Possible Involvement of Chlorinated Ethylenes in Arylhydrocarbon Receptor-Related Induction of Cytochrome P4501A1 (CYP1A1) in Human Hepatoma HepG2 Cells

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In our previous study, chlorinated ethylenes (CEs) such as tetrachloroethylene (PCE), trichloroethylene (TCE) and 1,1-dichloroethylene (1,1-DCE) were found to suppress CYP1A1 activity in rats. In the present study, the effects of CEs on CYP1A1 activity, CYP1A1 mRNA expression and the binding of nuclear proteins to the xenobiotic response element (XRE) were investigated in rat hepatocytes and human hepatoma HepG2 cells. Unexpectedly, CYP1A1 activity was enhanced in the cells cultured in the presence of CEs, suggesting that the suppressive effect of CEs in vivo is indirect. Well consistent with the enhanced enzymatic activity, the expression of CYP1A1 mRNA was increased by all CEs. Furthermore, the induction by 3-methylcholanthrene (3-MC) was reversed in the presence of individual CEs, implying cross-talk between the induction mechanisms of PAHs and CEs. In a luciferase reporter assay of transcription under the control of 4 repeats of XRE, a 2.5-fold increase was induced in HepG2 cells by CEs in the case of 1,1-DCE-treatment, while a 11-fold increase was observed in the cells treated with 3-MC. In HepG2 cells transfected with plasmids expressing aryl hydrocarbon receptor (AhR) antisense mRNA, in which the AhR-expression level was reduced by approximately 40%, no increase in luciferase activity was observed following the CE-treatment, though a 15-fold induction was observed in the presence of 3-MC. As for the nuclear XRE-binding proteins in the presence of CEs analyzed with the gel mobility shift assay, the band-intensity peaked 3 hr after the PCE-, TCE- and 3-MC-treatments. The metabolic activation might be responsible for the 3-hr delay to attain the maximal band-intensity in the case of 1,1-DCE.

Key words —— chlorinated ethylenes, CYP 1A1, rat hepatocyte, HepG2, aryl hydrocarbon receptor, xenobiotic response element

INTRODUCTION

Cytochrome P4501A1 (CYP1A1), a member of the CYP superfamily, metabolizes polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene and 3-methylcholanthrene (3-MC) to their polar derivatives.13 PAHs are recognized by their cytoplasmic aryl hydrocarbon receptor (AhR), which is a member of the basic helix-loop-helix/PER-ARNT-SIM superfamily (bHLH/PAS).2,3 After binding its ligand, AhR translocates into the nucleus where it dissociates from the chaperon proteins and dimerizes with ARNT.4,5 The binding of the AhR-ARNT heterodimer to the xenobiotic response element (XRE) enhances the transcription of the CYP1A1 gene.6 CYP1A1 and AhR have a wide range of substrates (ligand) which they share to some extent, as in the case of 3-MC, protecting host animals from the adverse effects of various xenobiotics such as food ingredients and environmental pollutants. CYP1A1 is also known to metabolically activate certain xenobiotics making them carcinogenic.

Recently, compounds without a planar structure such as benzimidazole and omeprazole have been reported to induce CYP1A1 expression by unknown mechanisms distinguishable from those of planar AhR-agonists represented by 3-MC.7,8 Chlorinated ethylenes (CEs) such as tetrachloroethylene (PCE), trichloroethylene (TCE) and 1,1-
dichloroethylene (1,1-DCE), known as environmental pollutants, are metabolized by CYPs to form epoxide intermediates, which are responsible for their toxicity and carcinogenicity. In our previous study, however, CEs were found to suppress hepatic ethoxyresorufin O-deethylase (EROD) activity (CYP1A) in rats. In the present study, the effects of CEs on the expression of CYP1A were examined in vitro using human hepatoma HepG2 cells and rat primary cultured hepatocytes in order to elucidate whether the suppressive effects in vivo are conferred directly or not.

**MATERIALS AND METHODS**

**Cell Culture Conditions and Treatments**

HepG2 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, GIBCO™/Invitrogen, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum and penicillin-streptomycin (50 U/ml-50 µg/ml) in humidified 5% CO₂ at 37°C. The treatments were performed when the cultures had reached 80% confluence. The primary cultured hepatocytes were separated from the livers of 7 to 8-week-old male Wistar rats (Clea, Tokyo, Japan) using the collagenase perfusion method. After enumeration of the cells by the trypan blue dye-exclusion method, the hepatocytes were plated in type I collagen-coated dishes (IWAKITM/Asahi Techno Glass, Chiba, Japan). The medium was exchanged with HepatoZYME-SFM (IWAKITM/Asahi Techno Glass, Chiba, Japan). The measurement of ethoxyresorufin O-deethylase (ECOD) activity was performed as described previously. HepG2 cells treated with CEs were incubated in 0.9 ml of Minimum Essential Medium (GIBCO™/Invitrogen) containing HEPES (final concentration, 12 mM), glucose (final 30 mM) and 7-ethoxycoumarin (final 1 mM) at 37°C for 3 hr, and the reaction was stopped with ice-cold acetone. After centrifugation of the reaction mixture, the pellet was used for determination of the protein concentration with Lowry’s method. The glucuronide of 7-hydroxyxoumarine in the supernatant was hydrolyzed by β-D-glucuronidase (Wako). The amount of 7-hydroxyxoumarine that formed was determined.
by measuring the intensity of fluorescence (λex = 370 nm, λem = 454 nm).

**Reporter Gene Expression** —— The XRE-pGL3 reporter plasmid was prepared by cloning the XRE sequence into the Bgl II site of the pGL3-Basic vector (Promega, U.S.A.) containing a firefly luciferase reporter gene. A double stranded XRE nucleotide sequence was prepared by annealing the following synthetic oligomers: XRE-Luc-1: 5'-GAT CGT CGG TGA CAA GCC TTG CGT GAC AAC CTT GCG TGA CAA GCT GTC GTG AC-3', and XRE-Luc-2: 5'-GAT CAC GCA AGC TTG TCA CGC AAG CTT GTC ACC AGG GC-3' (restriction sites are boxed and XRE pentanucleotide core sequences are underlined).

HepG2 cells were subcultured at 5 × 10⁵ cells/well on 12-well plates. The cells were transfected 48 hr after seeding with XRE-pGL3 and control plasmid phRL-SV40 Vector (Promega) containing the Renilla luciferase gene. A mixture (1 ml/well) of FuGENETM6 (Roche, U.S.A.) and OPTI-MEM (INVITROGEN) was used for transfection. After incubation in 5% CO₂ at 37°C for 5 hr, the medium was changed to DMEM. After incubation overnight, the reporter assay was performed using a Dual-Luciferase Reporter Assay System (Promega) and Turner Designs Luminometer (Model TD-20/20, Promega).

**Preparation of a Low AhR-Expressing Cell Line** —— Anti-sense AhR plasmid (pasAhR) was prepared using PCR-amplified AhR cDNA. The RT-PCR was performed using LA Taq (Takara, Japan). The primer pairs were designed to cover the whole coding sequence from the translational start site to the stop codon and tagged with the restriction sites. The expression vector, named pasAhR, was introduced into HepG2 cells. The selection of transfected cells was carried out in medium containing G418 (final concentration, 550 µg/ml). Selected single colonies were subcultured on 24-well plates. The low-level AhR-expression of the cell strains was confirmed by Western blotting and luciferase reporter assays.¹⁶,¹⁷)

**Preparation of the Nuclear Fraction for Gel Mobility Shift Assay** —— The nuclear fraction for the gel mobility shift assay was prepared from cells suspended in 100 µl of 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P40, 1 mM dithiothreitol (DTT), 0.5 mM PMSF and 2 µg/ml of leupeptin. The pellet from a centrifugation (4°C, 9000 × g, 1 min) was mixed with 100 µl of 50 mM HEPES, 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 20% glycerin, 1 mM DTT, 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 2 µg/ml of leupeptin. The mixture was gently rotated at 4°C for 30 min on a rotator and clarified by centrifugation (16000 × g, 4°C, 30 min) and the supernatant was stored at –80°C until used as a nuclear fraction.

**Western Blotting** —— An immunoblot analysis was performed to determine the AhR protein level. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out with 7.5% acrylamide, according to the method of Laemmli.¹⁸) The AhR proteins were transferred from the gel to the nitrocellulose sheet electrophoretically with the help of a blotting apparatus (BioRad, Transblot-SD). The sheet was treated with goat anti-human AhR (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) and peroxidase-labeled anti-goat IgG. Chemiluminescence was determined using the Western blotting detection reagent, ECL Plus (Amersham Biosciences). The protein band-images were read with Storm TM830 (Amersham Biosciences) and analyzed with Image Quant software.

**Gel Mobility Shift Assay (GMSA)** —— A double stranded oligonucleotide XRE-probe named XRE-1 was prepared by annealing the following synthetic oligomers and was labeled with ³²P using a DNA Polymerase I Large Fragment Mini Kit (Promega). The nucleotide sequences used were 5'-GAT CCC TCC AGG CTC TTC TGCA AAC CCG CAC CTT CTC-3' and 5'-GAT CGG AGT TGG AGA AGA GCC TGG AGG-3' (XRE pentanucleotide core sequences are boxed.). The nuclear protein (20 µg/5 µl) was incubated at 0°C for 10 min with 18 µl of binding buffer (50 mM HEPES, 250 mM KCl, 5 mM EDTA, 25 mM MgCl₂, 50% glycerol, 25 mM DTT, 10 µg/ml of leupeptin and 3.5 mM PMSF) containing 36 µg of poly(dl-dc), and incubated with the ³²P-labeled probe at 25°C for a further 20 min in advance of its application to a 4%-polyacrylamide gel for electrophoresis. The gel was dried with a gel drier and exposed to an imaging plate. The band image was ana-
lyzed with Storm TM830.

**Statistics** —— Statistical significance was determined using Student’s t-test. The 0.05 level of probability was adopted as the criterion of significance.

**RESULTS**

**Effects of CEs on CYP1A Activity in Rat Primary Hepatocytes and Human Hepatoma HepG2 Cells**

The effects of CEs on the expression of CYP forms were determined using rat primary hepatocytes in terms of ECOD activity, with 4.5-, 3.1- and 1.5-fold increases over the control level following PCE-, TCE- and 1,1-DCE-treatment, respectively (Fig. 1A). In human hepatoma HepG2 cells, 2.5-, 1.7- and 1.5-fold increases in ECOD activity, for which CYP1A1 was mainly responsible, were also observed in the presence of the respective CEs, in comparison with a 5.5-fold increase in the presence of 3-MC (Fig. 1B). The greatest increases were obtained with PCE in both cellular systems. Since HepG2 cells were superior to rat hepatocytes in terms of the level of ECOD activity and experimental reproducibility, subsequent experiments were conducted using HepG2 cells.

**Effects of CEs on CYP1A1 mRNA in HepG2 Cells**

The expression of CYP1A1 mRNA in HepG2 cells was measured using Northern blots. Well consistent with the data on ECOD activity, increased expression of CYP1A1 mRNA was observed after all the CE-treatments (Fig. 2A). Although an approximately 2-fold induction over the control was observed in the case of PCE, no significant increases were observed with TCE or 1,1-DCE.

The 28-fold increase observed with 3-MC (Fig. 2A) was partially reversed by the co-administered CEs as shown in Fig. 2B. The most significant reduction of CYP1A1 mRNA expression (35%) was by TCE, which was a less potent inducer than PCE when applied individually.

**Establishment of a Low AhR-Expressing HepG2 Cell Line**

HepG2 cells were transfected with pasAhR plasmids and selected in medium containing G418. Two-cell lines (referred to as asAhR3 and asAhR4) stably expressing antisense mRNA were established. About a 40% decrease in the expression of AhR protein in both these cells compared with the parental cells was observed. Similar results were obtained in the reporter assay.

**Effect of CEs on the Expression of the Reporter Gene with XRE as an Enhancer**

The effects of CEs on the activation of AhR were examined comparatively in asAhR3 cells and HepG2 cells using the luciferase assay. The reporter plasmid XRE-pGL3 consisting of 4 XRE responsive core pentanucleotides upstream of the firefly luciferase gene was introduced into asAhR3 and HepG2 cells. In the parental cells, a significant induction of luciferase activity was observed for all the treatments.
Fig. 2. Effect of CEs on Expression of CYP1A1 mRNA in HepG2 Cells and on 3-MC-Dependent CYP1A1 mRNA Expression in HepG2 Cells
(A) The CYP1A1 mRNA level was measured by Northern blotting 18 hr after the addition of individual CEs (1 mM), 3-MC (2.5 µM) or DMSO (0.5%) to the culture. Each lane contained 70 µg of total RNA. The expression of mRNA is presented relative to that for the control group (CONT). (B) The CYP1A1 mRNA level was measured by Northern blotting 18 hr after the addition of individual CEs (1 mM) simultaneously with 3-MC (2.5 µM) to the culture. Each lane contained 40 µg of total RNA. The expression of mRNA is presented relative to that for the 3-MC-treated group (3-MC). The data are shown as the mean ± S.E. for 3 plates with significant differences compared to the control at **: *p < 0.01 or the 3-MC-treatment at *: *p < 0.05, **: p < 0.01.

compared to the untreated control; 1.7, 2.0, 2.5 and 11-fold inductions were observed in the presence of PCE-, TCE-, 1,1-DCE and 3-MC, respectively (Fig. 3). In marked contrast, no significant effects of CEs were observed in asAhR3 cells, though the marked induction was reproducible in the presence of 3-MC.

When asAhR cells were cultured in the presence of both individual CEs and 3-MC, PCE, TCE and 1,1-DCE suppressed the 3-MC-induced luciferase activity by 50%, 25% and 12%, respectively (Fig. 4).

Time Course of the Effect of CEs on the Binding between the XRE Sequence and AhR-ARNT Heterodimer
A gel mobility shift assay (GMSA) using a 32P-labeled XRE-I double-stranded probe and nuclear extract was performed to detect the transacting AhR complex. Transcriptional activation was observed in the presence of CEs and 3-MC in terms of the luciferase activity in HepG2 cells (Fig. 3). Therefore, the nuclear accumulation of AhR in the presence of individual CEs or 3-MC was studied time-dependently.

The formation of the XRE-AhR-ARNT complex

Fig. 3. Effect of CEs on Luciferase Activity in asAhR3 and HepG2 Cells
asAhR3 and HepG2 cells transiently transfected with the XRE-pGL3 reporter plasmid were treated with individual CEs (1 µM) or 3-MC (2.5 µM). Normalized luciferase activity was measured 18 hr after treatment and presented relative to the value in HepG2 cells treated with the vehicle. The closed and open bars show the data for HepG2 and asAhR3 cells, respectively. The number on the column is a value relative to the untreated control level (CONT) in HepG2 (1.0). The data are shown as the mean ± S.E. for 7 plates with significant differences compared to the control at *: *p < 0.05, **: p < 0.01.
Fig. 5. Time–Dependent Effect of CEs on Formation of the XRE-AhR-ARNT Complex

CEs (1 µM) and 3-MC (2.5 µM) were added to the culture. The control cultures were incubated with DMSO (0.5%). The nuclear protein fractions were extracted 3(A), 6(B) and 12(C) hr after the treatment. Two micrograms of nuclear protein was loaded with a 32P-labeled XRE probe onto each lane of a 4% polyacrylamide gel for electrophoresis. The band corresponding to the XRE•AhR•ARNT complex is indicated by an arrow. A dose of moderate increases in band intensity with the PCE- or TCE-treatment and significant increases with the 3-MC-treatment compared to the time-matched vehicle control were observed 3 hr after treatment. However, no marked change was observed with the 1,1-DCE-treatment. Six hours after the treatment, however, a potent increase was observed in the presence of 1,1-DCE, while the band-intensity decreased to the control level following PCE-treatment, less extensively in the 3-MC-treated cells and negligibly in the case of TCE. After 12 hr, the band-intensity decreased to the control level in all the CE-treated cells, and that for 3-MC was below the control level.

**DISCUSSION**

CEs were metabolically activated by cytochrome P450 monoxygenases to form epoxide intermediates responsible for their cytotoxicity and carcinogenicity. CYP forms of families 1 through 4 are enzymes with a wide range of substrates which partially overlap, and the forms mainly responsible for the metabolic activation of individual CEs were divergent. When CEs were administered to the rats intraperitoneally, suppression of hepatic EROD activity was observed. In this paper, CEs were found to induce the expression of CYP1A in rat hepatocytes (Fig. 1A) and human hepatoma HepG2 cells both expressing AhR constitutively (Fig. 1B), in marked contrast with their in vivo suppressive effects.

Although the ECOD activity is attributable to several CYP forms, CYP1A1 is considered to be mainly responsible in HepG2 cells. Inflammatory cytokines interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF) were reported to be suppressive of the expression of certain CYP forms transcriptionally in cultured cells. Since the magnitude of the suppression of P450 activity by CEs well correlated with the inflammatory observations on biopsy, the inflammatory cytokines might play a key role in the in vivo effects of CEs on the expression of CYP forms. The hepatic CYP2B1 mRNA levels in the rats treated with CEs were determined time–dependently, showing an immediate increase at 6 hr after the treatment followed by a delayed decrease with trough values after 12–16 hr. The early increase in the level of CYP2B1 transcripts was a direct effect of CEs via unknown mechanisms and the delayed type of suppression might be an indirect effect supposedly mediated by inflammatory cytokines. The results obtained under the in vitro...
assay conditions employed in the present study could mimic the in vivo findings in the acute phase, due to the lack of any inflammatory cytokines. Therefore, the previous findings concerning the in vivo effect of CEs on the expression of CYP1A1 were attributable to the inflammatory nature of CEs at the doses employed, masking the enhancing effect of CEs reflected in the in vitro findings.

As shown in Fig. 2, CEs enhanced the expression of CYP1A1 transcriptionally. Due to the lack of a planar structure required for typical AhR agonists such as 3-MC, CEs were hardly recognized by AhR as agonists. However, CEs reversed the transcription of CYP1A1 enhanced by 3-MC, when the cells were treated simultaneously, implying competition at the ligand binding site of AhR. To verify the AhR-dependency of the transcriptional activation by CEs, a reporter assay based on the plasmid pXRE-luc was attempted, because the transcription of endogenous AhR-responsive genes represented by the CYP1A1 gene was not the only function of AhR as in the case of retinoids and primaquine. CEs transcriptionally activated the luciferase reporter gene as shown in Fig. 3, suggesting the involvement of AhR in the CE-induced activation of the CYP1A1 gene.

AhR is retained in the cytoplasmic compartment in a complex with chaperons and transported in a ligand-bound form into the nuclear compartment, where it dimerizes with ARNT. The AhR-ARNT heterodimers in turn interact with the cis-elements residing upstream of the target genes. If it were true that CEs modify the interaction between AhR and its partners or chaperons, the relative amount of AhR to the partners might affect the transcriptional activation. We established two cell lines named asAhR3 and asAhR4 stably expressing antisense AhR mRNA. The AhR protein levels in these cell lines decreased by about 40%. In the parental HepG2 and asAhR3 cells, CEs were tested for their activation potential normalized to the activity in untreated cells using the plasmid pXRE-luc. 3-MC enhanced the luciferase activity in asAhR3 cells at a comparable level of amplification to that in HepG2 cells. In contrast, the luciferase activity in asAhR3 cells was insensitive to the CE-treatment, although 3-MC was as effective as in HepG2 cells (Fig. 3). Although AhR plays a pivotal role in the transcriptional activation of the genes targeted by CEs, the reduction in AhR content relative to the background severely affected the CE-induced transcriptional activation in marked contrast to the almost negligible effect on the 3-MC-dependent one. Based on these observations, we propose that the molecular targets of CEs are the AhR-interacting proteins expressed more abundantly than AhR, by which ligand-free AhR was retained in the cytoplasm. The free form relatively abundant in asAhR3 cells as compared with HepG2 cells might be a preferable target of CEs to the AhR-complex form. In the presence of both 3-MC and CEs, the ligand-induced mechanism was overwhelmingly dominant over the CE-induced one in the process of AhR nuclear translocation. Proteins such as HSP90 and XAP2 would be candidates for the molecular targets of CEs.

The formation of the AhR-ARNT-XRE complex was evaluated by GMSA using an isotope-labeled XRE probe as summarized in Fig. 5. In the case of 3-MC, the band intensity of the AhR-ARNT-XRE complex increased immediately and ended within 12 hr by such mechanisms as nuclear export signal (NES)-mediated transport to the cytoplasmic compartment followed by ubiquitin-proteasome-catalyzed digestion. Likewise, the nuclear accumulation and then disappearance of the AhR-ARNT heterodimer were observed in the presence of CEs, suggesting the AhR-dependency of the CE-induced transcriptional activation. Based on the delayed time-course in the case of 1,1-DCE, the metabolites might be the active entities.

The negative correlation between the magnitude of the suppressive effect of CEs on the 3-MC-induced transactivation and the degree of ECOD activity induced by individual CEs (Figs. 1B and 4), implies a AhR-related behavior by the CEs. In the presence of 3-MC, however, the extent of the effect of CEs on the luciferase activity and on the CYP1A1 mRNA level was not consistent with each other (Figs. 2B and 4). The difference between endogenous and exogenous genes or the difference between mRNA and functional protein levels might be responsible for this discrepancy.

The retention of AhR in the cytoplasmic compartment might not be strict in HepG2, with a certain portion translocating into the nucleus even in the absence of the cognate ligands, though the transactivation potential was less than that of the ligand-bound form. Alternatively, the factor responsible for the background level in the GMSA in the untreated cells was the nuclear localized AhR repressor or inactive isoforms which had the ability to bind DNA. In the presence of CEs, AhR is translocated into the nucleus in a less potent ligand-free form via an unknown mechanism, which might be
elucidated in the near future.

REFERENCES

22) Mizuno, M., Nakahama, T. and Inouye, Y. (2001)
Comparative study on correlation between chemical structure and effect on expression of cytochrome P450 mRNAs in rat among chlorinated ethylenes, tetrachloroethylene, trichloroethylene, 1,1-dichloroethylene. *J. Health Sci.*, **47**, 373–377.


