Congener-specific metabolism and sequestration of dioxin-like compounds by cytochrome P450 1A induced in the liver of crows from Tokyo, Japan

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Introduction

Jungle crow (JC; *Corvus macrorhynchos*) is a useful bioindicator for monitoring contaminants in urban areas, because this species is residential, occupies a same habitat as human, and feeds variety of foods including domestic waste and garbage. Therefore, JCs may accumulate environmental contaminants such as polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and coplanar polychlorinated biphenyls (Co-PCBs), which are released by human activities.

Induction of cytochrome P450 (CYP) 1A is a responsive mechanism elicited by exposure to dioxin-like compounds including PCDDs/DFs and Co-PCBs. Toxicokinetic behavior of dioxin-like compounds in organisms is controlled by excretion, metabolism and absorption. These processes are, at least partly, dependent on CYP1A expression in addition to chemical structure and number of chlorine substitution of each congener. Low chlorinated congeners such as 2378-T_4CDD, 2378-T_4CDF, 12378-P_5CDD and 33'44'-PCB were easily metabolized by CYP1A1/2 in rat liver microsomes[1,2,3]. PCDDs/DFs accumulate in hepatic tissue to a greater extent than adipose tissue in rats and mice[4,5,6]. Recent study using transgenic CYP1A2 knockout mice demonstrated that CYP1A2 is responsible for the sequestration of 2378-T_4CDD and 23478-P_5CDF in hepatic tissue[7]. Therefore, CYP1A is considered as a key factor responsible for toxicokinetics of dioxin-like compounds. However, there's no comprehensive data on the contribution of CYP1A to the toxicokinetics of dioxin-like congeners in wild populations.

In this study, we investigated contamination levels of PCDDs/DFs and Co-PCBs in liver and breast muscle of JCs from Tokyo, Japan, and interactions of dioxin-like congeners with hepatic CYP to elucidate congener-specific toxicokinetics related to CYP expression in JC.

Materials and Methods

Thirteen JCs were collected by trapping from Tokyo, Japan in December 2002 under permission from the Tokyo Metropolitan Government. JCs were euthanized under deep anesthesia with ether, their biometry measured and dissected immediately. Breast muscle and liver samples for chemical analysis were stored in a freezer at -20 . Liver samples for enzyme assays were frozen in liquid nitrogen, and stored at -80 until microsomal preparation.

Chemical analysis of PCDDs/DFs and Co-PCBs was carried out following the standard method of the Environmental Agency of Japan[8] with some modifications. Liver and muscle samples of JCs were spiked with ^{13}C-labeled surrogate PCDDs/DFs and Co-PCBs standards, and extracted with 1.5 M ethanol-KOH for 1.5 hours. The extract was treated with sulfuric acid for cleanup and then applied to a multilayer silica gel column connected with graphite carbon column. The connected column was eluted with hexane, the multilayer silica gel column was removed and the graphite column was eluted with a mixture of 25% dichloromethane (DCM) in hexane with normal flow. The two fractions collected were pooled and concentrated, and passed through an activated basic alumina column. Mono-*ortho* Co-PCBs were eluted in the second fraction of alumina column with 5% DCM in hexane. The graphite column eluted with toluene in reverse flow contained PCDDs/DFs and non-*ortho* Co-PCBs. Identification and quantification of PCDDs/DFs and Co-PCBs were performed using HRGC/HRMS [Hewlett-Packard (HP) 5890 Series II / JEOL JMS SX-102A, HP 6890/JEOL JMS-700 or HP 6890/JEOL JMS-700D]. 2378-T_4CDD toxic equivalent (TEQ) was calculated by WHO bird-TEF[9].

Hepatic microsomal fractions were prepared according to the method of Guengerich[10]. Protein content was measured with the bicinchoninic acid assay. Measurements of methoxy-(MROD), ethoxy-(EROD), pentoxy-(PROD) and benzyloxyresorufin-*O*-dealkylation (BROD) activities, which were known to be CYP1A- or CYP2B-dependent enzyme activities in rodents were done by the method of Kennedy *et al*[11]. For inhibition test of PROD activity, hepatic microsomes were preincubated with polyclonal antibodies against rat CYP1A1, 2B1, 2C6, 3A2 or control sera for 30 min at room temperature before starting the PROD reaction by the addition of NADPH. Immunoblotting of liver microsomal fraction was conducted by western blot analysis using polyclonal antibodies against rat CYP1A1, 2B1, 2C6 and 3A2.

Correlation analyses were examined by Spearman's rank correlation. Mann-Whitney *U*-test was used for detection of statistical differences among groups. For samples with values below quantification limit, half of the respective limit of quantification was substituted to calculate mean concentrations, standard deviations and TEQs, and to perform statistical analysis. When more than half of the observations were below the quantification limit, statistical analyses were not conducted, and results were shown as "not data available (NA)". Specimens with values below quantification limit in both liver and breast muscle of JCs were not used for statistical analysis of relationships between ratios of congener concentrations in the liver to those in the breast muscle and CYP1A-like protein levels. Statistically significant *p*-value was regarded as $p < 0.05$.

Results and Discussion

Concentrations and liver-muscle concentration ratios

The concentrations of PCDDs/DFs in the liver and breast muscle of JCs were from 100 to 1,900, and from 21 to 180 pg/g lipid wt., respectively (Table 1). Concentrations of total Co-PCBs in the

Table 1. Concentrations and TEQs (pg/g lipid wt.) of PCDDs/DFs and Co-PCBs in the liver and breast muscle of JCs from Tokyo.

Tissue	Liver		Breast muscle	
lipid (%)	4.9 ± 0.73	(3.9-6.5)	2.8 ± 0.97	(1.5-4.5)
total PCDDs	470 ± 380	(42-1,200)	44 ± 34	(9.4-130)
total PCDFs	330 ± 230	(55-710)	27 ± 16	(11-56)
total non-*ortho* Co-PCBs	260 ± 200	(38-660)	77 ± 46	(23-170)
total mono-*ortho* Co-PCBs	65,000 ± 33,000	(16,000-130,000)	74,000 ± 53,000	(18,000-220,000)
PCDDs-TEQs	43 ± 35	(7.5-91)	13 ± 11	(1.6-42)
PCDFs-TEQs	82 ± 60	(11-190)	8.4 ± 5.3	(2.2-20)
non-*ortho* Co-PCBs-TEQs	2.7 ± 1.7	(0.93-7.1)	2.9 ± 1.7	(0.72-6.0)
mono-*ortho* Co-PCBs-TEQs	3.1 ± 1.5	(0.65-5.1)	3.6 ± 2.2	(0.74-8.9)
total TEQs	130 ± 96	(23-280)	27 ± 20	(5.6-78)

Values are means ± standard deviation. Numbers in parentheses indicate the range.

liver and muscle of JCs were 16-130, and 18-220 ng/g lipid wt., respectively. Congeners including 2378-T_4CDD, 123789-H_6CDD, 2378-T_4CDF, 12378-P_5CDF, 123789-H_6CDF, O_8CDF, CB-81 and CB-126, which were detected from a variety of avian species in Japan[12], were not detected in most of JC livers in this study (Fig.1). This implies high metabolic potential in JC. Comparison of congener concentrations between liver and breast muscle showed significant higher concentrations of seven PCDD/DF congeners, CB-169 and CB-123 in the liver than those in the breast muscle ($p < 0.05$; Fig.1). Lipid-normalized concentration ratios between liver and breast muscle (L/M) tended to increase with the number of chlorine substitution (Fig.2). In contrast, no statistical difference in concentration between liver and muscle was found for CB-77 and mono-*ortho* Co-PCBs except CB-123.

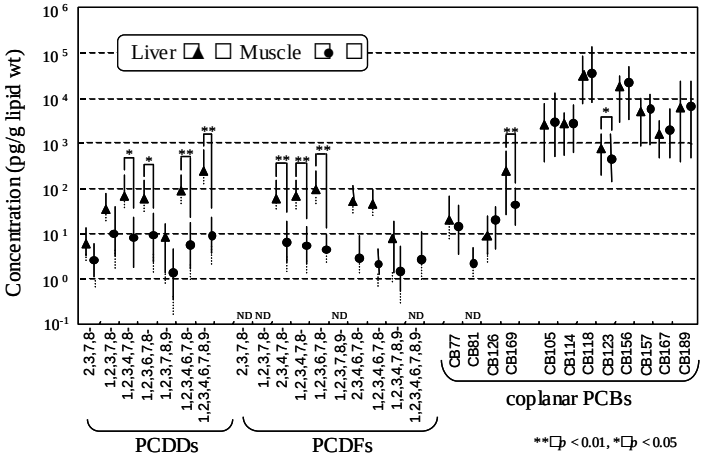

Figure 1. Concentrations of PCDDs/DFs and Co-PCBs in livers and breast muscle of JCs from Tokyo. Triangles and circles mean average concentrations in liver and muscle of JCs, respectively. Bars indicate the range. Dotted line means that there were specimens in which congeners were not detected. ND means that congeners were not detected in all specimens.

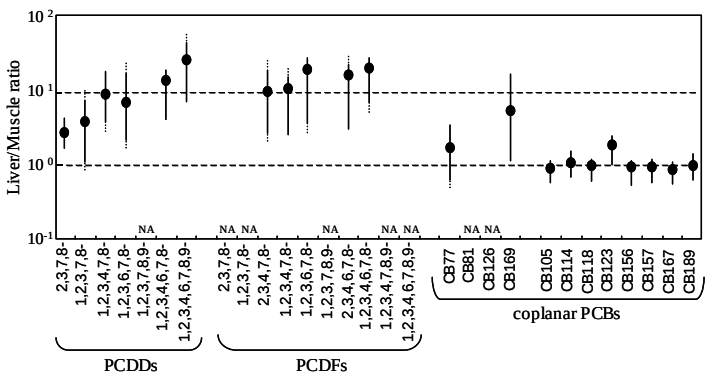

Figure 2. Concentration ratios of PCDD/DF and Co-PCB congeners in the livers to those in the muscle of JCs. Average (plots) and range (bars) are shown. Dotted line over and under the bars indicates that congeners were not detected in either muscle or liver.

TEQ

Total TEQ concentrations in the liver and muscle of JCs were 23-280, and 5.6-78 pg/g lipid wt., respectively. These TEQ concentrations in JCs were mostly lower than those in other bird species from North America, Asian and European developed countries, so far reported[12,13,14]. Sum of TEQ from both 12378-P_5CDD and 23478-P_5CDF (TEF = 1.0) contributed 33-75% and 26-70% in total TEQ in the liver and muscle of JCs, respectively.

Table 2. Spearman rank correlations between alkoxyresorufin-*O*-dealkylation activities and CYP1A-, 2B-, 2C- and 3A-like protein expression levels in liver microsomes of JCs.

	HMW CYP1A	LMW CYP1A	CYP2B	CYP2C	CYP3A
Alkoxyresorufin-*O*-dealkylation					
MROD	- 0.099	0.30	0.082	0.65	- 0.49
EROD	0.38	0.38	0.016	0.31	0.12
PROD	0.72	0.41	- 0.22	0.041	0.50
BROD	0.53	- 0.069	- 0.18	- 0.42	0.75

: $p < 0.05$; : $p < 0.01$

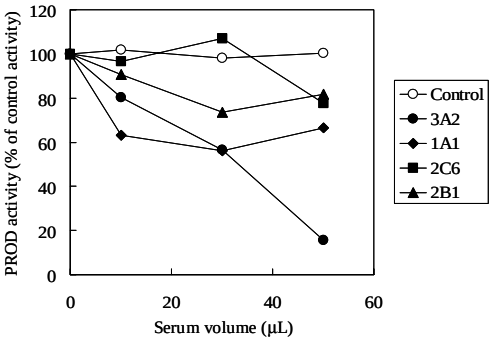

Figure 3. Antibody inhibition of PROD activity in hepatic microsomes of a JC. The reactions were carried out in the presence of varying volumes of control serum, anti-rat CYP1A1, 2B1, 2C6 and 3A2 serum.

AROD vs CYP protein expression

Alkoxyresorufin-*O*-dealkyration (AROD) activities in the liver microsomes were characterized by high M- and EROD activities followed by P- or BROD activities. CYP1A-, 2B-, 2C- and 3A-like proteins were immunochemically detected using anti-rat CYP polyclonal antibodies in hepatic microsomes of JCs. Regarding CYP1A-like proteins, two bands with higher (HMW) and lower molecular weight (LMW) were found, implying the presence of CYP1A5- and CYP1A4-like proteins as previously suggested in chicken[15]. To investigate which CYP subfamily is responsible for AROD activities, correlation analyses were conducted between CYP protein contents and AROD activities. It was found that P- and BROD activities had relatively high positive correlations with HMW CYP1A and 3A-like proteins (Table 2). Antibody inhibitions of PROD activity were studied using anti-rat CYP1A1, 2B1, 2C6 and 3A2 antisera to identify CYP subfamilies responsible for the catalytic activity (Fig.3). Magnitude of inhibition of PROD activity was in the following order: anti-rat CYP3A2 > 1A1 > 2B1 2C6, indicating that CYP3A and CYP1A may be responsible for PROD activity in JCs.

Induction of CYP

Although total TEQ in the liver of JCs were comparable or lower than the estimated lowest-observed-adverse-effect level (10 pg/g wet wt.) in white leghorn chicken embryo[13], hepatic TEQ in JCs exhibited a significant positive correlation with PROD and BROD activities (data not shown), and CYP1A/3A expression levels (Fig.4). This result suggests that JC is a sensitive species as for CYP

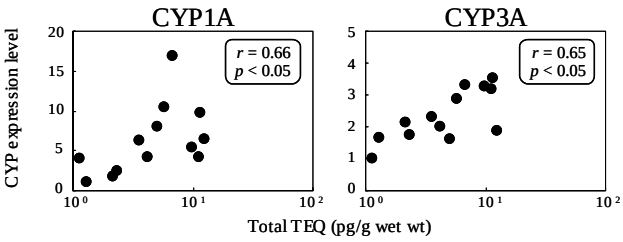

Figure 4. Relationships between TEQ and HMW CYP1A or 3A-like protein expression levels in JC livers.

induction. Induction of CYP3A by TEQ may be due to the parallel accumulation pattern between TEQ and unknown CYP3A inducers.

Hepatic metabolism of congeners

In order to investigate metabolic potential of CYP to each congener in the livers of JCs, we compared hepatic HMW CYP1A-like protein expression levels or AROD activities and the ratios of concentrations for PCDDs/DFs/Co-PCBs to CB-169 which is known to be almost metabolically resistant[16]. The ratios of several PCDD/DF and Co-PCB congeners revealed significant ($p < 0.05$) negative correlations with HMW CYP1A-like protein levels (Table 3), which implies a potential of CYP1A-like protein in JC to metabolize these congeners. The ratios of O_8CDD, however, showed no negative correlation with HMW CYP1A-like protein levels. O_8CDD is reported to be resistant to metabolism in rat due to complete chlorination and absence of carbon-hydrogen bonds limiting oxygenation by CYP[17].

Table 3. Relationships between CYP1A-like protein levels and concentration ratios of each congener to CB-169 in JC livers.

congener	*r*	*p*
PCDDs		
2,3,7,8-T_4CDD	NA	
1,2,3,7,8-P_5CDD	-0.57	0.048
1,2,3,4,7,8-H_6CDD	-0.26	0.37
1,2,3,6,7,8-H_6CDD	-0.47	0.10
1,2,3,7,8,9-H_6CDD	NA	
1,2,3,4,6,7,8-H_7CDD	-0.57	0.050
O_8CDD	0.005	0.98
PCDFs		
2,3,7,8-T_4CDF	NA	
1,2,3,7,8-P_5CDF	NA	
2,3,4,7,8-P_5CDF	-0.62	0.033
1,2,3,4,7,8-H_6CDF	-0.46	0.11
1,2,3,6,7,8-H_6CDF	-0.46	0.11
1,2,3,7,8,9-H_6CDF	NA	
2,3,4,6,7,8-H_6CDF	-0.70	0.016
1,2,3,4,6,7,8-H_7CDF	-0.71	0.013
1,2,3,4,7,8,9-H_7CDF	NA	
O_8CDF	NA	
Non-*ortho* PCBs		
3,3',4,4'-T_4CB (77)	-0.89	0.0020
3,4,4',5-T_4CB (81)	NA	
3,3',4,4',5-P_5CB (126)	NA	
3,3',4,4',5,5'-H_6CB (169)		
Mono-*ortho* PCBs		
2,3,3',4,4'-P_5CB (105)	-0.58	0.044
2,3,4,4',5-P_5CB (114)	-0.62	0.032
2,3',4,4',5-P_5CB (118)	-0.67	0.020
2',3,4,4',5-P_5CB (123)	-0.71	0.014
2,3,3',4,4',5-H_6CB (156)	-0.58	0.046
2,3,3',4,4',5'-H_6CB (157)	-0.56	0.055
2,3',4,4',5,5'-H_6CB (167)	-0.67	0.020
2,3,3',4,4',5,5'-H_7CB (189)	-0.27	0.35

Hepatic sequestration of congeners

The L/M ratios of multiple PCDD/DF congeners and CB-169 increased with an increase in hepatic HMW CYP1A-like protein expression levels (Table 4). The L/M ratios for higher chlorinated congeners revealed a tendency to be greater than those for lower chlorinated congeners. In contrast, no elevation was found in the L/M ratios for CB-77 and mono-*ortho* Co-PCBs. These results

Table 4. Relationships between the ratios of congener concentrations in the liver to those in the breast muscle, and HMW CYP1A-like protein levels in hepatic microsomes in JCs.

congener	a [#]	b	r^2	*p*
PCDDs				
2,3,7,8-T_4CDD	na	na	na	na
1,2,3,7,8-P_5CDD	0.35	1.5	0.48	0.033
1,2,3,4,7,8-H_6CDD	1.0	1.1	0.83	0.0023
1,2,3,6,7,8-H_6CDD	0.88	0.89	0.72	0.020
1,2,3,7,8,9-H_6CDD	na	na	na	na
1,2,3,4,6,7,8-H_7CDD	2.8	-1.3	0.81	0.0053
O_8CDD	2.3	9.4	0.63	0.0067
PCDFs				
2,3,7,8-T_4CDF	na	na	na	na
1,2,3,7,8-P_5CDF	na	na	na	na
2,3,4,7,8-P_5CDF	0.96	3.3	0.77	0.0036
1,2,3,4,7,8-H_6CDF	2.6	-3.2	0.77	0.0026
1,2,3,6,7,8-H_6CDF	2.8	3.6	0.72	0.014
1,2,3,7,8,9-H_6CDF	na	na	na	na
2,3,4,6,7,8-H_6CDF	na	na	na	na
1,2,3,4,6,7,8-H_7CDF	na	na	na	na
1,2,3,4,7,8,9-H_7CDF	na	na	na	na
O_8CDF	na	na	na	na
Non-*ortho* PCBs				
3,3',4,4'-T_4CB (77)	-0.020	1.6	0.012	0.75
3,4,4',5-T_4CB (81)	na	na	na	na
3,3',4,4',5-P_5CB (126)	na	na	na	na
3,3',4,4',5,5'-H_6CB (169)	0.85	0.17	0.77	0.0046
Mono-*ortho* PCBs				
2,3,3',4,4'-P_5CB (105)	-0.0024	0.93	0.0031	0.86
2,3,4,4',5-P_5CB (114)	0.0010	1.1	0.00033	0.78
2,3',4,4',5-P_5CB (118)	0.0081	0.90	0.042	0.56
2',3,4,4',5-P_5CB (123)	-0.013	1.9	0.021	0.59
2,3,3',4,4',5-H_6CB (156)	0.0013	0.93	0.00096	0.56
2,3,3',4,4',5'-H_6CB (157)	-0.0017	0.93	0.0016	0.37
2,3',4,4',5,5'-H_6CB (167)	0.011	0.79	0.099	0.46
2,3,3',4,4',5,5'-H_7CB (189)	0.010	0.92	0.044	0.53

#: Simple regression analysis was conducted; liver/muscle concentration (lipid wt) ratios = a x [relative expression levels of CYP1A] + b

suggest congener-specific hepatic sequestrations by the induced CYP1A-like proteins.

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References

1. Hu, K., Bunce, N.J. (1999) *J. Biochem. Mol. Tox.* 13, 307-315.
2. Murk, A., Morse, D., Boon, J., Brouwer, A. (1994) *Eur. J. Pharmacol.* 270, 253-261.
3. Tai, H.L., McReynolds, J.H., Goldstein, J.A., Eugster, H.P., Sengstag, C., Alworth, W.L., Olson, J.R. (1993) *Toxicol. Appl. Pharmacol.* 123, 34-42.
4. Chen, C.Y., Hamm, J.T., Hass, J.R., Birnbaum, L.S. (2001) *Toxicol. Appl. Pharmacol.* 173. 65-88.
5. DeVito, M.J., Ross, D.G., Dupuy, A.E. Jr., Ferrario, J., McDaniel, D., Birnbaum, L.S. (1998) *Toxicol. Sci.* 46. 223-234.
6. Körner, W., Golor, G., Schulz, T., Wiesmüller, T., Hagenmaier, H., Neubert, D. (2002) *Arch. Toxicol.* 75, 653-664.
7. Diliberto, J.J., Burgin, D.E., Birnbaum, L.S. (1999) *Toxicol. Appl. Pharmacol.* 159. 52-64.
8. Environmental Agency of Japan (2003) Manual on determination of dioxins in wildlife, in Japanese.
9. Van den Berg, M., Brinbaum, L., Bosveld, A.T.C., Brunström, B., Cook, P., Feeley, M., Giesy, J.P., Hanberg, A., Hasegawa, R., Kennedy, S.W., Kubiak, T., Larsen, J.C., Rolaf van Leeuwen, F.X., Liem, A.K.D., Nolt, C., Peterson, R.E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Wærn, F., Zacharewski, T. (1998) *Environ. Health Perspect.* 106, 775-792.
10. Guengerich, F.P. (1982) *Raven Press, NY,* 609-634.
11. Kennedy, S.W., Jones, S.P., Bastien, L.J. (1995) *Anal.Biochem.* 226, 362-370.
12. Senthilkumar, K., Iseki, N., Hayama,S., Nakanishi, J., Masunaga, S. (2002) *Arch.Environ.Contam.Toxicol.* 42, 244-255.
13 Giesy, J.P., Ludwig, J.P., Tillitt, D.E. (1994) *Environ. Sci. Technol.* 28, 128-135.
14. Koistein, J., Koivusaari, J., Nuuja, I., Vuorinen, P.J., Paasivirta, J., Giesy, J.P. (1997) *Environ. Toxicol. Chem.* 16, 1533-1544.
15. Gilday, D., Gannon, M., Yutzey, K., Bader, D., Rifkind, A.B. (1996) *J. Biol. Chem.* 271, 33054-33059.
16. Tanabe, S., Kannan, N., Subramanian, A., Watanabe, S., Tatsukawa, R. (1987) *Environ. Pollu.* 47. 147-163.
17. Van den Berg, M., De Jongh, J., Poiger, H., Olson, J.R. (1994) *Crit. Rev. Toxicol.* 24. 1-74.