	None	None*	None†	None
NADPH	None	1.13 ± 0.19	1.13 ± 0.44	2.8 ± 0.5
NADPH	CO (10 μM)	1.12 ± 0.21	1.27 ± 0.48	–
NADPH	CO (1 mM, saturated)	0.40 ± 0.11	–‡	–
NADPH	Metyrapone (5 mM)	0.64 ± 0.10	0.66 ± 0.23	1.9 ± 0.5
NADPH	DPPD/O_2 (1 μM, 1 mM)	0.48 ± 0.20	0.61 ± 0.33	None
Dithionite	None	1.23 ± 0.22	1.52 ± 0.52	2.9 ± 0.7
Dithionite	CO (1 mM, saturated)	0.48 ± 0.10	–‡	–

* Cytochrome P-450 content: 1.50 ± 0.16 mmol/mg microsomal protein.
† Total heme content: 2.38 ± 0.38 nmol/mg microsomal protein.
‡ Not measurable because of interference by CO. The incubation time was 20 min. The other experimental conditions are the same as in Fig. 1.

pretreatment, the rates of CO evolution always decreased during the course of the incubations.

DISCUSSION

Recently we have shown by experiments with rat liver microsomes under carefully-defined anaerobic conditions, that in the presence of NADPH/CCl_4, reactive CCl_4 metabolites are formed, which damage P-450 possibly by a direct attack on its heme moiety

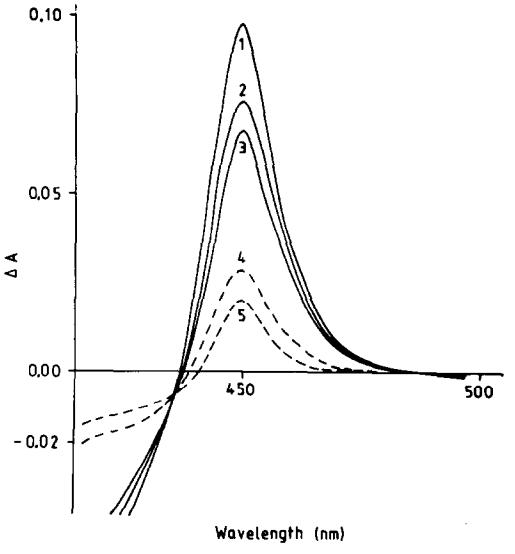

Fig. 2. Inhibitory effect of CO on the NADPH/CCl_4-dependent anaerobic loss of cytochrome P-450, measured by the CO difference spectra of the NADPH-reduced liver microsomes from phenobarbital-pretreated rats. The microsomes were incubated with NADPH/CCl_4 in the presence (1, 2, 3; scans at 0, 10 and 20 min) and absence (4, 5; scans at 10 and 20 min) of about 1 mM CO. In the latter case the sample cuvette was saturated with CO right before recording the CO spectra. Further experimental details are given in Fig. 1.

[13]. We show here that the P-450 content of anaerobic rat liver microsomes rapidly decreased not only in the presence of NADPH/CCl_4 but also in the presence of dithionite/CCl_4 (Fig. 1). Under both conditions the decrease of P-450 was accompanied by an equimolar loss of microsomal heme and by the formation of low amounts of CO, and was inhibited by saturating the incubation mixture with CO (Table 1). Though some P-450 was already damaged by dithionite in the absence of CCl_4 (Fig. 1), it appears possible that the mechanism by which CCl_4 damages P-450 is essentially the same in both NADPH- and dithionite-reduced microsomes. Thus not only NADPH- but also dithionite-reduced microsomes appear to be capable of catalysing the metabolism of CCl_4 to P-450-damaging products. This strengthens our contention that the inactivation of P-450 by CCl_4 *in vitro* can occur through an O_2-independent process, since the presence of dithionite, in addition to the careful deoxygenation procedure, ensures and maintains an O_2 concentration in the incubation mixture well below the sensitivity of the oxygen electrode (about 0.2 μM). The possibility of some involvement of O_2-dependent processes in P-450 inactivation by CCl_4 *in vivo* cannot be ruled out by these experiments.

It can be seen (Fig. 2) that the CO difference spectra of the anaerobic reduced microsomes showed no qualitative changes after incubation with NADPH/CCl_4 or dithionite/CCl_4. Therefore it is very unlikely that the inactivation of P-450 by CCl_4 is the result of the mere formation of a tight complex between P-450 and CCl_4 or its metabolites; because this complex should be characterised by an additional absorption at 454–460 nm [7]. Furthermore the CO spectra show that no P-420 could be detected as a possible product of the P-450 damage.

In an attempt to elucidate where the P-450-damaging CCl_4 metabolites are formed, we performed experiments with O_2 and P-450 inhibitors. The P-450 inhibitors CO and metyrapone, as well as O_2, bind on the reduced heme of P-450 [14, 17, 9]. All were

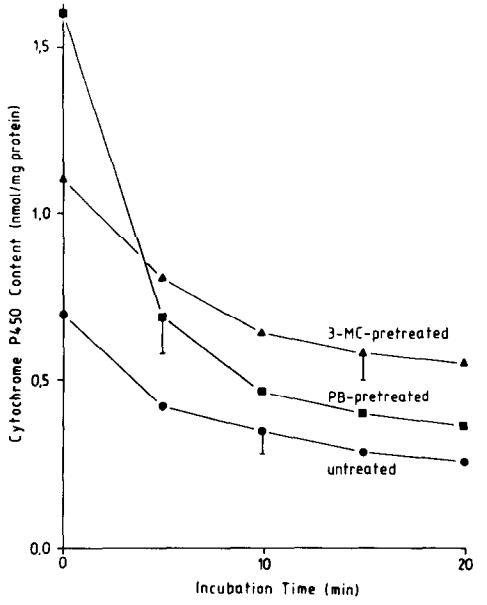

Fig. 3. NADPH/CCl_4-dependent anaerobic loss of cytochrome P-450 of liver microsomes from differently treated rats. The experimental details are given in Fig. 1.

able to inhibit the CCl_4-dependent decrease of P-450 (Table 1); the experiments with O_2 were performed in the presence of DPPD to suppress a CCl_4-independent peroxidative damage of P-450 [18]. Because of the obviously high affinity of P-450 for CCl_4, and because of their competitive mechanism, the inhibitors were effective only at relatively high concentrations (Table 1). This also means that the amounts of CO formed during the incubations (Fig. 4) were too low to be inhibitory. Though a shielding effect of the inhibitors against attack of CCl_4 metabolites formed for example by the P-450 reductase can not

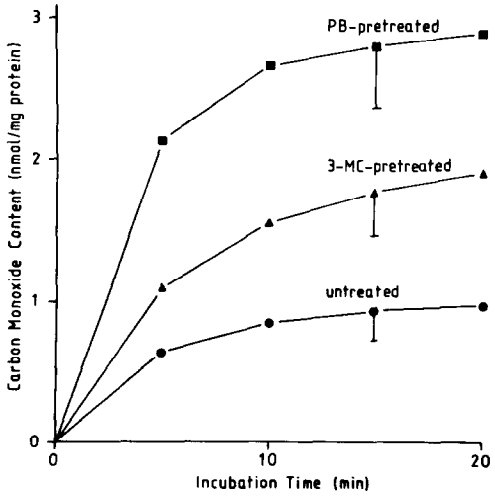

Fig. 4. NADPH/CCl_4-dependent formation of CO under anaerobic conditions by liver microsomes from differently treated rats. The experimental details are given in Figs. 1 and 3.

be excluded, these results suggest that the reactive P-450-damaging CCl_4 metabolites are formed by P-450 itself. Since the reductive metabolism of CCl_4 to CO is also inhibited by metyrapone [8] and furthermore the formation of CO declined (Fig. 4) at the time of nearly maximal P-450 loss (Fig. 3) it is likely that one or both of the reactive CCl_4 intermediates, $\dot{C}Cl_3$ or $|CCl_2$ [7, 8], are responsible for the inactivation of P-450 by CCl_4.

So far we did not regard that the microsomal P-450 represents a group of enzymes which are characterised by different, though overlapping, substrate specifities, and which are differentially inducible by lipophilic substances like phenobarbital or 3-methylcholanthrene [19]. As shown in Fig. 3, pretreatment of rats with phenobarbital, in contrast to pretreatment with 3-methylcholanthrene, greatly stimulated the inactivation of P-450 by CCl_4. In contrast to the formation of P-450-damaging CCl_4 metabolites, pretreatment of rats with either phenobarbital or 3-methylcholanthrene stimulated the NADPH/CCl_4-dependent formation of CO (Fig. 4). Thus, though a connection appears to exist between the metabolism of CCl_4 to P-450-damaging products and the CCl_4-dependent formation of CO (see above), the varying species of P-450 appear to differ not only in their ability to metabolise CCl_4 to damaging products but also in their ability to metabolise the reactive intermediates $\dot{C}Cl_3$ or $|CCl_2$ further to CO.

Remarkably, *in vivo* the same effects of pretreatment of rats with either phenobarbital or 3-methylcholanthrene were reported [20] concerning both CCl_4-dependent processes, the inactivation of P-450 as well as the formation of CO. But the CO formed was proposed to be a by-product of a CCl_4-stimulated lipid peroxidation, which is also held responsible for the inactivation of P-450 by CCl_4 [12]. Because of the striking similarities of these *in vivo* results with our *in vitro* results, we suggest that the CO formed *in vivo* after administration of CCl_4 is also a metabolite of CCl_4 and that the *in vivo* inactivation of P-450 by CCl_4 proceeds, in the same way as *in vitro*, self-catalysed, by direct attack of reactive CCl_4 metabolites, as already proposed by De Toranzo *et al.* [21].

Taken together, the results reported here supports our assumption [13] that the inactivation of P-450 by CCl_4 occurs *in vitro* as well as *in vivo* by direct attack of reactive CCl_4 metabolites on its heme moiety. Moreover the results suggest that mainly the phenobarbital-induced species of P-450 are involved in this damaging mechanism and that the P-450-damaging CCl_4 metabolites are probably $\dot{C}Cl_3$ or $|CCl_2$. These metabolites are formed by P-450 itself as intermediates of a reductive reaction pathway of CCl_4 to CO according to Wolf *et al.* [7]. Since carbenes are known to add to metalloporphyrins [22], it appears to be possible that the inactivation of P-450 by CCl_4 proceeds via alkylation of its heme moiety by $|CCl_2$.

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REFERENCES

1. R. O. Recknagel and E. A. Glende, *CRC Crit. Rev. Toxicol.* **2**, 263 (1973).
2. T. F. Slater, in *Biochemical Mechanisms of Liver Injury* (Ed. T. F. Slater). Academic Press, London (1978).
3. F. De Matteis, in *Heme and Hemoproteins* (Eds. F. De Matteis and W. N. Aldridge), pp. 95–127. Springer, Berlin (1978).
4. J. L. Poyer, P. B. McCay, E. K. Lai, E. G. Janzen and E. R. Davis, *Biochem. biophys. Res. Commun.* **94**, 1154 (1980).
5. A. Tomasi, E. Albano, K. A. K. Lott and T. F. Slater, *FEBS Lett.* **122**, 303 (1980).
6. T. C. Butler, *J. Pharmacol. exp. Ther.* **134**, 311 (1961).
7. R. C. Wolf, D. Mansuy, W. Nastainczyk, G. Deutschmann and V. Ullrich, *Molec. Pharmac.* **13**, 698 (1977).
8. H. J. Ahr, L. J. King, W. Nastainczyk and V. Ullrich, *Biochem. Pharmac.* **29**, 2855 (1980).
9. V. Ullrich, *Top. Curr. Chem.* **83**, 68 (1979).
10. T. F. Slater and B. C. Sawyer, *Biochem. J.* **123**, 815 (1971).
11. Y. Masuda and T. Murano, *Biochem. Pharmac.* **26**, 2275 (1977).
12. R. O. Recknagel, E. A. Glende and A. M. Hruszkewycz, in *Free Radicals in Biology*, Vol. 3 (Ed. W. A. Pryor), pp. 97–153. Academic Press, New York (1977).
13. H. de Groot and W. Haas, *FEBS Lett.* **115**, 253 (1980).
14. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
15. K. G. Paul, H. Theorell and A. Akeson, *Acta Chem. Scand.* **7**, 1284 (1953).
16. O. H. Lowry, N. J. Rosebrough. A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
17. H. H. Ruf and W. Nastainczyk, *Eur. J. Biochem.* **66**, 139 (1976).
18. W. Levin, A. Y. H. Lu, M. Jacobson, R. Kuntzman, J. L. Poyer and P. B. McCay, *Arch. biochem. Biophys.* **158**, 842 (1973).
19. F. P. Guengerich, *J. biol. Chem.* **252**, 3970 (1977).
20. T. D. Lindstrom and M. W. Anders, *Toxicol. Lett.* **1**, 307 (1978).
21. E. G. D. De Toranzo, M. J. Diaz Gomez and J. A. Castro, *Biochem. biophys. Res. Commun.* **64**, 823 (1975).
22. H. J. Callot and A. W. Johnson, *J. Chem. Soc. Perkin Trans.* **1**, 1424 (1973).