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Inducing Potency of Aryl Hydrocarbon Hydroxylase Activity in Human Lymphoblastoid Cells and Mice by Polychlorinated Dibenzofuran Congeners

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Aryl hydrocarbon hydroxylase (AHH)-inducing potency of eight polychlorinated dibenzofuran (PCDF) isomers, 3,4,5,3′,4′,5′-hexachlorobiphenyl (HCB) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxon (TCDD) in two inbred mouse strains (AHH responsive and nonresponsive mouse strains) and eight human lymphoblastoid cell lines (four males and four females) was investigated to evaluate their relative toxic potency. In AHH nonresponsive DBA mouse strain, only TCDD induced hepatic AHH activity at a dose of 30 μg/kg, while in AHH responsive C57 mouse strain, six PCDF isomers besides TCDD could enhance the enzyme activity significantly. 2,3,7,8-Tetrachlorodibenzofuran (2,3,7,8-TCDF), 1,2,3,7,8-pentachlorodibenzofuran (1,2,3,7,8-PCDF) and 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PCDF) showed the highest AHH inducing activity among the PCDF isomers tested. In contrast with the results obtained from the mouse experiments, in human lymphoblastoid cells, 2,3,4,7,8-PCDF, 1,2,3,4,6,7-hexachlorodibenzofuran (1,2,3,4,6,7-HCDF) and 1,2,3,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HCDF) elicited the highest AHH induction and were as potent AHH inducers as TCDD. These observations suggest that toxicities of 2,3,4,7,8-PCDF, 1,2,3,4,6,7-HCDF and 1,2,3,4,7,8-HCDF in human tissues may be comparable to that of TCDD.

It was also observed that in both male and female human cell lines, the degree of AHH inducibilities of these compounds were roughly parallel to that of 3-methylcholanthrene, possibly indicating that genetic susceptibility among human population to the toxic compounds are also present similar to those reported among mouse strains.

Introduction

Polychlorinated dibenzofurans (PCDFs) have already been determined in rice-bran oils which caused Yusho and Yu-Cheng in Japan in 1968 and in Taiwan in 1979, respectively, and several tissues from these patients (*1–3*). PCDFs have been considered most important causative agent of these diseases because of their high toxicity (*4,5*). PCDFs and polychlorinated dibenzo-*p*-dioxins (PCDDs) have been found at several hundred parts per billion levels in fly ash samples of municipal incinerators and industrial heating facilities (*6,7*). PCDFs have even been identified in fat samples from animal wildlife (*8*). Hence, the human environment seems to be contaminated with PCDFs and PCDDs as well as polychlorinated biphenyls (PCBs). Based on these findings, toxicity of individual PCDF isomers should be investigated more in detail.

It has been reported that aryl hydrocarbon (benzo[a]-pyrene) hydroxylase (AHH) inducing potency of PCBs, PCDFs or PCDDs correlates well with their toxic potency (*9,10*), and AHH responsiveness genetically segregates toxicity of TCDD in mice (*11*). In this study, therefore, the AHH-inducing potency (AHH inducibility i.e., induction ratio, induced/control) of eight PCDF isomers, HCB and TCDD was investigated in AHH-responsive and nonresponsive strains of mice, and also in human lymphoblastoid cell lines with different AHH inducibilities determined with 3-methylcholanthrene (3-MC), and then correlated with their toxic potency. In addition, genetic variations in susceptibility to the compounds and the difference in species to their toxicity were also evaluated.

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Methods

Animal Studies

Treatments of Animals. Two inbred strains of mice, DBA/2CrSlc(DBA) and C57BL/6N(C57), were purchased from Nippon Clea Co., Ltd., Osaka, Japan, housed in stainless steel cages (four or five mice/cage) with planed-chip bedding and kept in a temperature- and humidity-controlled room (22 ± 1°C, 55±5%). The animals were maintained on a diurnal cycle of 12 hr light/12 hr darkness and permitted water and food (CE-2, Nippon Clea Co., Ltd., Osaka, Japan) *ad libitum*. The birth dates of animals used in this experiment varied by not more than 3 days. At 10 weeks of age and 3 days before sacrifice, each test compound was administered in olive oil (0.2 mL/25 g of body weight) and given intraperitoneally in doses of 300 mg/kg (3-MC), 120 μg/kg (HCB) or 30 μg/kg (all other compounds). Control mice received vehicle alone in a similar volume and on the same time schedules as the corresponding experimental animals.

Enzyme Preparation and Assays. The animals were killed and the livers were washed in ice-cold 0.15 M KCl containing 0.02 *M* N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, weighed, minced and homogenized (Potter-Elvehjem homogenizer with a Teflon pestle) with a 9-fold volume of ice-cold 0.15 M KCl plus 0.02 M HEPES (pH 7.4). These homogenates were centrifuged at 9000*g* and 4°C for 15 min; the supernatant fluids were used for the AHH assay. AHH activity was determined as follows: The reaction mixture, in a total volume of 1.05 mL, contained 125 μmole HEPES (pH 7.5), 0.53 μmole NADPH, 3.6 μmole $MgCl_2$, 0.2 mL of 9000*g* supernatant fluid (containing approximately 8 mg protein/mL), and 100 μmole B(a)P in 50 μL of methanol (added just prior to incubation). The mixture was shaken at 37°C for 5 min in air. The reaction was stopped by addition of 1 mL of cold acetone; then 3.5 mL *n*-hexane was added and the preparation mixed and centrifuged at 3000 rpm for 2 min. The organic phase (1.0 mL) was extracted with 4.0 mL 1 *N* NaOH. After calibration of the fluorometer with standard solutions of 3-OH B(a)P, fluorescence of B(a)P metabolites in the NaOH layer was determined at an excitation wavelength of 395 nm and an emission wavelength of 522 nm using a Hitachi spectrophotofluorometer (Model 650-10S, Hitachi Ltd., Tokyo, Japan). AHH activity was expressed as the formation of hydroxylated metabolite with a fluorescence equivalent to pmole of 3-OH B(a)P per minute per milligram protein. The activity was compared to a blank to which acetone had been added prior to incubation. Duplicate determinations normally vary less than 10%. All the procedures were carried out in the dark. Protein concentration was determined according to Lowry et al. (*12*), bovine serum albumin being used as standard.

Statistical differences among AHH values were determined by the Student's *t*-test.

Cell Culture Studies

Cell Culture. Human lymphoblastoid cells obtained from apparently healthy volunteers who have never had habits of smoking and drinking were cultured in RPMI-1640 meduim containing 20% heat = inactivated fetal bovine serum (FBS); penicillin, 100 IU/mL; and streptomycin, 100 μg/mL. The cells were seeded at a density of approximately 3×10^5 cells/mL and the cultures were grown in an atmosphere of fully humidified air with 5% CO_2 at 37°C. Twenty-four hours later, enzymatic induction was performed by adding either 3-MC or the organochlorine compounds. 3MC (2.5 μM), PCDF isomers (7.5 ng/mL), TCDD (7.5 ng/mL) or HCB (95 ng/mL) in 5 μL of acetone was added to one flask (induced culture, 10 mL), and to the other flask 5 μL of acetone (control culture, 10 mL) was added. Incubation was continued for an additional 48-hr period, then the cells were harvested by centrifugation, and an aliquot was counted for determining the cell viability by the standard trypan blue dye exclusion procedure.

Enzyme Assay. The cells from each culture flask with over 90% in cell viability were harvested and assayed for AHH activity by the fluorometric procedure described by Gurtoo et al. (*13*). AHH activity was determined as follows. To reaction mixture (total volume, 1.0 mL) consisting of Tris (0.05 *M*): $MgCl_2$ (3 mM): sucrose (0.2 *M*) buffer mixture (pH 8.5), NADPH (1.7 mM), NADH (1.3 mM) and $2–4 \times 10^6$ viable cells was added B(a)P (0.1 mM) in 50 μL of acetone to start the reaction. The incubation was carried out at 37°C for 50 min in air. The reaction was stopped by addition of 1.0 mL ice-cold acetone, then the mixture in each tube was extracted with 0.5 mL of *n*-hexane. The organic phase (3.0 mL) was extracted with 0.5 mL of 1N NaOH. Fluorescence of the NaOH layer was determined with the same method as described in the animal studies. AHH activity was expressed as pmole equivalents of 3-OH B(a)P formed per minute per 10^6 viable cells. The results reported in this paper were obtained after subtracting the values for minus-cell blanks, which were treated under conditions identical with the control incubation but lacked cells. Each assay was performed twice, and the duplicate results normally varied less than 10%.

Results

Induction of AHH Activity by 3-MC in Mouse Livers

Hepatic AHH inducibility of 3-MC in two inbred mouse strains, DBA and C57, was examined at a dose of 300 mg/kg. These results are indicated in Table 1. DBA mice failed to respond to 3-MC and the enzyme activities were not enhanced in both males and females. However, C57 mice responded to the inducer, and hepatic AHH activities were significantly increased in both sexes;

i.e., induced AHH activities were about 10 times higher than control activities. Based on the results, DBA was regarded as the AHH-nonresponsive strain and C57 as an AHH-responsive strain, which was consistent data reported elsewhere (*14,15*).

Effects of PCDF Isomers, HCB and TCDD on Hepatic AHH Activity in DBA and C57 Mice

The hepatic AHH-inducing potency of eight PCDF isomers, HCB and TCDD was studied in DBA and C57 mouse strains to investigate genetic variations in response to the compounds. As shown in Table 2, in DBA mice, the AHH-nonresponsive strain, all PCDF isomers (30 μg/kg) and HCB (120 μg/kg) considered to be one of the most potent AHH inducers among PCB isomers, failed to enhance the enzyme activity; on the other hand, in C57 mice, the AHH-responsive strain, 2,3,6,7-tetrachlorodibenzofuran (2,3,6,7-TCDF), 2,3,7,8-TCDF, 1,2,3,7,8-PCDF, 2,3,4,6,7-pentachlorodibenzofuran (2,3,4,6,7-PCDF), 2,3,4,7,8-PCDF, and 1,2,3,4,7,8-HCDF significantly enhanced the activity, but HCB did not at the same doses. TCDD induced AHH activity at a dose of 30 μg/kg. The enzyme inducibilities (induced/control) were 15.7 and 15.4 in DBA and C57 mouse strains, respectively. 2,3,7,8-TCDF, 1,2,3,7,8-PCDF and 2,3,4,7,8-PCDF showed the highest AHH-inducing activity among PCDF isomers tested in C57 mice, and their enzyme activities were about 45% of that of TCDD. The order of the PCDF isomers for the enzyme inducing potency was as follows: 2,3,7,8-TCDF, 1,2,3,7,8-PCDF, 2,3,4,7,8-PCDF > 2,3,4,6,7-PCDF > 1,2,3,4,7,8-HCDF > 2,3,6,7-TCDF > 1,2,3,6,7-PCDF, 1,2,3,4,6,7-HCDF.

Table 1. Effects of pretreatment of mice with 3-MC on hepatic AHH activity.

Strain	Sex	*N*	3-OHB(a)p formed, pmole/min-mg protein[a]	
			Control	3-MC (300 mg/kg)
DBA	M	5	85 ± 7	77 ± 6
DBA	F	5	135 ± 18	138 ± 11
C57	M	5	282 ± 20	3069 ± 19
C57	F	5	401 ± 21	3792 ± 134

[a]Each value represents the mean ± standard error.
[b]Significantly different from the control group, $p < 0.01$.
[c]Significantly different from the male control group, $p < 0.05$.

Ranges of AHH Activity in Human Female Population

Table 3 shows the basal and 3-MC induced AHH activities in 23 individual lymphoblastoid cell lines obtained from women who never had habits of smoking and drinking and who ranged in age from 20 to 35 years with a mean value of 25.9 years. A very wide range in basal AHH activity (0.004–0.083 pmole/min/10^6 cells, mean value of 0.022 pmole/min/10^6 cells) was observed. The highest basal enzyme acitivity was about 20 times that lowest activity. Very wide ranges in induced AHH activity and AHH inducibility were also observed; i.e., treatment with 3-MC increased the enzyme activity from 2.3- to 16.4-fold (mean value, 5.7-fold), resulting in AHH activities of 0.021 to 0.543 pmole/min/10^6 cells (mean value, 0.135 pmole/min/10^6 cells).

Table 2. Effects of pretreatment of DBA and C57 mice with PCDF isomers, HCB and TCDD on hepatic AHH activity.

Compound	*N*	Dose, μg/kg body weight	AHH activity[a] (3-OHB(a)P formed, pmole/min-mg protein	
			DBA	C57
Control	4	–	79 ± 3[c]	282 ± 14
2,3,6,7-TCDF	4	30	72 ± 3[c]	448 ± 35[b,c,d,e]
2,3,7,8-TCDF	4	30	78 ± 4[c]	2067 ± 322[b,a]c
1,2,3,6,7-PCDF	4	30	74 ± 6[c]	241 ± 19
1,2,3,7,8-PCDF	4	30	77 ± 6[c]	1967 ± 131[b,c,e]
2,3,4,6,7-PCDF	4	30	85 ± 10[c]	1139 ± 72[b,c]
2,3,4,7,8-PCDF	4	30	63 ± 7[c]	1930 ± 116[b,c,e]
1,2,3,4,6,7-HCDF	4	30	84 ± 4[c]	274 ± 21
1,2,3,4,7,8-HCDF	4	30	82 ± 6[c]	679 ± 57[b,c,d,e]
HCB	4	120	83 ± 6[c]	259 ± 23
TCDD	4	30	1241 ± 62	4349 ± 123[b]

[a]Each value represents the mean ± standard error.
[b]Significantly different from control, 1,2,3,6,7-PCDF, 1,2,3,4,6,7-HCDF and HCB group, $p < 0.05$.
[c]Significantly different from TCDD group, $p < 0.01$.
[d]Significantly different from 2,3,7,8-TCDF, 1,2,3,7,8-PCDF, and 2,3,4,7,8-PCDF group, $p < 0.05$.
[e]Significantly different from 2,3,4,6,7-PCDF group, $p < 0.02$.

Table 3. Aryl hydrocarbon hydroxylase (AHH) activity and inducibility in human female lymphoblastoid cells.

Age,[b] yr	N[a]	Specific AHH activity, pmole/min × 10^{-6} cells		Induction ratio[b]
		Basal[b]	Induced[b]	
25.9 ± 0.9	23	0.022 ± 0.004	0.135 ± 0.033	5.7 ± 0.7
(20–35)[c]		(0.004–0.083)[c]	(0.021–0.543)[c]	(2.3–16.4)[c]

[a]N = number of lymphoblastoid cell lines used.
[b]Each value represents the mean ± standard error.
[c]Figures in parentheses indicate ranges of age, AHH activity and induction ratio.

Table 4. AHH inducibility of PCDF isomers, HCB and TCDD in human male lymphoblastoid cells.

Compound	Dose, ng/mL	Specific AHH activity as 3-OHB(a)P formed, pmole/min × 10^{-6} cells							
		19 yr, IR = 1.0[a,b]		4 yr, IR = 4.0[a,b]		12 yr, IR = 4.1[a,b]		40 yr, IR = 12.1[a,b]	
Control	—	0.006	(1.0)	0.005	(1.0)	0.017	(1.0)	0.024	(1.0)
2,3,6,7-TCDF	7.5	0.007	(1.2)	0.013	(2.6)	0.075	(4.4)	0.190	(7.9)
2,3,7,8-TCDF	7.5	0.007	(1.2)	0.014	(2.8)	0.072	(4.2)	0.165	(6.9)
1,2,3,6,7-PCDF	7.5	0.006	(1.0)	0.010	(2.0)	0.061	(3.6)	0.206	(8.6)
1,2,3,7,8-PCDF	7.5	0.011	(1.8)	0.017	(3.4)	0.118	(6.9)	0.272	(11.3)
2,3,4,6,7-PCDF	7.5	0.008	(1.3)	0.011	(2.2)	0.061	(3.6)	0.185	(7.7)
2,3,4,7,8-PCDF	7.5	0.012	(2.0)	0.021	(4.2)	0.138	(8.1)	0.335	(14.0)
1,2,3,4,6,7-HCDF	7.5	0.009	(1.5)	0.026	(5.2)	0.128	(7.5)	0.439	(18.3)
1,2,3,4,7,8-HCDF	7.5	0.016	(2.7)	0.028	(5.6)	0.184	(10.8)	0.336	(14.0)
HCB	95.0	0.005	(0.8)	0.006	(1.2)	0.016	(0.9)	0.040	(1.7)
TCDD	7.5	0.018	(3.0)	0.030	(6.0)	0.145	(8.5)	0.471	(19.6)

[a]IR = induction ratio (3-MC/C); measure against which compound IR is relative.
[b]Figures in parentheses indicate induction ratios (induced/control).

AHH Inducibility of PCDF Isomers, HCB and TCDD in Human Lymphoblastoid Cells

The AHH-inducing potency of eight PCDF isomers, HCB and TCDD was investigated in four male and four female human lymphoblastoid cell lines with various AHH inducibilities (1.0–15.9) determined by the enzyme induction with 3-MC. These results are shown in Table 4 and 5. Four male lymphoblastoid cell lines as well as female cell lines differently responded to 3-MC and had different AHH inducibilities (1.0–12.1). In both male and female cell lines, the degree of AHH inducibilities of these compounds were roughly paralleled to those of 3-MC. AHH inducibilities with 2,3,4,7,8-PCDF, 1,2,3,4,6,7-HCDF and 1,2,3,4,7,8-HCDF were much higher than that of 2,3,7,8-TCDF and were comparable to that of TCDD. HCB, one of the most potent AHH inducers and the most toxic isomers in PCB isomers, failed to induce AHH activity at a dose of 95 ng/mL, which was approximately 13 times the concentration of the other compounds.

Discussion and Conclusions

When certain inbred mouse strains are administered 3-MC, they respond with the induction of hepatic AHH activity (AHH responsive mouse strains); certain other inbred mouse strains when challenged with 3-MC fail to respond and their hepatic AHH activities are not induced (AHH-nonresponsive mouse strains) (*14,15*). The prototypical strain responsive to 3-MC is C57BL/6J and the prototypical nonresponsive strain is DBA/2J. As shown in Table 1, both C57 and DBA which have been kept in Japan maintain their original genetic AHH responsiveness. In a comparison of 3-MC and TCDD for their capacity to induce hepatic AHH activity in the rat, TCDD is 30,000 times as potent as 3-MC (*16*). Probably due to the extraordinary enzyme-inducing potency of TCDD relative to 3-MC, TCDD induced the hepatic enzyme activity in both mouse strains tested regardless of their response to 3-MC (Table 2), which was consistent with the results reported by Poland et al. (*17*). It is also shown in Table 2 that PCDF isomers tested failed to induce the hepatic enzyme activity in DBA mice, while in C57 mice, 2,3,7,8-TCDF, 1,2,3,7,8-PCDF and 2,3,4,7,8-PCDF significantly induced the enzyme activity. Induced AHH activities with these PCDF isomers were approximately 7 times that of the control activity. These results may indicate that AHH responsiveness segregates the toxic potency of these PCDF isomers; toxicity of the isomers in C57 mice may be much greater than that in DBA mice.

It is speculated that in human populations, AHH responsiveness may vary from person to person and may also segregate the toxicity of the organochlorine

Table 5. AHH inducibility of PCDF isomers, HCB and TCDD in human female lymphoblastoid cells.

Compound	Dose, ng/mL	Specific AHH activity as 3-OHB(a)P formed, pmole/min × 10^{-6} cells							
		26 yr, IR = 1.5[a,b]		46 yr, IR = 3.7[a,b]		24 yr, IR = 8.2[a,b]		46 yr, IR = 15.9[a,b]	
Control	—	0.011	(1.0)	0.007	(1.0)	0.088	(1.0)	0.024	(1.0)
2,3,6,7-TCDF	7.5	0.016	(1.5)	0.031	(4.4)	0.601	(6.8)	0.364	(15.2)
2,3,7,8-TCDF	7.5	0.017	(1.5)	0.024	(3.4)	0.523	(5.9)	0.428	(17.8)
1,2,3,6,7-PCDF	7.5	0.016	(1.5)	0.026	(3.7)	0.605	(6.9)	0.451	(18.8)
1,2,3,7,8-PCDF	7.5	0.020	(1.8)	0.052	(7.4)	0.684	(7.8)	0.660	(27.5)
2,3,4,6,7-PCDF	7.5	0.012	(1.1)	0.025	(3.6)	0.605	(6.9)	0.495	(20.6)
2,3,4,7,8-PCDF	7.5	0.028	(2.5)	0.052	(7.4)	1.020	(11.6)	0.855	(35.6)
1,2,3,4,6,7-HCDF	7.5	0.022	(2.0)	0.067	(9.6)	1.043	(11.9)	0.772	(32.2)
1,2,3,4,7,8-HCDF	7.5	0.025	(2.3)	0.074	(10.6)	0.964	(11.0)	0.667	(27.8)
HCB	95.0	0.009	(0.8)	0.006	(0.9)	0.122	(1.4)	0.039	(1.6)
TCDD	7.5	0.026	(2.4)	0.059	(8.4)	1.011	(11.5)	0.794	(33.1)

[a]IR = induction ratio (3-MC/C); measure against which compound IR is relative.
[b]Figures in parentheses indicate induction ratios (induced/control).

compounds. Hence, in this study, basal and 3-MC induced AHH activity in human lymphoblastoid cells were investigated, and the degree of AHH inducibility was determined to evaluate both toxic potencies and genetic susceptibilities of the compounds in human population. The data shown in Table 3 suggest that genetic variations among human population with respect to AHH responsiveness may be also present, similar to those reported among different inbred mouse strains. A person with higher AHH inducibility may be more sensitive to the toxic chemicals than a person with lower AHH inducibility.

In rat hepatoma cells (*10*), AHH-inducing potency of 2,3,7,8-TCDF was about 100 times less than that of TCDD, while in human lymphoblastoid cells, as shown in Table 4 and 5, AHH inducibility of 2,3,7,8-TCDF was about half that for TCDD. In human lymphoblastoid cells, 2,3,4,7,8-PCDF, 1,2,3,4,6,7-HCDF and 1,2,3,4,7,8-HCDF were more potent AHH inducers than 2,3,7,8-TCDF and were as potent an AHH inducer as TCDD (Table 4 and 5), whereas in rats and mice, 2,3,7,8-TCDF elicited the highest AHH induction and the greatest toxicity among PCDF isomers tested, including 2,3,4,7,8-PCDF (*18,19*). These observations suggest that the species difference may change the order of PCDF isomers for AHH-inducing activity and probably also for their toxic potency; i.e., toxicities of 2,3,4,7,8-PCDF, 1,2,3,4,6,7-HCDF and 1,2,3,4,7,8-HCDF in human tissues are possibly greater than that of 2,3,7,8-TCDF and may be comparable to that of TCDD.

PCDF isomers having no vicinal hydrogens in the dibenzofuran ring have been reported to be very persistent and accumulative in tissues of both non-domesticated animals and humans (*8,20*). Particularly, 2,3,4,7,8-PCDF and 1,2,3,4,7,8-HCDF are retained and accumulated and seem to be more resistant to the metabolic conversion, probably because arene oxide does not readily form *in vivo*. Furthermore, these two PCDF isomers have the highest AHH inducibility in human cells (Tables 4 and 5) and presumably a great toxicity in human bodies. Therefore, 2,3,4,7,8-PCDF and 1,2,3,4,7,8-HCDF should be given greater attention with regard to the possible etiology of Yusho and Yu-Cheng and the formation and/or accumulation in the environment.

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