Inhibition of the Induction and Activity of Hepatic P450IA Isozymes by *In Vivo* Administration of Carbon Tetrachloride to Rats

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The effect of carbon tetrachloride (CCl₄) on the induction and activity of 3-methylcholanthrene (MC)-inducible P450IA isozymes (P450IA1 and P450IA2) in the liver was examined by treating male Sprague-Dawley rats with simultaneous i.p. injections of MC and CCl₄. Following the treatment, the quantities of P450IA mRNAs determined by Northern blotting and P450IA proteins by Western blotting were significantly reduced as compared with those in rats treated with MC alone. As determined by a bacterial mutation test using carcinogenic aromatic amines as P450IA enzyme substrates, CCl₄ treatment selectively inhibited the activity of P450IA2 in the MC-induced isozymes. These results indicate that CCl₄ exerts an inhibitory effect on the induction of P450IA isozymes and that it selectively inhibits P450IA2 activity.

Keywords cytochrome P450; P450IA isozyme; carbon tetrachloride; rat; liver

Carbon tetrachloride (CCl₄) acts as a suicide-inhibitor of a certain cytochrome P450 (P450) isozyme¹⁻⁴⁾ and also inhibits the formation of polyribosomes and synthesis of several proteins.⁵⁾ We have demonstrated previously that treatment of MC-pretreated rats with CCl₄ leads to a selective destruction of P450IA2 in the MC-induced P450IA isozymes in the liver.^{6,7)} Recently, this finding was supported by Sesardic *et al.*⁸⁾ However, the effect of CCl₄ on the synthesis of P450 isozymes has not been reported. Therefore, we examined the effect of CCl₄ on the induction of P450IA isozymes and on their enzyme activities in rat liver.

We report here that *in vivo* administration of CCl₄ inhibits the induction of P450IA mRNAs and P450IA proteins and that it inactivates preferentially P450IA2 in MC-induced P450IA isozymes.

Materials and Methods

Chemicals 3-Amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) and 2-amino-6-methylpyrido[1,2-a:3',2'-d]imidazole (Glu-P-1) were kindly donated by Drs. T. Sugimura and M. Nagao, National Cancer Center Research Institute, Tokyo. MC and CCl₄ were obtained from Wako Pure Chemical Industries, Osaka, Japan.

Animal Treatment Male Sprague-Dawley rats, 7–9 weeks old, were obtained from Shizuoka Agricultural Corporation for Laboratory Animals Hamamatsu, Japan. Rats were treated with a single i.p. injection of MC (0.11 mmol/kg) or MC (0.11 mmol/kg) together with CCl₄ (5.2 mmol/kg) in corn oil and were killed at 6, 12 and 24 h after each chemical treatment. Control rats were given vehicle alone. Hepatic microsomes were prepared by differential centrifugations as described in our previous paper.⁹⁾ The amounts of P450 and protein in the microsomes were assayed by the methods of Omura and Sato¹⁰⁾ and Lowry *et al.*,¹¹⁾ respectively.

Mutation Assay Hepatic microsomal activities for mutagenic activations of Trp-P-2 and Glu-P-1 (each 2 nmol/plate) towards *Salmonella typhimurium* TA98 were assayed by the method described in our previous paper.⁷⁾ Throughout the present experiments, the number of spontaneous revertant colonies was in the range of 10–30 per plate. The data shown in this paper represent the values obtained from subtracting the number of spontaneous revertants from the total number of colonies with a substrate.

Northern Blot Analysis Cytoplasmic RNAs were prepared from liver tissue by the method of Chomczynski and Sacchi.¹²⁾ P450IA2 cDNA (pc-P-450-mc-3) was kindly donated by Dr. Y. Fujii-Kuriyama, Department of Chemistry, Faculty of Science, Tohoku University, Sendai, Japan. Analysis of P450IA mRNAs was performed by the method of Kawajiri *et al.*¹³⁾ with pc-P-450-mc-3, which is a cloned P450IA2 cDNA including the full length of the P450IA2 nucleotide sequence and cross-reactive with P450IA1 mRNA.

Immunochemical Analysis Hepatic microsomal P450 enzymes were characterized by means of protein A-enzyme-linked immunosorbent assay (protein A-ELISA) and Western blotting using mouse anti-rat P450IA enzyme monoclonal antibodies (MoAbs; APH-3, APL-1 and APL-2), as reported previously.^{6,14,15)} APH-3 and APL-1 MoAbs are selectively reactive with P450IA2 and P450IA1, respectively. APL-2 MoAb is reactive with both P450IA enzymes but only slightly with other P450 enzymes. All MoAbs used show no inhibitory effect on a P450IA enzyme-mediated reaction.

For protein A-ELISA, microsomes (100 µg/well) were fixed to wells of Costar No. 2590 polyvinyl chloride strips by incubating at 4 °C overnight and then successively treated with the following solutions (each 100 µl): (i) 1% bovine serum albumin (BSA) in PBS, pH 7.2, (ii) anti-P450 MoAb, (iii) rabbit anti-mouse immunoglobulin (Zymed Laboratory Inc., San Francisco, CA) in 1% BSA-PBS, (iv) horse-radish peroxidase-conjugated protein A, and (v) citrate buffer solution, pH 4.0, containing 0.05% 2,2'-azino-di(3-ethylbenzthiazole)-6-sulfonic acid (Sigma) and 0.01% H₂O₂. Absorbance of the developed color was measured at 414 nm using an InterMed NJ-200 automatic ELISA reader.

For Western blotting, microsomal preparations (40 µg protein/lane) were solubilized with sodium dodecyl sulfate (SDS) and developed by SDS-polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred from the gels to nitrocellulose sheets and then immunostained by means of protein A-ELISA using APL-2 MoAb and 0.05% 3,3'-diaminobenzidine tetrachloride (Sigma).

Results

Effect of CCl₄ on the Total Microsomal P450 Content and Activities of P450IA Isozymes Rats were treated with MC or MC plus CCl₄ (MC/CCl₄) and periodically monitored with respect to the total content of hepatic microsomal P450 and microsomal activity for the mutagenic conversion of Trp-P-2 and Glu-P-1. As shown in Table I, treatment with MC alone did not change the total P450 content, whereas a significant reduction in the content was observed with MC/CCl₄ and this manifested itself as early as 6 h after treatment.

The hepatic microsomal activities for the mutagenic activations of Trp-P-2 and Glu-P-1 were examined by means of the Ames mutation test using *Salmonella typhimurium* TA98 as test strain. Trp-P-2 is efficiently converted to mutagenic form(s) by either P450IA1 or P450IA2, while Glu-P-1 mutagenesis is catalyzed predominantly by P450IA2, but only slightly by P450IA1.⁷⁾ The microsomal activity for both Trp-P-2 and Glu-P-1 mutageneses was induced by MC-treatment and this induction was suppressed

TABLE I. Effects of MC- and MC/CCl₄-Treatments on the Microsomal Activities for the Mutageneses of Trp-P-2 and Glu-P-1 in Rat Liver

Chemical treatment	Time (h) after treatment	P450 content (nmol/mg protein)	Microsomal activity; No. revertants/mg protein		Substrate-specificity (Trp P-2/Glu-P-1)
			Substrates		
			Trp-P-2	Glu-P-1	
None (control)	—	0.64 (1.0) ^{a)}	2100 (1.0)	1300 (1.0)	1.5
MC	6	0.64 (1.0)	6800 (2.4)	2100 (1.7)	3.2
MC + CCl ₄	6	0.14 (0.2)	2900 (1.3)	400 (0.3)	7.3
MC	12	0.58 (0.9)	32000 (15.2)	7700 (5.9)	3.4
MC + CCl ₄	12	0.15 (0.2)	13700 (6.5)	1600 (1.2)	8.5
MC	24	0.70 (1.1)	85500 (40.7)	26700 (20.5)	3.2
MC + CCl ₄	24	0.37 (0.6)	61900 (29.5)	7700 (5.9)	8.0

Hepatic microsomes were prepared from the pooled livers of 3 rats in each group and were used as enzyme sources. Values shown represent the means of triplicate samples and the standard errors of the means (SEMS) were less than 15% of the means. a) Values shown in parentheses represent the ratios with respect to the corresponding controls.

Fig. 1. Northern Blotting of P450IA mRNAs from the Livers of MC- and MC/CCl₄-Treated Rats

RNA was extracted from the pooled livers of three rats in each group. An aliquot of the RNA (20 and 5 µg/lane for untreated rats and rats treated with chemical, respectively) was subjected to Northern blot analysis using a P450IA2 cDNA probe, as described in Materials and Methods. Lane a, untreated (control) rats; lanes b, d and f, MC-treated rats; lanes c, e and g, MC/CCl₄-treated rats.

by simultaneous treatment with CCl₄ (Table I). As reported previously,¹⁶⁾ the ratio between the activities for Trp-P-2 and Glu-P-1 mutageneses represents the involvement of either P450IA1 or P450IA2 activity in the assay. At any time (6, 12 and 24 h) after drug treatment, this ratio (Trp-P-2/Glu-P-1) was increased about 2-fold by MC and about 5-fold by MC/CCl₄ as compared with the values in the untreated (control) rats. This finding indicates that CCl₄ preferentially inhibits P450IA2 activity compared with P450IA1 activity.

Effect of CCl₄ on the Induction of P450IA mRNAs
Hepatic RNAs were prepared from control rats and rats treated with MC or MC/CCl₄ and the amounts of P450IA mRNAs were monitored by Northern blotting using of pcP-450 mc-3 as a probe. The results are shown in Fig. 1. P450IA2 mRNA, but not P450IA1 mRNA, was detected in untreated control rats. When rats were treated with MC, the mRNAs corresponding to both P450IA1 and P450IA2 were increased in a time-dependent fashion. Additional treatment with CCl₄ retarded and suppressed the induction of both P450IA mRNAs compared with MC treatment alone.

Effect of CCl₄ on the Amount of P450IA Proteins
Changes in the amounts of P450IA1 and P450IA2 protein in hepatic microsomes were determined by means of protein

Fig. 2. Time-Dependent Changes in P450IA2 and IA1 in MC- and MC/CCl₄-Treated Rat Liver Microsomes

Microsomes were prepared from the pooled livers of three rats in each group. An aliquot of the microsomes (100 µg/well) was fixed to wells of Costar no. 2590 polyvinyl chloride strips and assayed from their reactivities of APH-3 (for P450IA2) and APL-1 (for P450IA1) MoAbs by protein A-ELISA. ○, untreated rats; □, MC-treated rats; △, MC/CCl₄-treated rats. Detectable amount of each purified P450IA isozyme was > 2 pmol (OD₄₁₄, 0.05). Each symbol shown represents the mean of triplicate samples and the bars, the standard error of the mean (SEM).

Fig. 3. Western Blotting of Hepatic Microsomes from MC- and MC/CCl₄-Treated Rats

Microsomes were prepared from the pooled livers of three rats in each group, and an aliquot of the microsomes (40 µg/lane) was subjected to Western blotting. Proteins were immunostained with APL-2 MoAb. Lane a, untreated rats; lanes b, d and f, MC-treated rats; lanes c, e and g, MC/CCl₄-treated rats.

A-ELISA and Western blotting using anti-rat P450IA MoAbs. Results obtained by the protein A-ELISA with APH-3, and APL-1 MoAbs, which react specifically with P450IA2 and P450IA1, respectively, are shown in Fig. 2. In accordance with the results of assays for activity and mRNA, the increase in P450IA proteins, especially P450IA2 protein, produced by MC-treatment was suppressed by an additional treatment with CCl₄. In MC-treated rats, both P450IA1 and IA2 levels were increased as early as 6 h after drug treatment. Whereas in MC/CCl₄-treated rats, P450IA2 was not detected at 6 h. After 24 h, levels of

P450IA2 and IA1 in MC/CCl₄-treated rats were 25% and 70% of those in MC-treated rats, respectively.

To clarify the molecular character of CCl₄-sensitive P450 enzymes, we performed further Western blotting of hepatic microsomes obtained from MC- or MC/CCl₄-treated rats. The profiles of the blotting using APL-2 MoAb are shown in Fig. 3. In control rat liver, the APL-2-reactive component (mol. wt. 54000), which corresponds to P450IA2, was detected but P450IA1 (mol. wt. 56000) was not. The components corresponding to P450IA1 and P450IA2 proteins were increased by MC-treatment and this induction was suppressed by simultaneous treatment with CCl₄.

Discussion

We have demonstrated here that CCl₄ inhibited the MC-mediated induction of P450IA isozymes with respect to both their mRNA and protein levels. Although MC-treatment did not affect significantly the amount of P450 per microsomal protein in rat liver, it increased the microsomal activities per protein for Trp-P-2 and Glu-P-1 mutageneses as early as 6 h after treatment. The increase in the microsomal activities was dependent on those in MC-induced P450IA mRNAs and proteins which were determined by means of Northern and Western blotting, respectively. On the other hand, simultaneous treatment of rats with MC and CCl₄ (MC/CCl₄) decreased the total amount of microsomal P450; the amount at 6 h was 20% of the control, about 20% at 12 h, and 60% at 24 h after treatment. At any time (6, 12 and 24 h) after chemical treatment, the microsomal activities per P450 for the mutageneses of Trp-P-2 and Glu-P-1 in MC/CCl₄-treated rats were lower than in the corresponding MC-treated rats but higher than in the control rats, suggesting that MC/CCl₄-treatment also resulted in an increase in the amount of MC-inducible P450IA isozymes. However, the increment was smaller than in rats treated with MC alone. This was demonstrated by Northern and Western blot analyses for MC- and MC/CCl₄-treated rat livers. Thus, simultaneous treatment with CCl₄ inhibited the production of P450IA isozymes by reducing the amount of MC-induced P450 mRNAs. In addition, the substrate-specificity (Trp-P-2/Glu-P-1 ratio) of the hepatic microsomes differed between MC- and MC/CCl₄-treated rats, and at any time (6, 12 and 24 h) after chemical treatment, the ratio in the latter was more than twice that in the former. The difference in the substrate-specificity of the microsomes was also demonstrated by means of Protein A-ELISA with anti-P450IA MoAbs, and this may be due to preferential degradation of P450IA2 by CCl₄ in MC-induced P450IA isozymes.⁶⁻⁸⁾

CCl₄ was able to non-selectively decrease the production of MC-induced P450IA mRNAs, suggesting that CCl₄ decreased the total amount of P450 below control levels by reducing the production of both P450IA and constitutive P450 mRNAs in MC/CCl₄-treated rats. The mechanism of the CCl₄-mediated reduction in P450 mRNAs is still uncertain, although it is known that CCl₄ inhibits the formation of polyribosomes,⁵⁾ which are necessary for protein synthesis.

Although administration of CCl₄ to MC-pretreated rats leads to a selective degradation of P450IA2 in MC-induced P450IA isozymes,⁶⁻⁸⁾ we have demonstrated in this experiment that *in vivo* treatment with CCl₄ inhibits not only the activity of P450IA2 in MC-induced P450IA isozymes but also the induction of both P450IA isozyme mRNAs by MC in the rat liver.

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