Specificity in the Metabolic Activation of Chlorinated Ethylenes by Cytochromes P450 in Primary Rat Hepatocytes

Takayuki Nakahama,* Ikuyo Maruyama, Mami Endo, and Yoshio Inouye

School of Pharmaceutical Sciences, Toho University, 2–2–1 Miyama, Funabashi, Chiba 274–8510, Japan

(Received September 18, 2000; Accepted Octobar 13, 2000)

The manifestation of toxicity by certain environmental compounds requires enzymatic activation. It is well known that chlorinated ethylenes (CEs) are metabolized by intracellular enzymes represented by cytochromes P450 (CYPs) to the urinary secreted forms via epoxide intermediates, which are responsible for their toxicities by forming complexes with intracellular components including CYPs. In order to study the roles of CYPs in the metabolic activation of tetrachloroethylene (PCE), trichloroethylene (TCE) or 1,1-dichloroethylene (1,1-DCE), the cytotoxicity of individual CEs were tested in primary hepatocyte cultures established from animals treated with various CYP-inducers such as 3-methylchoranthrene (inducer of CYP1A1/2), phenobarbital (CYP2B1/2) and pyridine (CYP2E1). The cytotoxicity of CEs measured by lactate dehydrogenase leakage after 24 hr was enhanced in different fashions, depending on the CYP inducers used. The results are summarized as follows: CYP1A1/2 and 2B1/2 raised the cytotoxicity of PCE; CYP2B1/2 was exclusively associated with the enhanced cytotoxicity of TCE; and CYP2E1 and 2B1/2 were proved to potentiate 1,1-DCE. Based on these observations, CEs differing in the number of chlorine substitutions were disclosed to have divergent preferences for CYPs, by which they were metabolically activated.

Key words —— tetrachloroethylene, trichloroethylene, 1,1-dichloroethylene, cytotoxicity, hepatocyte, cytochrome P450

INTRODUCTION

Highly chlorinated ethylenes (CEs), *i.e.*, tetrachloroethylene (PCE) and trichloroethylene (TCE), are widely used in industrial fields, mainly as degreasing solvents for metals, dry cleaning solvents, thinners for paints/varnishes, *etc.* 1,1-Dichloroethylene (1,1-DCE, vinylidene chloride), with a much lower boiling point than those of PCE and TCE, is used as a raw material for polymer products. Due to environmental stabilities, they pose a threat to human health as pollutants of soil, ground water and especially the domestic and industrial atmosphere. The liver and kidney are major target organs for CE-induced toxicities. Although CEs are not apt to accumulate in a whole body, their carcinogenicity has been reported in animals and is suspected in human beings.

Metabolic activation of CEs is considered an obligatory pathway for other known toxicities such as hepatotoxicity, nephrotoxicity and carcinogenicity. This pathway is triggered by cytochromes P450 (CYPs), leaving epoxides as unstable active intermediates, these are then detoxified in the succeeding metabolic steps. TCE was metabolized by CYPs to trichloroacetic acid (TCAA) and trichloroethanol (TCOH). In isolated rat hepatocytes, TCE was more toxic than TCAA or TCOH, causing cellular dysfunction in advance of the appearance of the latter metabolites.[1] The reactions between epoxides and cellular macromolecules would reportedly account for the toxic or carcinogenic outcomes.[2] Besides epoxides, free radical species were generated by CYPs as active metabolites of CEs, contributing the cytotoxicity and genotoxicity, associated with the metabolic activation by CYPs.[3,4] It is known that bioactivation and cytotoxicity are enhanced by CYP induction[5] and by active intermediates originated from CEs bound to DNA, resulting in DNA-adduct formation.[6]

CEs characteristically function as initiators and promoters in carcinogenesis, in good accordance with the fact that CEs caused tumors in animal models. Epidemiological evidence also indicates in-

*To whom correspondence should be addressed: School of Pharmaceutical Sciences, Toho University, 2–2–1 Miyama, Funabashi, Chiba 274–8510, Japan. Tel.: +81-47-472-2548; Fax: +81-47-472-2548; E-mail: nakahama@phar.toho-u.ac.jp

creased cancer-risks in human beings exposed to TCE.[7,8]

Channel *et al*. reported that TCE enhanced the proliferation of peroxisomes[4] and hepatocytes, caused apoptosis and generated 8-hydroxyguanosin. Constan *et al*. reported that apoptosis caused by environmental pollutants, including TCE, were directly associated with the enhanced proliferation of hepatocytes.[9] In the case of cocaine treatment, the preceding phenobarbital (PB)-treatment (or the induction of CYP2B1/2) enhanced lactate dehydrogenase (LDH) leakage, and decreased both the glutathione level and the production of lipid peroxide in rat hepatocytes.[10] Although CEs are metabolized by CYP1A1/2, CYP2B1/2, CYP2E1 and CYP2C11, it remains to be determined which forms of CYP are mainly associated with the metabolic activation of individual CEs differing in the number of chlorine substitutions.

We previously observed divergent effects of CEs on the enzyme activity of CYPs in vivo.[11] In order to approach the causes of these findings, the roles of CYPs in the metabolic activation of CEs have been the focus of the present study.

MATERIALS AND METHODS

Reagents —— PCE, TCE, 3-methylchoranthrene (3-MC), PB and pyridine (PY) are products of Wako Pure Chemical Ind. (Osaka, Japan) 1,1-DCE was purchased from Aldrich Chemical Co. (U.S.A.). 1-Amino-benzotriazole was obtained from Sigma Chemical Co. (Germany). Collagenase solution (Liver Digest Medium), Minimum Essential Medium (MEM) and modified Chee's medium (HepatoZYM-SFM) were purchased from Gibco BRL.

CYP-Induction, and Isolation and Culture of Hepatocytes —— Male Wistar rats were inoculated with 3-MC, PB or PY (80 mg/kg body weight) by the i.p. route. Hepatocytes were isolated from rat livers using collagenase perfusion 24 hr after the injection of CYP inducers. Briefly, the livers were perfused with collagenase solution, excised, filtered, and washed four times in MEM. Viability of hepatocytes was determined by a Trypan blue dye exclusion test, and only those preparations in excess of 80% viable cells were used. Hepatocytes were plated at 1.0×10^4 cells/well onto 96-well plates coated with type I collagen, then cultured in HepatoZYM-SFM in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

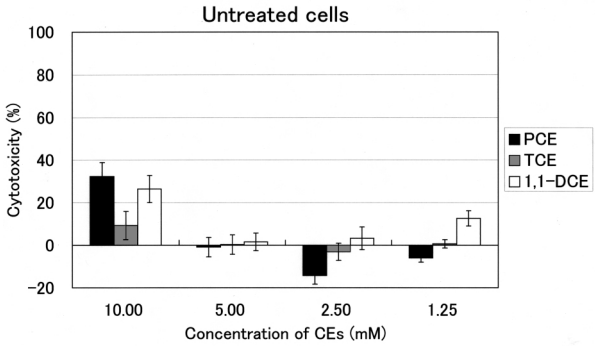

Fig. 1. Cytotoxicity of CEs in the Cultured Hepatocytes from Untreated Rat

Cytotxicity was measured by the LDH release into the medium during 24 hr culture and expressed as a percentage relative to the LDH released from the detergent disrupted cells. Vertical bars represent ±S.E.

Cytotoxicity Measurement —— Hepatocytes were cultured for 24 hr in the presence of serial 2-fold dilutions of PCE, TCE or 1,1-DCE, from 10 down to 1.25 mM in a 0.5% DMSO solution. LDH activity released into the culture supernatant was determined using a Cytotoxicity Detection Kit (Rosh) and microplate reader (BioRad) at 490 nm.

RESULTS

Cytotoxicity of CEs in Rat Hepatocytes

The cytotoxicity of CEs was measured using hepatocytes prepared from mock-treated rats. Low-level cytotoxicities were observed at 10 mM PCE and 1,1-DCE, but no marked cellular damage was noted under the other conditions (Fig. 1). When cultured in the coexistence of a nonspecific CYP inhibitor, 1-amino-benzotriazol, at 10 mM, the cytotoxicity of 10 mM PCE and 1,1-DCE was decreased by approximately 80%, implying the role of CYPs in their cytotoxicities (data not shown).

Effects of CYP-Induction on Cytotoxicity of CEs

CEs were assessed for their cytotoxic effects on rat hepatocytes which originated from the animals inoculated with one of the three well-known CYP inducers: 3-MC was used for the induction of CYP1A1/2, PB for CYP2B1/2 and PY for CYP2E1.

Hepatocytes from 3-MC-treated rats were more susceptible to the cytotoxicity of PCE and 1,1-DCE, in a descending order, when compared with the control cells, whereas no marked effect was observed in the case of TCE on the LDH leakage from either 3-MC-treated or mock-treated hepatocytes (Figs. 1

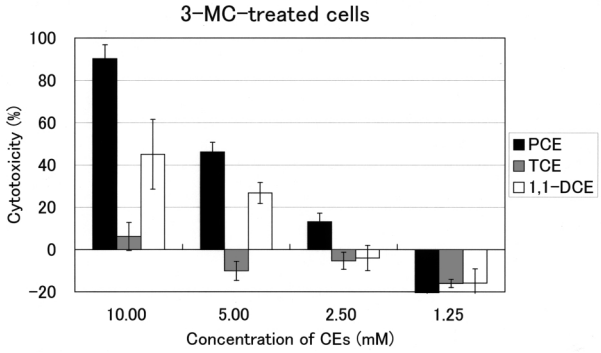

Fig. 2. Cytotoxicity of CEs in the Cultured Hepatocytes from 3-MC-treated Rat

Cytotoxicity was measured and expressed as described in the legend to Fig. 1.

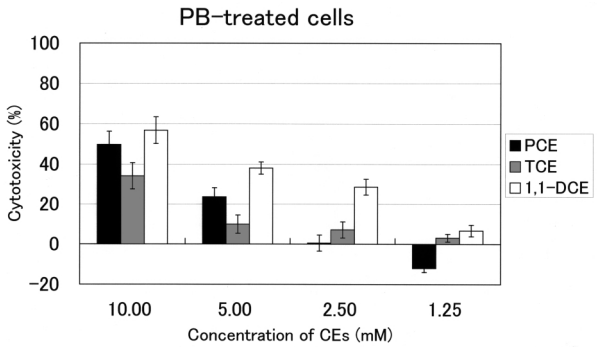

Fig. 3. Cytotoxicity of CEs in the Cultured Hepatocytes from PB-treated Rat

Cytotoxicity was measured and expressed as described in the legend to Fig. 1.

and 2) .

Hepatocytes of PB-treated rats showed rather nonspecifically enhanced susceptibility to the CE-dependent cytotoxicity, especially that of TCE in terms of the ratio to the control value. Although the cytotoxicity of TCE was negligible in the control culture, it became detectable only in hepatocytes derived from PB-treated rats, attributing the metabolic activation to the function of CYP2B1/2 (Fig. 3). In fact, the enzymatic activity of CYP2B1/2 was hardly detected in the hepatic microsomal fractions of the control rats.[12] The enhancing effect of PY-treatment was only significant in the case of 1,1-DCE, suggesting the specificity of CYP2E1 for 1,1-DCE among three CEs tested (Fig. 4). Thus, there seemed to exist some correlation between the function of CYPs and the cytotoxicity of CEs, as summarized in the followings: 3-MC-treatment potentiated mainly the cytotoxicity of PCE. The effect of

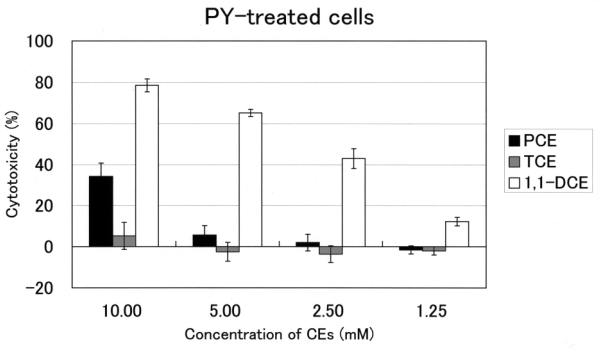

Fig. 4. Cytotoxicity of CEs in the Cultured Hepatocytes from PY-treated Rat

Cytotoxicity was measured and expressed as described in the legend to Fig. 1.

PB-treatment was general, but exclusive, if the cytotoxicity of TCE was concerned. The cytotoxicity of 1,1-DCE was selectively amplified by PY-treatment.

DISCUSSION

3-MC, PB and PY, well known inducers of CYP1A1/2, CYP2B1/2 and CYP2E1, respectively, were used in order to study the engagement of these phase I enzymes in the cytotoxicity of CEs in rat hepatocytes. From our results, it was found for the first time that three structurally closely-related CEs had differential preferences for their metabolizing CYP species. CYP1A1/2, CYP2B1/2 and CYP2E1 were found to mainly contribute to the cytotoxicity of PCE, TCE and 1,1-DCE, respectively. There have been many reports concerning the bioactivation of CEs by CYP2E1. Griffin *et al*. found in rat hepatocytes a few TCE-protein adducts, with one of them co-migrating with CYP2E1 on SDS-PAGE.[13] They also observed the co-migration of TCE with an unidentified protein in human samples. In the present study, TCE showed higher preference to CYP2B1/2 than CYP2E1. However, the basal expression of CYP2B1/2 in terms of monooxygenase activity was negligible in untreated rats, in marked contrast to the constitutive expression of CYP2E1 activity. These findings would account for the discrepancy between our findings and those of Griffin *et al*.[13]

Dahlstrom *et al*. investigated the cytotoxicity of CEs in rat hepatocytes immediately after isolation by the release of alaninaminotransferase.[5] They reported the cytotoxicity of CEs in the descending order of PCE, TCE and 1,1-DCE. In contrast, we ob-

served previously that 1,1-DCE showed the highest hepatotoxicity.[11] Since an unanchored culture was not suitable for the hepatocytes to temporarily maintain their *in vivo* properties, the results of Dahlstrom *et al*. could not necessarily reflect the *in vivo* findings.[14]

Likewise, differences in the profiles of expressed CYP species among organs could be the basis for the differences in target organs among these environmentally hazardous compounds, and the same is applicable to the sexual difference in susceptibility to these compounds.

REFERENCES

1) Kadry, A. M., Farghali, H., and Abdel Rahman, M. S. (1989) Toxicity and metabolism of trichloroethylene in rat hepatocytes. *Arch. Environ. Contam. Toxicol*., **18**, 888–894.
2) Miller, R. E. and Guengerich, F. P. (1983) Metabolism of trichloroethylene in isolated hepatocytes, microsomes and reconstituted enzymes systems containing cytochrome P-450. *Cancer Res*., **43**, 1145–1152.
3) Mingxue, Y., Yunda, L. and Yugu, L. (1992) Potential use of luminol-dependent chemiluminescence for estimation of free radicals produced in hepatic microsomes and reconstituted cytochrome P450 systems. *Biomed. Environ. Sci*., **5**, 336–348.
4) Channel, S. R., Latendresse, J. R., Kidney, J. K, Grabau, J. H., Lane, J. W., Steel-Goodwin, L. and Gothaus, M. C. (1998) A subchronic exposure to trichloroethylene causes lipid peroxidation and hepatocellular proliferation in male B6C3F1 mouse liver. *Toxicol. Sci*., **43**, 145–154.
5) Dahlstrom, K. L., Couture, J., Lamoureux, C., Vaillancout, T. and Plaa, G. L. (1990) Dose-dependent cytotoxicity of chlorinated hydrocarbons in isolated rat hepatocytes. *Fundam. Appl. Toxicol*., **14**, 833–841.
6) Dekant, W., Schulz, A., Metzler, M. and Henshler, D. (1986) Absorption elimination and metabolism of trichloroethylene: A quantitative comparison between rats and mice. *Xenobiotica*, **16**, 143–152.
7) Anttila, A., Pukkala, E., Sallmen, M., Hernberg, S. and Hemminki, K. (1995) Cancer incidence among Finishworkers exposed to halogenated hydrocarbons. *J. Occup. Environ. Med*., **37**, 797–806.
8) Lynge, E., Anttila, A. and Hemminki, K. (1997) Organic solvents and cancer. *Cancer Causes and Control*, **8**, 406–419.
9) Constan, A. A., Benjamin, S. A., Tessari, J. D., Baker, D. C. and Yang, R. S. H., (1996) Increase rate of apoptosis correlates with hepatocellular proliferation in Fischer-344 rats following long-term exposure to a mixture of groundwater contaminants. *Toxicol. Pathol*., **24**, 315–322.
10) Jover, R., Ponsoda, X., Gomez-Lechon, J. and Castell, J. V. (1993) Cocain hepatotoxicity: Two different toxicity mechanisms for phenobarbital-induced and non-induced rat hepatocytes. *Biochem. Pharmacol*., **46**, 1967–1974.
11) Nakahama, T., Sarutani, S. and Inouye, Y. (2000) Effects of chlorinated ethylenes on expression of rat CYP forms: Comparative study on correlation between biological activity and chemical structures. *J. Health Sci*., **46**, 251–258.
12) Nakahama, T., Sarutani, S. and Inouye, Y. (1999) Comparative study on the effect of trichloroethylene on the expression of P450 isoforms in rat lung and liver. *J. Health Sci*., **45**, 8–14.
13) Griffin, J. M., Lipscomb, J. C. and Pumford, N. R. (1998) Covalent binding of trichloroethylene to proteins in human and rat hepatocytes. *Toxicol. Lett*., **95**, 173–181.
14) Kefalas, V. and Stacey, N. H. (1993) Use of primary cultures of rat hepatocyte to study interactive toxicity: Carbon tetrachloride and trichloroethylene. *Toxicol. in Vitro*, **7**, 235–240.