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Metabolic pathways of dioxin by CYP1A1: species difference between rat and human CYP1A subfamily in the metabolism of dioxins

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Abstract

Metabolism of polychlorinated dibenzo-*p*-dioxins by CYP1A subfamily was examined by using the recombinant yeast microsomes. In substrate specificity and reaction specificity, considerable species differences between rats and humans were observed in both CYP1A1- and CYP1A2-dependent metabolism of dioxins. Among four CYPs, rat CYP1A1 showed the highest activity toward dibenzo-*p*-dioxin (DD) and mono-, di-, and trichloroDDs. To reveal the mechanism of dioxin metabolism, we examined rat CYP1A1-dependent metabolism of 2-chloro-dibenzo-*p*-dioxin. In addition to hydroxylation at an unsubstituted position, hydroxylation with migration of a chloride substituent, hydroxylation with elimination of a chloride substituent, and cleavage of an ether linkage of the dioxin ring were observed. In particular, the cleavage of an ether linkage of the dioxin ring appeared most important for the detoxication of dioxins. Based on these results, the metabolic pathways of 2-chloro-dibenzo-*p*-dioxin by rat CYP1A1 were proposed. The metabolic pathways contain most of the metabolites observed in vivo using experimental animals, suggesting that P450 monooxygenase systems including CYP1A1 are greatly responsible for dioxin metabolism in vivo.

Keywords: Dioxin; Metabolism; Cytochrome P450; Recombinant yeast; CYP1A

Polychlorinated dibenzo-*p*-dioxins (PCDDs)[1] are known as environmental contaminants. The family contains 75 species of PCDDs. Of these compounds, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TetraCDD) is known to be the most toxic one. Chlorination at lateral positions (C-2, 3, 7, and 8) appears essential for the toxicity of PCDDs, and all PCDDs conferred toxic equivalency factors (TEFs) are chlorinated at lateral positions [1]. Until now, the metabolism of PCDDs has been studied in vivo using experimental animals and in vitro using liver microsomal fractions [2–9]. The metabolic reactions that were observed in vivo include hydroxylation at an unsubstituted position, hydroxylation with migration of a chloride substituent, hydroxylation with elimination of a chloride substituent, and cleavage of an ether linkage of dioxin ring. These reactions are suited for the detoxication of PCDDs.

Recently, we examined the catalytic activities toward PCDDs by monooxygenase systems dependent on 12 forms of human cytochrome P450. Remarkable metabolism by the multiple CYPs was observed toward dibenzo-*p*-dioxin (DD), mono-, di-, and trichloroDDs (unpublished results). In particular, CYP1A1 and CYP1A2 showed a high catalytic activity toward di- and trichloroDDs. In addition, our recent studies revealed that yeast expressing recombinant rat CYP1A1 or

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[1] *Abbreviations used:* P450 or CYP, cytochrome P450; PCDD, polychlorinated dibenzo-*p*-dioxin; MCDD, monochloro-dibenzo-*p*-dioxin; DCDD, dichloro-dibenzo-*p*-dioxin; TriCDD, trichloro-dibenzo-*p*-dioxin; TetraCDD, tetrachloro-dibenzo-*p*-dioxin.

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CYP1A2 showed a remarkable metabolic efficiency toward di- and trichloroDDs [10].

In this report, substrate specificity and reaction specificity of CYP1A subfamily for dioxin metabolism were compared between human and rat, expecting to reveal a remarkable species difference between human and rat on a half-life time of 2,3,7,8-TetraCDD. In addition, we examined the metabolism of 2-chloroDD to understand the mechanism of metabolism of PCDDs by P450 monooxygenase system.

Materials and methods

Materials

Dibenzo-*p*-dioxin was purchased from Wako Pure Chemical Industries (Osaka, Japan). 1-MonochloroDD (1-MCDD), 2-MCDD, and 2,3-dichloroDD (2,3-DCDD) were purchased from AccuStandard (New Haven, CT). 2,7-DichloroDD (2,7-DCDD), 2,3,7-trichloroDD (2,3,7-TriCDD), and 2,3,7,8-tetrachloroDD (2,3,7,8-TetraCDD) were purchased from Cambridge Isotope Lab. (Andover, MA). The yeast microsomes containing recombinant human CYP1A1 or human CYP1A2 were purchased from Sumika Chemical Analysis Service (Osaka, Japan) [11]. Specific content of P450 (pmol/mg protein) and standard activity (mol/min/mol P450) of each microsomal fraction measured in Sumika Chemical Analysis Service were as follows:

CYP1A1, 16.2 (pmol/mg protein), 9.3 (mol/min/mol P450) for 7-ethoxyresorufin O-deethylation; CYP1A2, 40.0 (pmol/mg protein), 2.4 (mol/min/mol P450) for 7-ethoxyresorufin O-deethylation. NADPH was purchased from Oriental Yeast (Tokyo, Japan). All other chemicals were of the best commercially available grade.

Preparation of microsomal fractions from the recombinant Saccharomyces cerevisiae cells

The recombinant *S. cerevisiae* AH22/pAMR2 cells [12] expressing rat CYP1A1 and AH22/pGR1A2 cells expressing rat CYP1A2 [10] were cultivated in a synthetic minimal medium containing 8% glucose, 5.4% yeast nitrogen base without amino acids, and 160 mg/L histidine. Microsomal fractions of AH22/pAMR2 cells and AH22/pGR1A2 cells were prepared as described previously [10]. The contents of CYP1A1 and CYP1A2 in the corresponding microsomal fractions were 130 and 231 pmol/mg protein, respectively.

Measurement of reduced CO difference spectra and 2,3,7,8-tetraCDD-induced difference spectra

The reduced CO difference spectra were measured with a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) according to the following procedure as described previously [13–17]. The concentration of CYPs was determined from the reduced CO difference spectrum using a difference of the extinction coefficients at 446 and 490 nm of 91 $mM^{-1} cm^{-1}$ [18]. The substrate-induced difference spectra of the microsomal fraction containing 0.33 μM P450 were measured in the presence of 3.1 μM 2,3,7,8-TetraCDD. The solution contains dimethylsulfoxide at a final concentration of 2.0%.

Measurement of catalytic activity toward PCDDs

The substrates DD, 1-MCDD, and 2-MCDD were each dissolved in ethanol, 2,3-DCDD, 2,7-DCDD, and 2,3,7-TriCDD were dissolved in acetone, 2,3,7,8-TetraCDD was dissolved in dimethylsulfoxide. The reaction mixture contained 0–10 μM PCDDs with 1% of the organic solvent and the microsomes containing 7.5–100 nM each of the CYPs in 50 mM potassium phosphate buffer, pH 7.4. The reaction was initiated by the addition of NADPH at a final concentration of 0.5 mM. Aliquots of the reaction mixture were collected after varying time intervals and were extracted with 4 volumes of chloroform/methanol (3:1. v/v). The organic phase was recovered and carefully dried in a vacuum evaporator centrifuge (Sakuma Seisakusyo, Tokyo, Japan) without evaporating substrates and metabolites. The recovery of the substrates and the metabolites was estimated to be approximately 80%. The resulting residue was solubilized with acetonitrile and applied to HPLC performed on a YMC-Pack ODS-AM [4.6 mm (inner diameter) × 300 mm] (YMC, Kyoto, Japan). A linear gradient of 20–100% acetonitrile aqueous solution containing 0.01% trifluoroacetic acid for 25 min followed by 100% acetonitrile for 10 min was used as a mobile phase. The flow rate was 1.0 ml/min, and the elution was detected by measuring absorption at 227 nm. The column temperature was maintained at 40 °C.

Mass spectrometric analysis of the metabolites

Isolated metabolites from HPLC effluents were subjected to GC-mass spectrometric analysis using a Finnegan mat Thermo Quest GC with EI mode. Chrompack Cp-Sil 24CB-MS (0.32 mm × 30 m) was used as a GC column.

Other methods

The concentrations of PCDDs and their metabolites with the dioxin ring were estimated by using a molar extinction coefficient of $4.12 \times 10^4 M^{-1} cm^{-1}$ at 227 nm on the basis of spectral analysis of PCDDs. Protein concentration was determined by the method of Lowry et al. [19], using bovine serum albumin as a standard.

Results

Rat CYP-dependent metabolism of PCDDs

The yeast microsomal fraction containing each of the recombinant CYPs was examined for the metabolism of PCDDs. The control microsomes with overexpression of yeast NADPH-P450 reductase [12] showed no activity toward PCDDs. Each form of rat CYP showed catalytic activities toward DD, 1-MCDD, 2-MCDD, 2,3-DCDD, 2,7-DCDD, and 2,3,7-TriCDD (Table 1). Among the four CYPs, rat CYP1A1 showed the highest activities toward all the PCDDs examined in this study. Among the PCDDs examined, rat CYP1A1 showed the highest activity toward 2-MCDD. Significant species differences between rat and human CYP1A subfamilies were observed in the metabolism of PCDDs (Table 1). For example, the activity of rat CYP1A1 toward DD was 45-fold higher than that of human CYP1A1 (Table 1). It was noted that activities of human CYP1A1 toward PCDDs increased with increasing number of chloride substituents from 0 to 3. On the other hand, no significant correlation was observed between activities of rat CYP1A1 toward PCDDs and number of chloride substituents. Toward 2,3,7-TriCDD, both rat and human CYP1A1 showed similar enzymatic properties as shown in Table 2 [10]. In CYP1A2-dependent metabolism of 1-MCDD, a significant species difference was observed as shown in Fig. 1. As reported previously [10], rat CYP1A2 showed two major metabolites formed from 2,7-DCDD and 2,3,7-TriCDD. Rat CYP1A2 can catalyze hydroxylation with elimination of a chloride substituent toward those substrates. Similar metabolic pattern was also observed in human CYP1A2. On the other hand, no CYPs showed detectable catalytic activities (less than 0.005 (mol/min/mol P450)) toward 2,3,7,8-TetraCDD.

Metabolism of 2-MCDD by rat CYP1A1

The yeast microsomal fraction containing recombinant rat CYP1A1 was examined for the analysis of metabolic pathways of 2-MCDD. Fig. 2 shows HPLC analysis of metabolite of 2-MCDD by rat CYP1A1.

Table 2
Kinetic parameters for 2,3,7-TriCDD 8-hydroxylation activity by rat and human CYP1A subfamily

CYP	K_m (μM)	V_{max} (mol/min/mol P450)
Rat CYP1A1[a]	0.20 ± 0.03	63.6 ± 6.5
Human CYP1A1	0.30 ± 0.08	50.8 ± 2.7
Rat CYP1A2[a]	0.34 ± 0.01	1.1 ± 0.2
Human CYP1A2	0.23 ± 0.04	3.9 ± 0.7

The K_m and V_{max} values represents the mean ± SD from three separate experiments.
[a] The values were reported in our previous report [10].

Approximately 70% of the substrate was converted into the metabolites designated M1, M2, M3, M4, M5, M6, and M7 at 2 min of the reaction, and no substrate was observed at 8 min. M1 and M2 were major metabolites at 2 min, while M6 and M7 were major metabolites at 8 min. It is noted that extinction coefficients of M6 and M7 at 227 nm are 11.4 and 7.4% of that of 2-MCDD, respectively, as described in the next section. Fig. 3 shows time course of 2-MCDD metabolism, suggesting that most of the 2-MCDD is first converted into M1 and M2. When M1 and M2 collected from HPLC effluents were added to the reaction mixture as the substrates, the metabolites M5, M6, and M7 were observed (data not shown). These results strongly suggest that most of the 2-MCDD was first converted into the metabolites M1 and M2, and then M1 and M2 were further metabolized to M5, M6, and M7. The recovery of the substances was decreased sequentially as shown in Fig. 3. After 6 min, the recovery was less than 50%, suggesting that more hydrophilic metabolites than M7 were produced.

Identification of the metabolites by GC-MS analysis

The metabolites collected from HPLC effluents were subjected to GC-mass spectrometric analysis. The mass spectrum of P1 showed molecular ions at m/z 234 (M with $^{35}Cl)^+$, 236 (M with $^{37}Cl)^+$, 205 (M–COH)$^+$, and 178 (M–2CO)$^+$ (Fig. 1C), suggesting that the metabolite contained a hydroxyl group. On the other hand, the mass spectrum of P2 showed molecular ions at m/z 234 (M with $^{35}Cl)^+$, 236 (M with $^{37}Cl)^+$, 199 (M–Cl)$^+$, and 171 (M–COCl)$^+$ (Fig. 1D). Although the mass spectra

Table 1
Metabolism of PCDDs by rat and human CYP1A subfamily

Activity (mol/min/mol P450)							
CYP	DD	1-MC	2-MC	2,3-DC	2,7-DC	2,3,7-TC	2,3,7,8-TC
Rat 1A1	44.6	28.2	72.6	31.5	27.2	60.2	—
Human 1A1	1.0	3.4	10.3	18.4	20.0	44.7	—
Rat 1A2	2.7	0.6	1.5	0.4	0.8	0.9	—
Human 1A2	5.6	0.2	5.0	1.6	2.2	5.5	—

Each value represents the total activity including multiple metabolities at substrate concentration of 10 μM except for 2,3,7,8-TetraCDD (0.78 μM) as described under Materials and methods. Value are means of triplicate determinations; —, not detected (less than 0.005).

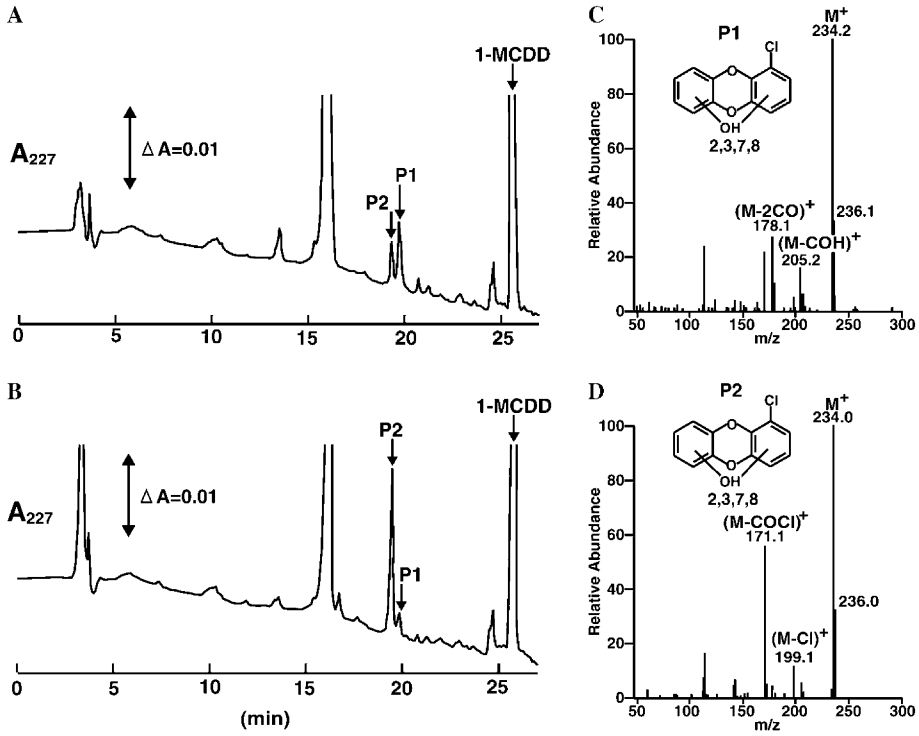

Fig. 1. HPLC profiles of 1-MCDD and its metabolites by human CYP1A2 (A) and rat CYP1A2 (B) and mass spectra of the metabolites P1 (C) and P2 (D). Following 10 min of incubation with 10 μM 1-MCDD, each reaction mixture was extracted and analyzed by HPLC as described under Materials and methods. P1 and P2 appear to have a hydroxyl group at a distinct site in a lateral (C-2,3,7,8) position as described under Results.

of P1 and P2 were clearly different, the mass spectra of methylated compounds of both metabolites showed molecular ions at m/z 248 (M with $^{35}Cl)^+$, 233 (M–$CH_3)^+$, and 205 (M–$COCH_3)^+$ (data not shown). The presence of (M–$CH_3)^+$ fragment indicates that the methoxy group is in a lateral (C-2, C-3, C-7, and C-8) position as described by Tulp and Hutzinger [20]. These results suggest that P1 and P2 have a hydroxyl group at a distinct site in a lateral position.

The broad peak including M1 and M2 in HPLC (Fig. 2) was clearly separated into two peaks designated M1 and M2 in GC profile (Fig. 4A). The mass spectrum of M1 showed molecular ions at m/z 234 (M with $^{35}Cl)^+$, 236 (M with $^{37}Cl)^+$, 199 (M–Cl)$^+$, and 171 (M–COCl)$^+$. On the other hand, the mass spectrum of M2 showed molecular ions at m/z 234 (M with $^{35}Cl)^+$, 236 (M with $^{37}Cl)^+$, 205 (M–COH)$^+$, and 178 (M–2CO)$^+$, suggesting that M2 has a hydroxyl group at a site different from that of M1. Mass spectra of methylated compounds of both M1 and M2 showed (M–$CH_3)^+$ fragment, indicating the presence of the methoxy group in a lateral position (data not shown). Thus, M1 and M2 appear to have a hydroxyl group at a distinct site in a lateral position. Based on the GC profile shown in Fig. 4A, the amount of M1 was estimated to be approximately 50% of that of M2.

The mass spectrum of M3 showed molecular ions at m/z 234 (M with $^{35}Cl)^+$, 236 (M with $^{37}Cl)^+$, 199 (M–Cl)$^+$, and 171 (M–COCl)$^+$. The elution time of M3 in HPLC coincided with that of the metabolite of 1-MCDD with a hydroxyl group in a lateral position (data not shown). These results strongly suggest that M3 is 2-hydroxy-1-MCDD arising via an NIH shift (Fig. 5A).

The mass spectrum of M4 showed molecular ions at m/z 200 (M)$^+$, 171 (M–COH)$^+$, and 144 (M–2CO)$^+$, suggesting that the metabolite contained a hydroxyl group with an elimination of a chloride substituent of 2-MCDD (Fig. 5B).

The mass spectrum of M5 showed molecular ions at m/z 250 (M with $^{35}Cl)^+$, 221 (M–COH)$^+$, 215 (M–Cl)$^+$, 194 (M–2CO)$^+$, and 187 (M–COCl)$^+$, suggesting that the metabolite contained two hydroxyl groups (Fig. 5C).

The mass spectrum of M6 showed molecular ions at m/z 144 (M with $^{35}Cl)^+$ and 146 (M with $^{37}Cl)^+$ (Fig. 5D). The elution time of M6 in HPLC was identical with that of 4-chlorocatechol. These results strongly suggest that M6 is 4-chlorocatechol. On the other hand, the mass spectrum and HPLC profile of M7 suggest that M7 is catechol (data not shown). As mentioned above, the extinction coefficients of authentic 4-chlorocatechol and catechol at 227 nm are 11.4 and 7.4% of 2-MCDD, respectively. Thus, the amounts of M6 and M7 were estimated by HPLC analysis on the basis of the peak areas of known amounts of authentic 4-chlorocatechol and catechol, respectively. Based on these results, metabolic pathways of 2-MCDD by rat CYP1A1 are summarized in Fig. 6.

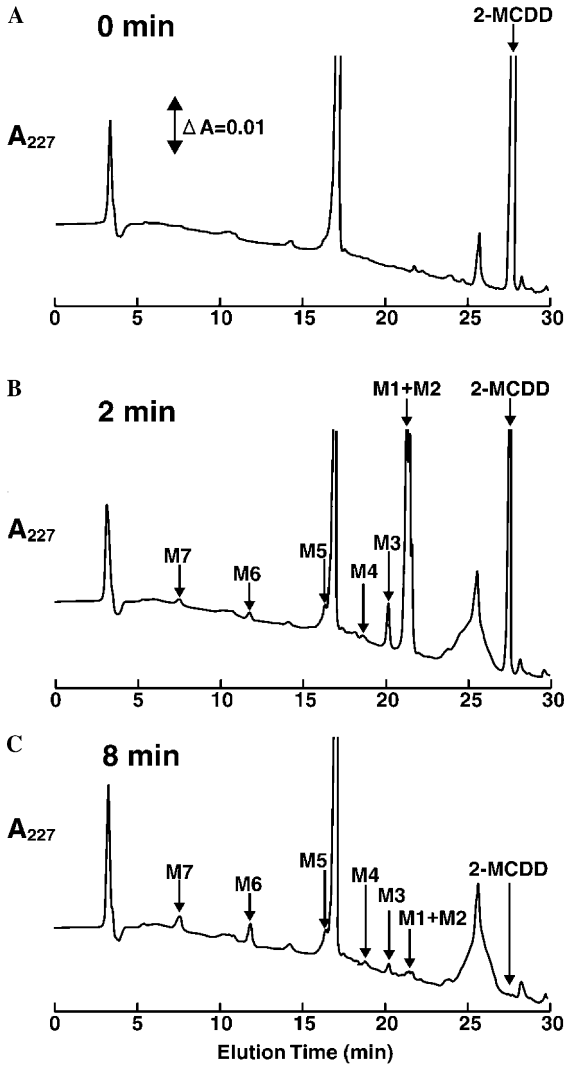

Fig. 2. HPLC profiles of 2-MCDD and its metabolites by rat CYP1A1. Following 0 min (A), 2 min (B), and 8 min (C) of incubation with 10 μM 2-MCDD, each of reaction mixtures was extracted and analyzed by HPLC as described under Materials and methods.

Kinetic analysis of hydroxylation of 2-MCDD by rat CYP1A

The initial rates of the formation of M1 and M2 were measured at various concentrations of 2-MCDD. The apparent kinetic parameters, K_m and V_{max}, were estimated to be 0.53 ± 0.01 μM and 83.6 ± 7.8 (mol/min/mol P450), respectively, by Hanes–Woolf (S/v versus S) plots.

2,3,7,8-TetraCDD-induced difference spectrum of CYP1A1

Addition of 2,3,7,8-TetraCDD to the microsomal fraction containing rat CYP1A1 (Fig. 7) or human CYP1A1 (data not shown) induced a typical Type I spectrum, indicating a change of the heme iron of

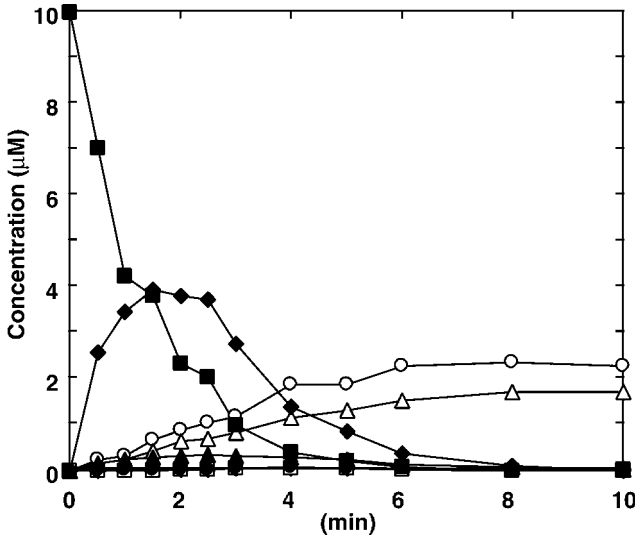

Fig. 3. Time course of 2-MCDD metabolism by rat CYP1A1. The substrate 2-MCDD was added to the reaction mixture at a final concentration of 10 μM. Concentrations of 2-MCDD (■), M1 + M2 (◆), M3 (▲), M4 (●), M5 (◊), M6 (△), and M7 (○) were estimated on the basis of HPLC analysis.

CYP1A1 from a low-spin state to a high-spin state upon binding of 2,3,7,8-TetraCDD. It should be noted that 2,3,7,8-TetraCDD bound to the substrate-binding pocket of rat and human CYP1A1, whereas no detectable activity of CYP1A1 was observed toward 2,3,7,8-TetraCDD. On the other hand, no spectral change of CYP1A2 was observed with the addition of 2,3,7,8-TetraCDD, probably due to a high-spin form of CYP1A2 even in the absence of a substrate.

Discussion

We examined the metabolism of the PCDDs by rat and human CYP1A subfamily and revealed a significant species difference in dioxin metabolism. The similarity of amino acid sequences in human CYP1A1 and rat CYP1A1 is 79%, and the similarity between human CYP1A2 and rat CYP1A2 is 73%. Thus, the significant species difference in dioxin metabolism might be accountable. Spectral analysis clearly demonstrated that both rat and human CYP1A1 can bind 2,3,7,8-TetraCDD. Although we have not detected the metabolic activities of CYP1A subfamily toward 2,3,7,8-TetraCDD, a remarkable species difference between humans (7.1 years) and rats (a few weeks) in a half-life time of 2,3,7,8-TetraCDD [21–23] might originate from the species differences in enzymatic properties and the expression levels of CYP1A1 and CYP1A2.

Among the CYPs examined, rat CYP1A1 showed the highest catalytic activity toward DD, mono-, di-, and trichloroDDs. Our previous study demonstrated a

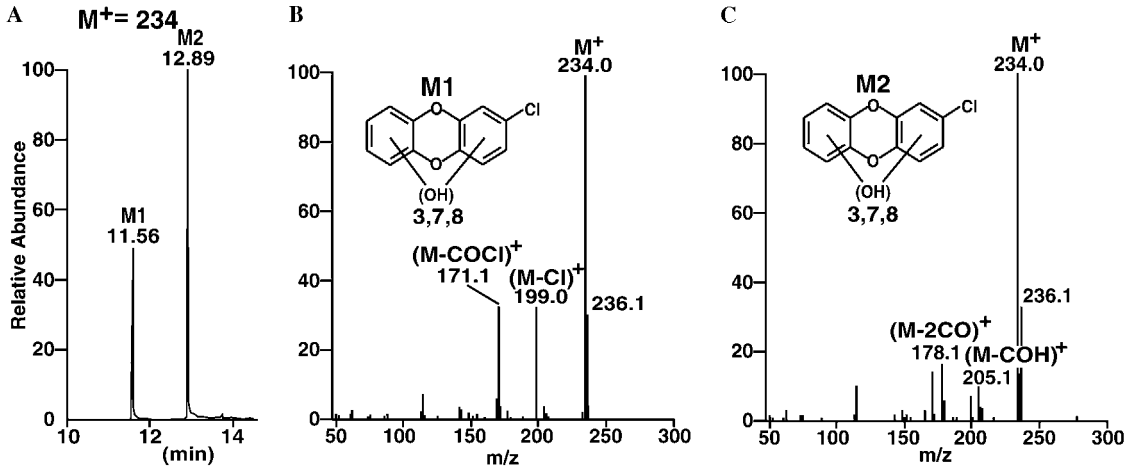

Fig. 4. GC profile ($m/z = 233.5$–234.5) of the metabolites of 2-MCDD collected from HPLC effluent (A) and mass spectra of M1 (B) and M2 (C) in Fig. 3. M1 and M2 appear to have a hydroxyl group at a distinct site in a lateral (C-2,3,7,8) position as described under Results.

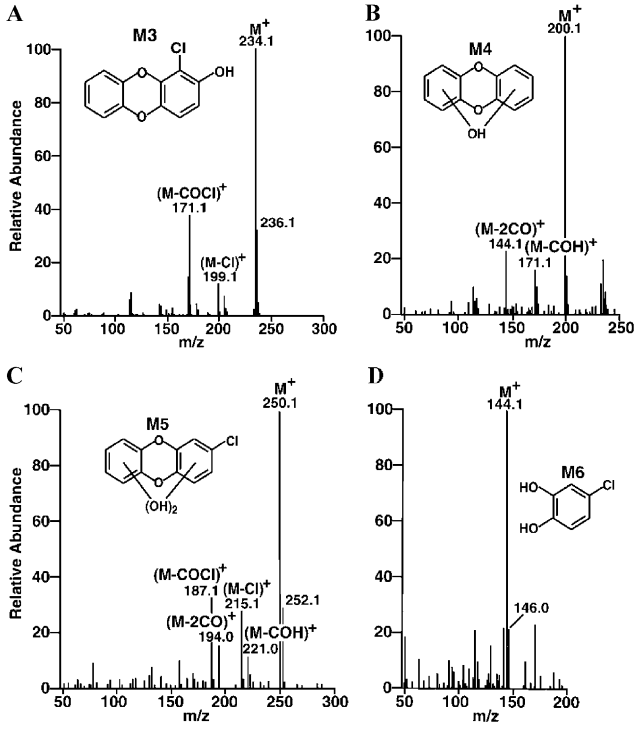

Fig. 5. Mass spectra of M3 (A), M4 (B), M5 (C), and M6 (D) in Fig. 2. Possible structures of M3, M4, M5, and M6 are shown.

remarkable metabolism of 2,7-DCDD and 2,3,7-TriCDD by yeast cells expressing recombinant rat CYP1A1 [10]. In this study, we tried to reveal metabolic pathways of PCDDs by CYP1A1 using microsomal fractions. Among the PCDDs examined, rat CYP1A1 showed the highest activity toward 2-MCDD (Table 1), and many metabolites were identified with HPLC and GC-MS analysis. It is noteworthy that catechols and a two-hydroxylated compound were identified as metabolites with GC-MS analysis. Two major metabolites of 2-MCDD, M1 and M2, were observed at initial stage of the metabolism. It is noted that the ratio of M1 to M2 by rat CYP1A1 was approximately 1:2 (Fig. 4A), while the ratio by human CYP1A1 was approximately 1:10 (data not shown). These results indicate the difference of reaction specificity between rat and human CYP1A1. The metabolite M3 was suggested to be 2-hydroxy-1-MCDD arising via an NIH shift. In vivo studies using rats showed the presence of NIH-shifted metabolites [3,5]. Our results demonstrated that P450 monooxygenase could catalyze this reaction probably via formation of epoxide as shown in Fig. 6. The metabolite M4 was produced by hydroxylation with elimination of a chloride substituent of 2-MCDD, probably via 2,3-epoxide or 1,2-epoxide formation. Poiger et al. [3] observed 8-OH-2,3,7-TriCDD as a metabolite of 2,3,7,8-TetraCDD in rats. This reaction seems quite essential for detoxication of PCDDs. The metabolite M5 was formed as a result of two-step sequential hydroxylation of the substrate. Judging from the fact that M5 was observed even at an initial stage of metabolism, at least a part of M5 was produced by two-step sequential hydroxylation without being released from the substrate-binding pocket of CYP1A1 [24]. The metabolites M6 and M7 were identified as 4-chlorocatechol and catechol, respectively. The cleavage of ether linkages of dioxin ring is considered to be the most important reaction for detoxication of dioxins. The putative mechanism of opening of dioxin ring is shown in Fig. 6. Epoxide formation by CYP1A1 would be followed by hemiacetal formation to yield 4′- or 5′-chloro-2,2′-dihydroxy-diphenylether spontaneously. The same reactions toward 4′- or 5′-chloro-2,2′-dihydroxy-diphenylether would produce 4-chlorocatechol and catechol. The epoxide and hemiacetal could not be detected probably due to their unstability. When M1 and M2 collected from HPLC effluents were added to the

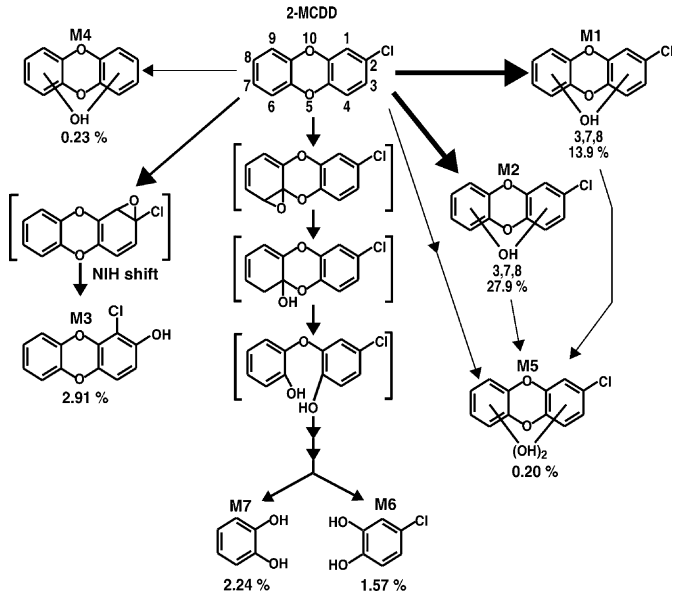

Fig. 6. Metabolic pathways of 2-MCDD by rat CYP1A1. The values present the ratio of each metabolite at 2 min after the reaction started. The putative intermediates are given in brackets.

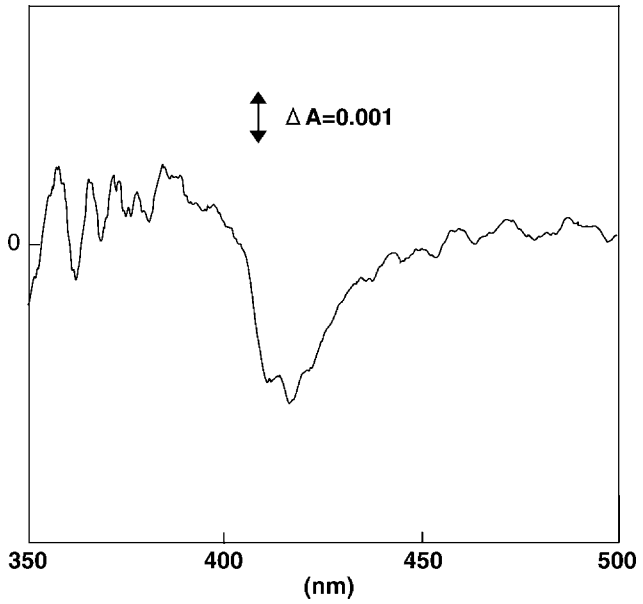

Fig. 7. 2,3,7,8-TetraCDD induced difference spectrum of rat CYP1A1. The difference spectrum of the microsomal fraction containing 0.33 μM rat CYP1A1 was measured in the presence of 3.1 μM of 2,3,7,8-TetraCDD as described under Materials and methods.

reaction mixture as the substrates, metabolites M5, M6, and M7 were observed. If the ether linkages of M1 and M2 cleaved via the pathway described in brackets in Fig. 6, the metabolites with three hydroxy groups should be observed in addition to M6 and M7. However, we could not detect metabolites with three hydroxyl groups. High polarities of the metabolites might disturb the extraction of the metabolites to organic phase. Fig. 6 summarizes the metabolic pathways of 2-MCDD by rat CYP1A1. It is noteworthy that Fig. 6 contains almost all of the metabolic reactions toward dioxins observed in vivo using experimental animals except for conjugation with glucuronide and sulfate [1–8]. These results strongly suggest that P450 monooxygenases, particularly in CYP1A subfamily, are greatly responsible for metabolism of dioxins. Nevertheless, we could not detect CYP1A-dependent activity toward 2,3,7,8-TeraCDD. Extremely low activity of CYP1A toward 2,3,7,8-TetraCDD, which tightly binds to Ah receptor, appears to result in the extreme toxicity of 2,3,7,8-TetraCDD.

Recently, Parikh et al. [25] have succeeded in the construction of mutants of human CYP1A2 whose activities toward 7-ethoxyresorufin have increased. Carmichael and Wong [26] have changed substrate specificity of CYP102 by protein engineering, and the mutant showed high activity toward polycyclic aromatic hydrocarbons that were more bulky than original substrates. As rat CYP1A1 showed a remarkable metabolism of 2,3,7-TriCDD, it might be possible to construct a mutant CYP1A1 with a high catalytic activity against 2,3,7,8-TetraCDD by site-directed mutagenesis.

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