THE APPARENT LOSS OF CYTOCHROME P-450 ASSOCIATED WITH METABOLIC ACTIVATION OF CARBON TETRACHLORIDE

Yasushi YAMAZOE, Masahiko SUGIURA, Tetsuya KAMATAKI and Ryuichi KATO

Department of Pharmacology, School of Medicine, Keio University, Shinanomachi, Shinjuku-ku, Tokyo 160, Japan

Accepted May 2, 1979

Abstract—Carbon monoxide inhibited the carbon tetrachloride-induced NADPH oxidation rate. The addition of methylviologen to the incubation mixture under the atmosphere of nitrogen resulted in the enhancement of the reductase activity of microsomes for carbon tetrachloride, as determined by chloroform formation. The addition of methylviologen also enhanced the carbon tetrachloride-induced loss of cytochrome P-450, while the apparent content of cytochrome b5 and the activity of NADPH-cytochrome c reductase remained unchanged. Under a strong inhibition of lipid peroxidation by addition of EDTA, carbon tetrachloride induced a clear loss of cytochrome P-450 to the extent similar to that seen in the absence of EDTA. These results indicate that cytochrome P-450 is directly degraded in association with the reductive metabolism of carbon tetrachloride by cytochrome P-450.

The in vivo administration and in vitro addition of carbon tetrachloride to the incubation mixture decrease the apparent content of cytochrome P-450 in liver microsomes (1, 2). The decrease in the cytochrome P-450 content has been attributed to one or two possibilities. One is that cytochrome P-450 heme is degraded by a peroxidative reaction initiated by the trichloromethyl radical, a reactive intermediate formed during the reduction of carbon tetrachloride (2, 3). The other is that the trichloromethyl radical binds covalently to cytochrome P-450 in a chemical manner via reductive dehalogenation (4). However, no direct evidence has been reported for the latter mechanism. To examine the possibility of the latter mechanism, we studied the reduction of carbon tetrachloride and subsequent loss of cytochrome P-450 under an anaerobic condition.

MATERIALS AND METHODS

Male Wistar rats weighing about 100 g were used throughout this study. The animals were given phenobarbital as a 0.1% solution in drinking water for 3 days, but were fasted for 18 hours prior to sacrifice. They were stunned by a blow on the head and decapitated. The livers were immediately perfused with 1.15% potassium chloride solution from the inferior vena cava to portal vein (5). Liver microsomes were washed once by homogenization and centrifugation. The washed microsomes were stored in a refrigerator at -80° under an atmosphere of nitrogen. The incubation mixture for determination of carbon tetrachloride-induced NADPH oxidation rate contained 3 mg protein of microsomes and 150
moles of sodium potassium phosphate (pH 7.4) in a final volume of 3.0 ml. The mixture was bubbled for 5 min with oxygen-free nitrogen, followed by the addition of NADPH (0.3 μmole) and carbon tetrachloride (2 μmoles). The NADPH oxidation rate was measured by recording the absorption decrease at 340 nm using a glass cuvette sealed with a rubber cup. When necessary, carbon monoxide was bubbled through the mixture for 30 seconds after introducing the nitrogen gas. NADPH oxidation rate was calculated using a molar extinction coefficient of 6.2 mM⁻¹ cm⁻¹. The incubation mixture for the assay of the reductive dehalogenation of carbon tetrachloride to chloroform contained 2 mg protein of microsomes, 0.4 μmole of NADP, 8 μmoles of glucose 6-phosphate, 2 IU of glucose 6-phosphate dehydrogenase, 6 μmoles of MgCl₂, 50 μmoles of sodium potassium phosphate (pH 7.4) in a final volume of 1.0 ml. The incubation tubes were sealed with a rubber serum cup and the mixture was bubbled through a needle with oxygen-free nitrogen gas for 5 min. To maintain the anaerobicity in the tube, the incubation tube was sealed by covering the rubber cup with Parafilm®. NADP was added after bubbling with the nitrogen gas. The reaction was started by addition of carbon tetrachloride (1 μmole/10 μl ethanol) after pre-incubation at 37° for 2 min. Incubations were carried out at 37° for 2 min with appropriate shaking and stopped by dipping in dry ice-acetone. A 1.5 ml of n-heptane was injected through the rubber cup, shaken for 2 min and then centrifuged for 3 min at 3000 rpm. A portion (1.0 ml) of n-heptane layer was transferred to another tube and chloroform was analyzed by gas chromatography. Halothane (50 nmoles) was added as an internal standard, and an aliquot (1 μl) was injected into a gas chromatograph (Hewlett-Packard, Model 5840A) equipped with a 63Ni electron capture detector using an automatic sampler. The other conditions for the gas chromatographic analysis are as follows: column; glass (6 ft × 3 mm id) packed with 5% SE-30 on chromosorb W HP (80–100 mesh), temperatures; injection 150°, column 40° and detector 200°, carrier gas; 5% methane in argon. The incubation mixture for examination of the effect of carbon tetrachloride on the activities of microsomal enzymes contained 10 mg of microsomes, 0.8 μmole of NADP, 8 μmoles of glucose 6-phosphate, 2 IU of glucose 6-phosphate dehydrogenase, 6 μmoles of MgCl₂, 150 μmoles of sodium potassium phosphate (pH 7.4) and 1 μmole of carbon tetrachloride (in ethanol, 10 μl) in a final volume of 3.0 ml. In some experiments, methylviologen (3 μmoles) was added to the incubation mixture. The incubation conditions were almost the same as for the assay of chloroform, except that the incubation was carried out for 20 min. The reaction was terminated as described above, then the microsomes were washed by suspending with 6 ml of cold, distilled water and centrifugation at 105,000 g for 30 min. The contents of cytochromes P-450, P-420 and b₅ in the washed microsomes were measured by the method of Omura and Sato (6) and the activity of NADPH-cytochrome c reductase by the method of Phillips and Langdon (7) using cytochrome c as an electron acceptor.

NADPH, NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Japan. Cytochrome c was supplied by Boehringer Mannheim. Carbon tetrachloride, ethanol and n-heptane were of spectral grade. Other reagents were purchased from commercial routes but were of the highest grade.
RESULTS

Effect of carbon monoxide on the metabolism of carbon tetrachloride determined as NADPH oxidation rate: It was reported that NADPH-dependent reductive dehalogenation of carbon tetrachloride occurs in microsomes from phenobarbital-treated rabbits (8). To obtain further evidence supporting the idea that the reduction of carbon tetrachloride is mediated by cytochrome P-450, the effect of carbon monoxide on the carbon tetrachloride-induced change in the rate of NADPH oxidation was measured. Fig. 1 shows the effect of carbon monoxide on the rate of NADPH oxidation in microsomes from phenobarbital-treated rat livers. In the anaerobic condition, the initial rate of NADPH oxidation due to the addition of carbon tetrachloride was 7.25 nmoles per mg of protein per min. The linearity of the reaction was, however, lost within the first 2 min of incubation and the rate was decreased with time. The addition of oxygen-free carbon monoxide in place of nitrogen resulted in the decreased rate of NADPH oxidation. The rate was 0.73 n mole per mg of protein per min, representing that approximately 90% of the activity was inhibited by carbon monoxide.

Reducive dehalogenaion as determined by chloroform formation: Chloroform is reported to be formed as a reductive dehalogenation product of carbon tetrachloride (8). The formation of chloroform as well as the cytochrome P-450-mediated reductive reactions such as reduction of tertiary amine N-oxides and benzo(a)pyrene 4,5-oxide (9, 10) is inhibited by oxygen. The inhibition is probably due to the competition between the substrate and oxygen to bind to the ligand of the cytochrome heme. Methylviologen is assumed to enhance the reductive reactions of tertiary amine oxide and arene oxide by transferring electrons from NADPH-cytochrome c reductase to cytochrome P-450 (11) since the electron transfer from NADPH-cytochrome c reductase to cytochrome P-450 limits the rate of drug metabolism (12). In addition, methylviologen in the reaction medium enhanced the reduction rates since the reduced methylviologen consumes oxygen and produces almost complete anaerobicity. Therefore, the effect of methylviologen on the reduction of carbon
tetrachloride was determined as a chloroform formation rate. As can be seen in Fig. 2, the addition of methylviologen to the reaction mixture resulted in about 2.3-fold increase in the activity. The activity reached plateau level when the concentration of methylviologen was higher than 0.5 mM. The enhancement of chloroform formation by methylviologen was further examined as a function of the incubation time (Fig. 3). The addition of 0.5 mM methylviologen enhanced the formation of chloroform, however, the ratio of the activity in the presence to the absence of methylviologen was nearly constant during the incubation period of 10 min.

Effects of methylviologen on the carbon tetrachloride-induced loss of cytochrome P-450: As described above, the reduction of carbon tetrachloride as determined by chloroform formation was enhanced by addition of methylviologen. Thus, if the loss of cytochrome P-450 is closely associated with the reduction reaction, it seems possible to assume that carbon tetrachloride-induced loss of cytochrome P-450 is enhanced by methylviologen. As shown in Fig. 4, the addition of methylviologen resulted in the increase in the loss of cytochrome P-450. To determine whether or not other microsomal enzymes are impaired by carbon tetrachloride, microsomes were incubated with carbon tetrachloride in the presence and absence of methylviologen (Table 1). As shown in Fig. 4, the addition of methylviologen produced the larger decrease in the cytochrome P-450 content. Carbon tetrachloride also decreased the apparent content of cytochrome P-420, however, the extent of the decrease was not enhanced by methylviologen. It was reported that the activity of NADPH-cyto-
The content of cytochrome b₅ and the activity of NADPH-cytochrome c reductase were not affected by carbon tetrachloride, even in the presence of methylviologen. To further confirm that the carbon tetrachloride-induced loss of cytochrome P-450 is caused by the direct attack of a reactive intermediate formed during carbon tetra-chloride reduction, but is not caused by the indirect oxidative reaction, lipid peroxidation, the effect of EDTA, a potent inhibitor of lipid peroxidation, on the carbon tetrachloride-induced loss of cytochrome P-450 was examined (Fig. 5). The content of cytochrome b₅ was not affected by aerobic incubation (13). In our experiments, the content of cytochrome b₅ and the activity of NADPH-cytochrome c reductase were not affected by carbon tetrachloride, even in the presence of methylviologen.

Table 1. Effects of methylviologen on the content of microsomal enzymes

|                | Cyt P-450 (a) | Cyt P-420 (a) | Cyt b₅ (a) | NADPH-cytochrome c reductase (b) |
|----------------|--------------|--------------|------------|---------------------------------|
| MV            | 0.43         | 0.64         | 0.67       | 0.151                           |
| MV            | 0.45         | 0.64         | 0.67       | 0.157                           |

Table 1. Effects of methylviologen on the content of microsomal enzymes

|                | Cyt P-450 (a) | Cyt P-420 (a) | Cyt b₅ (a) | NADPH-cytochrome c reductase (b) |
|----------------|--------------|--------------|------------|---------------------------------|
| MV            | 0.43         | 0.64         | 0.67       | 0.151                           |
| MV            | 0.45         | 0.64         | 0.67       | 0.157                           |

% changed: -54.7 - 58.1 - 57.8 0 0 -5.3 - 8.3

a) nmole/mg protein  b) µmole cytochrome c reduced/mg protein/ml/min  c) Ethanol (10 µl) was added as the control. d) methylviologen. Each value represents the mean of duplicate determinations. Experimental details are the same as described in Fig. 4.
was also determined as a reference. Control experiments were carried out in the absence of carbon tetrachloride. As can be seen, the content of cytochrome P-450 was decreased by the incubation with carbon tetrachloride and EDTA did not effect any protection towards the loss. This result indicates that in our experimental conditions, cytochrome P-450 was lost solely by a reactive intermediate formed during reductive metabolism of carbon tetrachloride, but not by the formation of lipid peroxides.

DISCUSSION

As proposed by Uehleke et al. (8), it can be confirmed that chloroform is produced through reductive dehalogenation of carbon tetrachloride since chloroform was formed by an anaerobic incubation and, further, the activity was enhanced by addition of methylviologen.

Glende et al. (3) demonstrated that cytochrome P-450 was not degraded by carbon tetrachloride after anaerobic incubations in the presence of EDTA. However, as shown in Fig. 4 and Table 1 in this paper, cytochrome P-450 was markedly diminished by the anaerobic incubation. Data obtained using methylviologen strongly supported the idea that cytochrome P-450 is degraded by reductive reaction(s). The discrepancy between the results of Glende et al. and those of the present studies are probably caused by the difference of the methods for determination of cytochrome P-450. We measured the cytochrome content after removing carbon tetrachloride by washing since cytochrome P-450-CCl₄ complex interferes the determination of cytochrome P-450 content, whereas Glende et al. (3) measured the content without washing the microsomes. Based on such evidence, it can be concluded that the formation of chloroform and degradation of cytochrome P-450 are associated with a common chemical step. The first of the two electrons received by cytochrome P-450 may be transferred to carbon tetrachloride in order to produce trichloromethyl radical and chloride. The trichloromethyl radical thus formed may be able to bind cytochrome P-450 directly.

Glende et al. (3) proposed that the degradation of cytochrome P-450 was induced solely by lipid peroxidation which was initiated by the trichloromethyl radical. As stated above, our results provide further evidence that cytochrome P-450 is degraded directly by interacting with the reductive product. To eliminate the possibility of involvement of lipid peroxidation in the degradation of cytochrome P-450 in our experimental conditions, we examined the effects of methylviologen on the degradation of cytochrome P-450 and lipid peroxidation. Although the formation of lipid peroxides was hardly detected as malondialdehyde (data not shown), probably owing to a complete consumption of remaining oxygen by reduced methylviologen, the degradation of cytochrome P-450 was rather enhanced by the presence of methylviologen.

It was reported that certain peroxides or reactive intermediates formed during aerobic incubations degraded cytochrome P-450 even in the absence of carbon tetrachloride (14). Therefore, lipid peroxidation is assumed to be one of the causes for the degradation of cytochrome P-450 in aerobic environments.
REFERENCES

1) SASAME, H.A., CASTRO, J.A. AND GILLETTE, J.R.: Studies on the destruction of liver microsomal cytochrome P-450 by carbon tetrachloride administration. Biochem. Pharmacol. 17, 1759-1768 (1968)

2) REINER, O., ATHANASSOPOULOS, S., HELLMER, K.H., MURRAY, R.E. AND UEHELEKE H.: Bildung von Chloroform aus Tetrachlorkohlenstoff in Lebermikrosomen, Lipid peroxidation und Zerstorung von Cytochrom P-450. Arch. Toxicol. 29, 219-233 (1972)

3) GLENDE, J.R., HRUSZKIEWYCZ, A.M. AND RECKNAGEL, R.O.: Critical role of lipid peroxidation in carbon tetrachloride-induced loss of aminopyrine demethylase, cytochrome P-450 and glucose 6-phosphate. Biochem. Pharmacol. 25, 2163-2170 (1976)

4) DE TORANZO, E.G.D., DIAZ GOMZ, M.I. AND CASTRO, J.A.: Mechanism of in vivo carbon tetrachloride-induced liver microsomal cytochrome P-450 destruction. Biochem. biophys. Res. Commun. 64, 823-828 (1975)

5) KAMATAKI, T. AND KITAGAWA, H.: Effects of lyophilization and storage of rat liver microsomes on activity of aniline hydroxylase, contents of cytochrome b5 and cytochrome P-450 and aniline-induced P-450 difference spectrum. Japan. J. Pharmacol. 24, 195-203 (1974)

6) OMURA, T. AND SATO, R.: The carbon monoxide-binding pigment of liver microsomes I. Evidence for its hemoprotein nature. J. biol. Chem. 239, 2370-2378 (1964)

7) PHILLIPS, A.H. AND LANGLEY, R.G.: Hepatic triphosphopyridine nucleotide-cytochrome c reductase: Isolation, characterization, and kinetic studies. J. biol. Chem. 237, 2652-2660 (1962)

8) UEHELEKE, H., HELLMER, K.H. AND TABARELLI, S.: Binding of 14C-carbon tetrachloride to microsomal protein in vitro and formation of CHCl3 by reduced liver microsomes. Xenobiotaica 3, 1-11 (1973)

9) SUGIURA, M., IWASAKI, K. AND KATO, R.: Reduction of tertiary amine N-oxides by liver microsomal cytochrome P-450. Mol. Pharmacol. 12, 322-334 (1976)

10) KATO, R., IWASAKI, K., SHIRAGA, T. AND NOGUCHI, H.: Evidence for the involvement of cytochrome P-450 in reduction of benzo(a)pyrene 4,5-oxide by rat liver microsomes. Biochem. biophys. Res. Commun. 70, 681-687 (1976)

11) KATO, R., IWASAKI, K. AND NOGUCHI, H.: Reduction of tertiary amine N-oxides by cytochrome P-450. Mechanism of the stimulatory effect of flavins and methylviologen. Mol. Pharmacol. 14, 654-664 (1978)

12) MIWA, G.T., WEST, S.B. AND LU, A.Y.H.: Studies on the rate-limiting enzyme component in the microsomal monooxygenase system. Incorporation of purified NADPH-cytochrome c reductase and cytochrome P-450 into rat liver microsomes. J. biol. Chem. 253, 1921-1929 (1978)

13) MASUDA, Y. AND MURANO, T.: Carbon tetrachloride-induced lipid peroxidation of rat liver microsomes in vitro. Biochem. Pharmacol. 26, 2275-2282 (1977)

14) LEVIN, W., LU, A.Y.H., JACOBSON, M., KUNTZMAN, R., POYER, J.L. AND MACAY, P.B.: Lipid peroxidation and the degradation of cytochrome P-450 heme. Arch. Biochem. Biophys. 158, 842-852 (1973)