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A Study for Regulation of Ethanol-inducible P_{450}(CYP2E1) on CCl_4-induced Hepatic Damage

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Previous study showed that CCl_4 administration evoked a rapid decrease in cytochrome P_{450} 2E1 protein soon after the exposure due to posttranslational inhibition(Biochem. Biophys. Res. Commun. 179:449-454, 1991). In this report, aniline hydroxylase and the amounts of immunoreactive P_{450} 2E1 were rapidly decreased during day 1 to 2 and recovered during day 3 to 4 after a single dose of CCl_4. The activity of pentoxyresorufin-0-dealkylase was also suppressed at day 1 and began to repair from day 2. However, the decrease in immunoreactive P_{450} 2C content was not observed. The decreases in P_{450} 2E1 enzyme activity and immunoreactive protein by acute CCl_4 treatment were accompanied by a decline in P_{450} 2E1 mRNA level. The data thus suggested a pretranslational reduction of P_{450} 2E1 during day 1 to 2 after acute CCl_4 treatment.

Key words : Carbon tetrachloride, Cytochrome P_{450} 2E1, Cytochrome P_{450} 2C, Aniline hydroxylase, Pentoxyresorufin-0-dealkylase

INTRODUCTION

Carbon tetrachloride is widely used as the hepatotoxic agent. Carbon tetrachloride is metabolized to trichloromethyl radical ($\cdot CCl_3$) in rat liver microsomes both in vitro (Poyer *et al.*, 1978) and in vivo (Lai *et al.*, 1979). This radical quickly destroys only a specific form of liver microsomal cytochrome P_{450}, ethanol-inducible cytochrome P_{450} (P_{450} 2E1), which is an isozymic form of cytochrome P_{450}s associated with metabolism of ethanol, acetaminophen, carcinogen as well as CCl_4 (Noguchi *et al.*, 1982; Koop and Coon, 1987). It is suggested that trichloromethyl radical ($\cdot CCl_3$) formed during metabolism of CCl_4 may attack haem moiety and apoprotein of cytochrome P_{450} (Manno *et al.*, 1988). However, it is still controversial which reactive metabolism is responsible for cytochrome P_{450} destruction. Previous study (Sohn *et al.*, 1991) suggested that a rapid decrease in cytochrome P_{450} 2E1 soon after acute CCl_4 treatment was due to posttranslational inhibition of P_{450} 2E1. Using monoclonal antibodies and cDNA probe for P_{450} 2E1, we have examined molecular regulation of P_{450} 2E1 during polyclonal after acute treatment of rats with carbon tetrachloride. In this communication, we demonstrated a specific reduction of P_{450} 2E1 protein and enzyme activity by CCl_4 which is accompanied by an unexpected concomitant decrease in its mRNA, indicating a pretranslational destruction.

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MATERIALS and METHODS

Chemicals

CCl_4 was purchased from Sigma Chemical Co. (USA). Gene Screen membranes were purchased from NEN (USA) and nitrocellulose membranes from Millipore (USA). All other chemicals were obtained from commercial sources and were the highest grade.

Treatment of animals. Male outbred Sprague-Dawley rats (4 per group, weighing 190-210 gram) were obtained from SamYuk Animal Company. Animals were kept in a 12 h light-dark cycle with food and water. After a single intraperitoneal injection of CCl_4 (2 g/kg body weight diluted in corn oil, 1:1), the animals were sacrificed at the times as described. The liver was rapidly removed, frozen in liquid nitrogen, and stored at -80°C until processed further.

Preparation of microsomal fractions and enzyme assays

Microsomal fractions from the liver of control and CCl_4-treated animals were prepared by calcium aggregation method (Ravindranath and Anandatheerthavarada, 1990) and resuspended in 0.1M Tris, pH 7.4,

containing 20% glycerol, flushed with liquid nitrogen and stored at -80°C. Protein concentration was determined by Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as standard. Aniline hydroxylase activity was determined by measuring p-aminophenol formation (Kim *et al.*, 1988). Pentoxyresorufin-0-dealkylase activity were measured by the method of Lubet et al (Lubet *et al.*, 1985).

Immunoblot analyses

Immunoblot analyses following SDS-polyacryl-amide gel electrophoresis of the samples were performed as previously described (Song *et al.*, 1987). .Polyclonal antibodies against P_{450} 2E1 and P_{450} 2C which were either purchased from the Oxford Biochemicals (Oxford, MI) or kindly provided by Dr. B. J. Song (National Institute of Health, U. S. A.).

Northern mRNA blot analysis

Total RNA was isolated from starved rat liver. Isolated RNA was reverse transcribed by RT-PCR Kit. P_{450} 2E1 cDNA was amplified in PCR reaction solution and two primers such as 5'ATG GCG GTT CTT GGC ATC ACC 3' and 5'TCA TGA ACG GGG AAT GAC ACA 3'. Total liver cytoplasmic RNA from control and treated animals were prepared by the acid guanidine isothiocyanate-phenol-chloroform method (Chemozynski and Sacchi, 1987). Total RNA was separated on 0.66 M formaldehyde 1% agarose gels and electrically transferred to Gene Screen membranes. cDNA probe of P_{450} 2E1 was labeled by random priming method using a kit and [α-^{32}P]dCTP. Prehybridization and hybridization were performed at 63°C overnight by a method of Virca et al (Virca *et al.*, 1990) with a slight modification. The hybridization buffer contained 50mM PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid]), 100mM NaCl, 50mM sodium phosphate, 1mM EDTA, 5% SDS. The washed membranes were subjected to autoradiography by exposure to X-ray film with intensifying screens at -80°C.

RESULTS

In order to study the molecular mechanism of P_{450} 2E1 in acute exposure of CCl_4, the activity of aniline hydroxylase was measured at different times after a single injection of CCl_4. The activity of P_{450} 2E1 was changed by CCl_4 treatment in a time dependent manner (Table I). At 48h after a single injection of CCl_4, the activity of aniline hydroxylase was only 11% of the control, but 72% of the control after 96 h exposure to CCl_4. The activity of pentoxyresorufin-0-dealkylase (P_{450} 2C-related) by CCl_4 was also changed in a time-dependent manner (Table I). P_{450} 2C catalytic activity (pentoxyresorufin-0-dealkylase) was reduced after a single injection of CCl_4 to 3.3% at 24 h, but increased to 51% at 96 h. These results indicate

Table I. Effect of CCl_4 on aniline hydroxylase and pen-

CCl_4 Treatment	Aniline hydroxylase activity[1] (% control)	Pentoxyresorufin-O-dealkylase activity[2] (% control)
Time Oh	0.36±0.07(100)	11.50±3.87(100)
24 h	0.05±0.03(13.9)**	0.38±0.38(3.3)*
48 h	0.04±0.01(11.1)**	2.37±0.03(20.6)
72 h	0.20±0.04(55.6)	4.57±1.45(39.7)
96	0.26±0.02(72.2)	5.86±2.18(51.0)

Data are presented as mean±SEM, n=4.
[1]nmoles p-aminophenol formed/min/mg protein at 37°C.
[2]pmoles resorufin formed/min/mg protein at 25°C.
The significance of differences as compared with control group *p<0.05, **p<0.01.

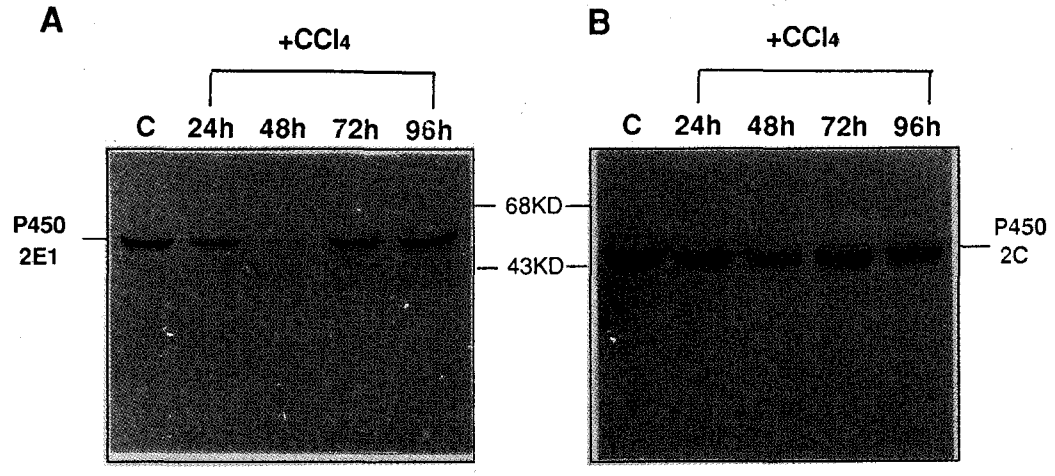

Fig. 1. Western immunoblot analyses for P_{450} 2E1 (A), P_{450} 2C (B) in rat liver microsomes after acute CCl_4 treatment. Equal amounts of microsomal protein (30 μg/well) from control (C) and CCl_4-treated animals at different times as indicated were subjected to immunoblot analyses using specific polyclonal antiserum against P_{450} 2E1 and P_{450} 2C. The molecular size markers designated in the figure were bovine serum albumin (68 kDa) and ovalbumin (43 kDa).

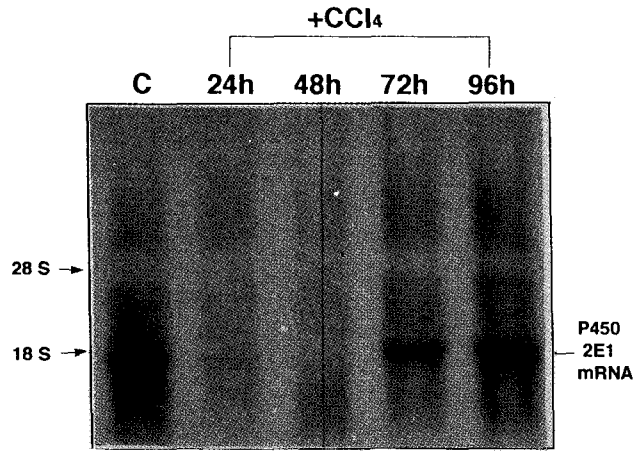

Fig. 2. Northern blot analyses for hepatic P_{450} 2E1 mRNA after acute CCl_4-treatment. Total cytosolic RNA (20mg/well) were subjected to northern blot analyses using [^{32}P]-labeled cDNA probe for P_{450} 2E1. The arrows on the left represent the ribosomal RNA subunits (28 S and 18 S).

that CCl_4 causes a significant reduction in the catalytic activity of P_{450} 2C as well as P_{450} 2E1. Reduced enzyme activity of P_{450} 2E1 and P_{450} 2C by acute CCl_4 treatment was reversible.

The immunoreactive amounts of P_{450} 2E1 (Mr 52, 000) was rapidly decreased in CCl_4-treated rats within 48 h of exposure and recovered from 72 h (Fig. 1). This was further confirmed by the inhibitory pattern in the activity of aniline hydroxylase after CCl_4 administration. In contrast, the immunoreactive protein contents of P_{450} 2C (Mr 53,000) was not changed by the same treatment (Fig. 1). CCl_4 reduced the level of immunoreactive P_{450} 2E1 selectively, while CCl_4 changed both P_{450} 2E1 and P_{450} 2C in the catalytic activity. Also, these results indicate that the inhibition of P_{450} 2E1 by CCl_4 shown here could be due to the destruction of the enzyme molecule by its substrate and that the inhibition of P_{450} 2C without apparent changes in its protein content might be due to possible conversion to apoenzyme after destruction of heme molecule by CCl_4 (Levin *et al.,* 1972).

To further study the molecular mechanism of the decrease in P_{450} 2E1-mediated catalytic activity and immunoreactive protein by CCl_4, the levels of P_{450} 2E1 mRNA in control and CCl_4-treated rats were measured by northern blot analyses using a cDNA probe specific for P_{450} 2E1 (Fig. 2). A significant decline in P_{450} 2E1 mRNA level was observed during 24 h to 48 h after CCl_4 administration while a significant increase was observed during 72 h and 96 h. These data thus confirm the pretranslational reduction of P_{450} 2E1 by CCl_4 with changing its mRNA level.

DISCUSSION

The cytochrome P_{450} isozymes are microsomal mixed function oxidases metabolizing xenobiotics as well as endogeneous substances. Xenobiotics include drugs, environmental toxic materials and carcinogens, while endogeneous materials comprise prostaglandin, fatty acid, and steroids. Cytochrome P_{450} exits in various isozymes with overlapping specificity on substrates. These enzymes can be induced by those substrate for those own P_{450} isozymes. Most of P_{450}s are induced through transcriptional activation by their own inducers, while CYP2E1 appears to be rather complex. These multiple mechanisms include transcriptional activation during normal development, pretranslational activation observed in diabetes, fasting and a high fat diet feeding, posttranslational activation by ethanol, pyridine and pyrazole, protein stabilization by acetone. In contrast to the induction mechanism CYP2E1 was suppressed by the administration of growth hormone (Umeno *et al.,* 1988; Kim and Novak, 1990; Kubota *et al.,* 1988; Song *et al.,* 1989).

The response pattern on acute exposure of carbon tetrachloride appears to be diverse according to the period after the damage. Posttranslational reduction was operated initially as previously reported (Song *et al.,* 1987), while pretranslational reduction with a decrease in CYP2E1 mRNA was unexpectedly observed from 24 hr to 48hr after the acute administration of CCl_4. CCl_4 causes haem loss through radical formation and the resulting apo-CYP2E1 was recognized and degraded by Mg^{++} ATP-activated reaction, while cAMP-dependent reaction also activates proteolytic degradation. Hormone- and substrate-regulated intracellular degradation of cytochrome P_{450} 2E1 was said to be done by proteolytic system within the ER membrane. Biphasic degradation has been reported for CYP2E1 (7, 37 h) and acetone knockouts the fast phase component of CYP2E1 degradation (Umeno *et al.,* 1988).

However, no one has reported that CCl_4 causes specific decrease in CYP2E1 mRNA. Tho ugh the mechanism is not clear at this moment, secondary events, which is evoked by initial CCl_4 metabolism through CYP2E1, might be reponsible for this phenomenon. Lipid peroxidation and alteration of Ca^{++} ion, which was caused by CCl_4 metabolism, might be key events for the cascades of secondary mechanism. Relatively long-lived toxic materials might diffuse from the ER to other parts of the cell. Further study is needed for the better understanding the specific degradation mechanisms of CYP2E1 mRNA.

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REFERENCES CITED

Chemozynski, P. and Sacchi, N., Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* 162, 156-159 (1987).

Kim, S. G., Williams, D. E., Schuetz, E. G., Guzelian, P. S. and Novak, R. F., Pyridine induction of cytochrome P_{450} in the rat. *J. Pharmacol. Exp. Ther.,* 246, 1175-1182 (1988).

Kim, S. G. and Novak, R.F., Induct ion of rat hepatic P_{450} 2E1 (CYP2E1) by pyridine: Evidence for a role of protein synthesis in the absence of transcriptional activation. *Biochem. Biophys. Res. Commun.,* 166, 1072-1079 (1990).

Koop, D. and Coon, M. J., Alcohol-inducible cytochrome P_{450} (P_{450} ALC). *Arch. Toxicol.,* 60, 16-21 (1987).

Kubota, S., Lasker, J. M. and Lieber, C. S., Molecular regulation of ethanol-inducible cytochrome P_{450} 2E1 in hamsters. *Biochem. Biophys. Res. Commun.,* 150, 304-310 (1988).

Lai, E. K., McCay, P. B., Noguchi, T. and Fong, K. L., In vivo spin-trapping of trichloromethyl radicals formed from carbon tetrachloride. *Biochem. Pharmacol.,* 2, 2231-2235 (1979).

Levin, W., Jacobson, M. and Kuntzman, R., Incorporation of radioactive-delta-aminovulinic acid into microsomal cytochrome P_{450}. *Arch. Biochem. Biophys.,* 148, 262-269 (1972).

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* 193, 265-275 (1951).

Lubet, R. A., Mayer, R. T., Cameron, J. W., Nims, R. W., Burke, M. D., Wolff, T. and Guengerich, F. P., Dealkylation of pentoxyresorufin: A rapid and sensitive assay for measuring induction of cytochrome (s) P_{450} by phenobarbital and other xenobiotics in the rat. *Arch. Biochem. Biophys.,* 238, 43-48 (1985).

Manno, M., De Matteis, F. and King, L. J., The mechanism of suicidal reductive inactivation of microsomal cytochrome P_{450} by carbon tetrachloride. *Biochem. Pharmacol.,* 38, 1001-1007 (1988).

Noguchi, T., Fong, K. L., Lai, E. K., Alexander, S. S., King, M. M., Olson, L., Poyer, J. L. and McCay, P. B., Specificity of a phenobarbital induced cytochrome P_{450} for metabolism of carbon tetrachloride to r the trichloromethyl radical. *Biochem. Pharmacol.,* 31, 615-624 (1982).

Poyer, J. L., Floyd, R. A., McCay, P. B., Janzen, E. G. and Davis, E. R., Spin-trapping of the trichloromethyl radical produced during enzyme NADPH oxidation in the presence of carbon tetrachloride or bromotrichloromethane. *Biochem. Biophys. Acta.,* 539,402-409 (1978).

Ravindranath, V. and Anandatheerthavarada, H. K., Preparation of brain microsomes with cytochrome P_{450} activity using calcium aggregation method. *Anal. Biochem.,* 187, 310-313 (1990).

Sohn, D. H., Yun, Y. P., Park, S. S., Veech, R. L. and Song, B. J., Post-translational reduction of cytochrome P_{450} 2E1 by CCl_4, its substrate. Biochim. *Biophys. Res. Commun.,* 179, 449-454 (1991).

Song, B. J., Matsunaga, T., Hardwick, J. P., Park, S. S., Veech, R. L., Gelboin, H. V. and Gonzalez,F. J., Stabilization of CYP P_{450}j messenger ribonucleic acid in the diabetic rat. *Mol. Endocrinol.,* 1, 542-547 (1987).

Song, B. J., Veech, R. L., Park, S. S., Gelboin, H. V. and Gonzalez, F. J., Induction of rat hepatic N-nitrosodimethylamine demethylase by acetone is due to protein stabilization. *J. Biol. Chem.,* 264, 3568-3572 (1989).

Umeno, M., Song, B. J., Kozak, C., Gelboin, H. V. and Gonzalez, F. J., The rat P_{450} 2E1 gene:Complete intron and exon sequence, chromosome mapping, and correlation of developmental expression with specific 5'cytosine demethylation. *J. Biol. Chem.,* 263, 4956-4962 (1988).

Virca, G. D., Northemann, W., Shiels, B. R., Widera, G. and Broome, S., Simplified northern blot hybridization using 5% sodium dodecyl sulfate. *Biotechniques,* 8, 37 0-371 (1990).