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In vivo selection of a low spin form of cytochrome P-448 from 3-methylcholanthrene-induced rat cytochrome P-450 isozymes by carbon tetrachloride

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Cytochrome P-450 isozymes catalyze oxidative metabolism of a variety of chemicals with different substrate specificities in each isozyme. They are inducible by treatment of animals with chemicals called drug metabolizing enzyme inducers. Proportions of cytochrome P-450 isozymes present in drug-induced microsomes are also different with different inducers. To investigate the precise role of a cytochrome P-450 isozyme in drug metabolism, it is desirable to obtain selective inducers for a certain cytochrome P-450 isozyme. We previously demonstrated that carcinogenic aromatic amines such as 4-aminoazobenzene derivatives and pyrolysate components of amino acids are converted to mutagenic or carcinogenic metabolites via the help of cytochrome P-448H, which is selectively inducible by treatment of rats with these carcinogenic aromatic amines [1, 2]. In contrast to aromatic amines, the activation of polycyclic aromatic hydrocarbons including 3-methylcholanthrene (MC) is found to be mainly mediated by cytochrome P-448L. However, selective inducers of cytochrome P-448L have not been found. Recently, Enosawa and Nakazawa [3] have shown that the treatment of MC-pretreated rats with carbon tetrachloride (CCl_4) results in a striking decrease of the activities of aminopyrine *N*-demethylase and aniline *p*-hydroxylase, but little affects benzo[*a*]pyrene hydroxylase. These data suggest that CCl_4 administration to MC-pretreated rats may cause selective degradation of cytochrome P-448H (Levin's cytochrome P-450d), leading to selective retention of cytochrome P-488L (Levin's cytochrome P-450c) in the hepatic microsomes. However, cytochrome P-450 species changed by the CCl_4 administration has not been characterized. In the present paper, we examined the effect of CCl_4 administration on the composition of microsomal cytochrome P-450 isozymes in MC-pretreated rats by means of mutation test with two substrates having different specificity to MC-induced cytochrome P-450 isozymes and further by immunochemical analyses using anti-cytochrome P-448 monoclonal antibodies.

Materials and methods

Chemicals. 3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole acetate (Trp P-2) and 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*] imidazole acetate (Glu P-1) were kindly donated by Drs. T. Sugimura and M. Nagao, National Cancer Center Research Institute, Tokyo. 3-Methylcholanthrene (MC) and carbon tetrachloride (CCl_4) were obtained from Wako Pure Chemical Industries, Osaka, Japan.

Animal treatment. Male ACI/N rats of 7–9 weeks old obtained from Hoshino Laboratory Animal Co. (Saitama, Japan) were used. Treatment of rats with MC and/or CCl_4 were performed as described by Enosawa and Nakazawa [3] but with a slight modification. Briefly, rats were pretreated by i.p. injection of a single dose of MC in corn oil (0.11 mmoles/kg). Forty-eight hours after the MC treatment, CCl_4 (5.2 mmoles/kg) dissolved in corn oil were given i.p. In prior to the CCl_4 administration, the animals were fasted overnight. Control animals were given the vehicle only.

Preparation of hepatic microsomes. Hepatic microsomes were prepared by differential centrifugation of the liver homogenates as described in a previous paper [4]. Protein and cytochrome P-450 contents in the microsomes were assayed by the methods of Lowry *et al.* [5] and Omura and Sato [6], respectively.

Mutation assay. Mutagenicities of Trp P-2 and Glu P-1 (each 2 nmoles/plate) toward *Salmonella typhimurium* TA98 were assayed as reported previously [4]. Throughout the present experiment, the number of spontaneous revertant colonies was in the range of 10–30 per plate.

Immunochemical characterization of cytochrome P-450 isozymes. Liver microsomal cytochrome P-450 isozymes were characterized by means of both protein A-enzyme-linked immunosorbent assay (Protein A-ELISA) and immuno-blotting using anti-cytochrome P-448 monoclonal antibodies (APH-3, APL-1 and APL-2) as reported previously [7]. APL-1 and APL-2 are newly obtained from hybridoma clones established by a fusion between P3×63Ag8.653 mouse myeloma cells and spleen cells of a BALB/c mouse hyperimmunized with partially purified cytochrome P-448L (details of their characteristics will be reported elsewhere). APH-3 and APL-1 are selectively reactive with cytochrome P-448H and P-448L, respectively. APL-2 is reactive with both forms of cytochrome P-448 but hardly reactive with phenobarbital-induced cytochromes P-450. Cytochrome P-448L and P-448H are suggested to be identical with Levin's P-450c and P-450d, respectively [8, 9].

Results and discussions

We first examined the effect of CCl_4 administration on microsomal cytochrome P-450 content in MC-pretreated rats and the enzyme activities of microsomes for mutagenic activations of Trp P-2 and Glu P-1 (Table 1). The liver microsomal cytochrome P-450 content at the 48th hour after MC-treatment was increased to the 1.3-fold of control ($P < 0.01$). Thirty minutes after treatment of the MC-treated rats with CCl_4, cytochrome P-450 content was decreased to about 60% of that of CCl_4-untreated rats, and the decreased cytochrome P-450 content was not recovered at least for 6 hr. The microsomal enzyme activity was then assayed by mutation test using two substrates, Trp P-2 and Glu P-1. The Trp P-2 mutagenesis is efficiently mediated by either cytochrome P-448H or P-448L, however, Glu P-1 mutagenesis efficiently mediated by the former but inefficient by the latter isozyme [1, 2, 8]. When the microsomal activities in mutagenic activation of Trp P-2 and Glu P-1 were examined, the microsomes from MC-treated rats showed much greater activity than those of untreated control rats (99-fold of the control for Trp P-2 mutagenesis and 29-fold for Glu P-1 mutagenesis). The microsomal activity to Glu P-1 mutagenesis was strikingly decreased (12–14% of that of MC-pretreated rats) as early as 30 min after administration of CCl_4 to the MC-treated rats. The microsomal activity to Trp P-2 mutagenesis, however, was not significantly decreased by the CCl_4 treatment. These results suggest that the CCl_4 administration to MC-pretreated rats may lead to selective degradation of cytochrome P-448H in the MC-induced cytochrome P-448 isozymes.

To confirm this assumption, we further examined immunological properties of hepatic cytochrome P-450 isozymes retained after CCl_4 treatment by means of protein A-ELISA and immuno-blotting using anti-cytochrome P-448 monoclonal antibodies (APH-3, APL-1 and APL-2). As to the specificity of anti-cytochrome P-448 monoclonal antibodies, APH-3 and APL-1 are selective to cytochrome P-448H and P-448L, respectively. APL-2 is reactive with both the cytochromes and but not with phenobarbital-

Table 1. Effect of CCl_4 administration on cytochrome P-450 isozymes in the liver microsomes of MC-pretreated rats

Chemical treatment	Time after CCl_4 treatment (hr)	Cytochrome P-450 content (nmoles/mg protein)	Mutagenic activation (No. revertants/pmole cytochrome P-450) Substrate		Protein A-ELISA (OD, 414nm) Monoclonal antibody	
			Trp P-2	Glu P-1	APH-3	APL-1
—	—	0.96 ± 0.03	6 ± 1	3 ± 1	0.01 ± 0.01	0.03 ± 0.04
MC	—	1.25 ± 0.00	493 ± 67	87 ± 2	0.33 ± 0.03	0.55 ± 0.04
MC + CCl_4*	0.5	0.75 ± 0.02†	451 ± 33	10 ± 2‡	0.09 ± 0.02†	0.51 ± 0.02
MC + CCl_4	1	0.67 ± 0.02‡	469 ± 29	12 ± 0‡	0.05 ± 0.02†	0.45 ± 0.02
MC + CCl_4	3	0.79 ± 0.06§	414 ± 36	12 ± 1‡	0.06 ± 0.04†	0.62 ± 0.05
MC + CCl_4	6	0.76 ± 0.03†	406 ± 36	10 ± 1‡	0.03 ± 0.01‡	0.61 ± 0.05

Cytochrome P-450 levels represent the mean ± SEM for three animals. Mutation assay and Protein A-ELISA were performed using microsomal protein containing about 30 pmoles of cytochrome P-450. These results represent the mean ± SEM of triplicate samples using a pooled microsomes from three rat livers. The mutagenic data were calculated from the values subtracted No. of the spontaneous revertants from total colony number.

* CCl_4 was administered to rats pretreated with MC 48 hr before.

†‡§ Statistical difference to MC-treatment; † $P < 0.01$; ‡ $P < 0.001$; § $P < 0.05$.

induced cytochrome P-450s. As shown in Table 1, the component reactive to APH-3 was markedly decreased to about 30% of that of MC-treated rats as fast as 30 min after the CCl_4 administration. This was in accordance with the result of mutagenicity test using Glu P-1 as a substrate. On the other hand, the CCl_4 treatment little affected the component reactive to APL-1. Figure 1 shows the results of immuno-blotting with a monoclonal antibody, APL-2. In the liver microsomes from untreated control rats, the component reactive to APL-2 was undetectable (see lane a). The MC-treatment resulted in a striking increase of two components which migrated at positions of cytochrome P-448L (m.w. 56,000) and cytochrome P-448H (m.w. 54,000) (see lane b). When CCl_4 was administered to the MC-treated rats, the component of m.w. 54,000 was gradually decreased in time-dependent fashion after the CCl_4 treatment, and it was disappeared after 6 hr, but the amount of the component of m.w. 56,000 was little changed (see lanes c, d, e and f).

Although an MC-induced cytochrome P-448H component detected by APL-2 retained even 3 hr after the CCl_4 treatment, but the binding capacity of a microsomal cytochrome P-448H component to the selective APH-3 antibody and the microsomal enzyme activity responsible for the mutagenic activation of Glu P-1 (selective substrate for cytochrome P-448H-mediated mutagenesis) were lost as early as 30 min after the CCl_4 treatment (see Table 1 and Fig. 1). This discrepancy can be attributable to the stability of the antigenic determinants to these monoclonal antibodies and of the active center of the cytochrome; probably the CCl_4 treatment exerts rapid conformational change of the cytochrome molecule, which may result in hindrance of the reactive site for APH-3 reactive site and results in the loss of the enzyme activity. By contrast, the antigenic determinant to APL-2 antibody in the same cytochrome molecule may be retained longer after the CCl_4 treatment.

The administration of CCl_4 to MC-pretreated rats reduced only cytochrome P-448H and retained cytochrome P-448L, as indicated by bacterial mutation assays using two substrates showing different substrate-specificity to each cytochromes and by immunochemical analyses using anti-cytochrome P-448 monoclonal antibodies.

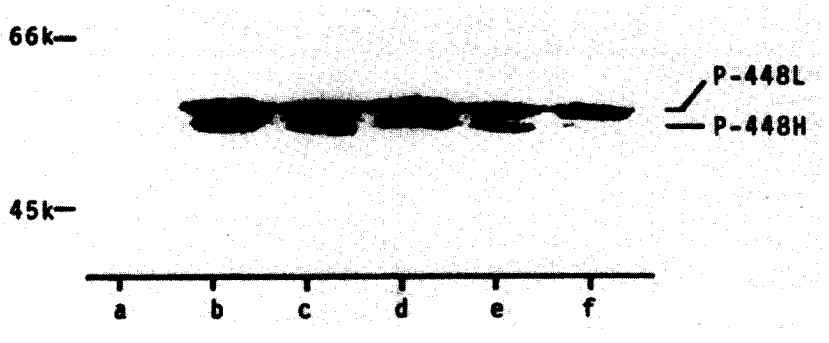

Fig. 1. Immuno-blots of hepatic microsomes. After sodium dodecylsulfate–acrylamide gel electrophoresis of the liver microsomes (about 30 pmoles of cytochrome P-450 per lane) from untreated rats and rats treated with MC only or with MC followed by CCl_4, the cytochrome P-450 components were transferred from the gel to a nitrocellulose sheet and then immunostained with a use of APL-2, mouse peroxidase anti-peroxidase and 0.05% 3,3′-diaminobenzidine tetrachloride. Lane a, untreated microsomes. Lane b, microsomes from rats treated with MC only. Lanes c–f, microsomes from rats treated with MC followed by CCl_4: c, 0.5 hr; d, 1 hr; e, 3 hr, f, 6 hr after the CCl_4 treatment.

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In vitro conversion of pyrazinamide into 5-hydroxypyrazinamide and that of pyrazinoic acid into 5-hydroxypyrazinoic acid by xanthine oxidase from human liver

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An unknown, called compound II, found in human urine containing pyrazinamide and its metabolites, was identified recently by the authors as 5-hydroxypyrazinamide [1], thus establishing that the pathway described by Weiner and Tinker [2] (conversion of pyrazinamide into Compound II) is the same as that described by Pitre *et al.* [3] (conversion of pyrazinamide into 5-hydroxypyrazinamide). However, few *in vitro* studies on the oxidation of pyrazinamide or pyrazinoic acid in human liver have been reported. Considering this need for further study, the investigation of the oxidation of pyrazinamide and pyrazinoic acid by xanthine oxidase found in human liver was undertaken and is herein described.

Materials and methods

A normal human liver was obtained by operative resection following traumatic injury. It was immediately frozen to −80° and stored until used. The homogenates were subjected to differential centrifugation to obtain the cytosol fraction according to the method of Wilgram and Kennedy [4]. The cytosol fraction, precipitated with 30–50% saturated ammonium sulfate, was dialyzed against 10 mM potassium buffer (pH 7.4) containing 1 mM EDTA and 1 mM GSH. Next, using the batch method described previously [5], the dialyzed fraction was treated with hydroxyapatite. After concentrating the post-treated dialyzed supernatant 2-fold, it was incubated by the method of Hande *et al.* [6] except for the use of 1.5 mM dithiothreitol. This dialyzed supernatant fraction was used as the partially purified xanthine oxidase fraction (XO fraction). The specific activity of xanthine oxidase in this fraction was 12.7 nmol uric acid formed · min^{-1} · (mg protein)$^{-1}$, having increased 32.4-fold compared with that in the cytosol fraction. Each reaction mixture consisting of 200 μl of the XO fraction and 100 μl of 50 mM potassium phosphate buffer (pH 7.4) containing the substrate (pyrazinamide or pyrazinoic acid) was incubated at 37° after 10 min of preincubation without substrate. After incubation, extraction was performed by the modified method of Yamamoto *et al.* [7]. The measurements of 5-hydroxypyrazinamide and pyrazinoic acid using HPLC were performed by the method of Yamamoto *et al.* [7] except that the detection of fluorescence was 410/310 nm, the pH of the mobile phase was 2.5, and the flow rate was 1.6 ml/min. Polyacrylamide disc gel electrophoresis was performed by the method of Davis [8] and staining was by the method of Holmes *et al.* [9] using 1 mM hypoxanthine, 80 mM pyrazinamide, 50 mM pyrazinoic acid, 0.5 mM allopurinol, 0.5 mM oxypurinol, 20 mM benzaldehyde or 1.5 mM *N'*-methylnicotinamide (NMN) as the substrate. Xanthine oxidase was measured as described previously [10]. Protein was determined by the method of Lowry *et al.* [11].

Results and discussion

Xanthine oxidase was stained most markedly by hypoxanthine after 60 min of staining. It was also stained by allopurinol, which has been shown to be a powerful and specific inhibitor of xanthine oxidase [12]. It was not stained at all, however, by oxypurinol. The resulting intensity of staining of xanthine oxidase with 1 mM hypoxanthine was inhibited considerably by 0.5 mM oxypurinol over a 10-min staining period, performed after disc gels had been preincubated in the solution with 0.5 mM oxypurinol for 30 min. Furthermore, xanthine oxidase could be stained by benzaldehyde but not by *N'*-methylnicotinamide. It was also stained by pyrazinamide and pyrazinoic acid, though faintly. The addition of 0.5 mM NAD proved to have no effect on the staining patterns of the disc gels. As conversion of pyrazinamide into 5-hydroxypyrazinamide and that of pyrazinoic acid into 5-hydroxypyrazinoic acid, respectively, increased linearly over a 60-min incubation period in the XO fraction, the K_m value of xanthine oxidase for pyrazinamide or pyrazinoic acid was determined after the reaction mixtures were incubated for 40 min. The K_m value for pyrazinamide was 2.4 mM and that for pyrazinoic acid was 0.7 mM. The double-reciprocal plots from experiments on the XO fraction were linear for both pyrazinamide and pyrazinoic acid [Y (1/μmol 5-hydroxypyrazinamide formed per h per mg protein) =0.017 + 0.0412 X (1/mM pyrazinamide), r = 0.99, and Y (1/μmol 5-hydroxypyrazinoic acid