COMPCRISON OF OVERALL METABOLISM OF 2,3,7,8-TCDD IN CYP1A2 (-/-) KNOCKOUT AND C57BL/6N PARENTAL STRAINS OF MICE

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Introduction

The most toxic dioxin congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is sequestered to a high degree in the mammalian liver to yield liver-to-fat ratios, in excess of unity.[1-6] TCDD induces hepatic cytochrome P450-1A2 (CYP1A2) to which it subsequently binds,[2-4] resulting in whole body half lives of 5-11 years in humans and ~30 days in rats. In addition, TCDD is metabolized very slowly in all systems tested.[7-9] Systematic *in vitro* metabolism rate studies have not been conducted with TCDD and the individual cytochromes; therefore, it has not been firmly established whether TCDD is a poor substrate for metabolizing enzymes, or whether TCDD is unavailable for metabolism due to its strong affinity to CYP1A2. The purpose of the present study was to quantify the extent of overall metabolism of TCDD in mice, which possess or lack the CYP1A2 gene.

Materials and Methods

Chemical

[^{14}C]TCDD was purchased from ChemSyn (Lenexa, KS; 63.5 µCi/µmole, 0.2 µCi/µg) and was used without dilution with unlabeled TCDD. Radiochemical purity of the TCDD (>98 %) was determined by silica gel TLC (1:1 hexane:methylene chloride) and HPLC (C18, DeltaPak, 8x40mm, H_2O:MeOH gradient, 5 % to 100 % MeOH over 60 min). The TCDD was administered as a single oral dose in 0.1 ml of peanut oil (1.2 µCi/mouse, 156 µg/kg body weight).

Animals

Nine male C57BL/6N mice were purchased from Taconic Labs (Germantown, NY). Eleven CYP1A2 (-/-) knockout mice (KO) were obtained from US EPA (RTP, NC)[3, 4]. The mice were housed for 96h in Plexiglas® metabolism cages with separate collection of urine and feces every 24h. Four days after dosing, the mice were killed by cervical dislocation, and tissues (kidney, liver, lung, epididymal fat, heart, GI tract, thymus, testes, spleen and carcass) were collected, immediately weighed and frozen at -28EC.

Analysis of tissues and excreta

A longisimus dorsi muscle and overlying skin sample were removed prior to carcass homogenization, and analyzed for ^{14}C by combustion analysis (Packard Model 307 sample oxidizer). The remaining carcass was diluted with hamburger (73 % lean), and homogenized three times with a hand grinder. Three aliquots (0.5 g) were removed and analyzed for radioactivity by combustion analysis. Radioactivity in feces and the remaining tissues were quantified by combustion of dried

samples (0.5 g). ^{14}C in the urine was quantified by liquid scintillation counting (Ecolite, ICN, Costa Mesa, CA). Urine was incubated with ?-glucuronidase (recombinant *E. coli*; Sigma, St. Louis, MO), and partitioned with toluene. The resulting aqueous layer was treated with aryl sulfatase (*Patella vulgata*, Sigma) and partitioned with ethyl acetate.

Chromatography

Air dried feces were pulverized via mortar and pestle, and extracted successively with hexane, ethyl acetate, and methanol. Extracts were chromatographed by silica gel TLC (solvent system: 1:1 hexane:methylene chloride) and the bands were quantitated with a System 2000 Imaging Scanner (Bioscan, Inc., Washington, D. C.).

Results and Discussion

Tissue distribution results are presented in Table 1 and are in agreement with previous studies[4]. Liver:fat ratio of the two groups was vastly different, i.e. 4.09 (C57BL/6N) vs. 0.57 (KO). Again, these data are in agreement with earlier results[3-4] and further establish the hepatic CYP1A2 enzyme as the TCDD sequestering species. In KO mice, the lack of the CYP1A2 sequestering species allowed for a short residence time in the liver and subsequent repartitioning into lipophilic tissues.

Slightly higher levels of ^{14}C-derived TCDD were excreted in the urine and feces of the parental strain at each time point when compared to KO mice (Table 1). All of the urinary ^{14}C from both study groups existed as metabolites as determined by silica gel TLC (data not shown). Glucuronide ether and sulfate ester conjugates were indicated in the urine. Solvent partitioning, following hydrolysis of 72-96h KO mouse urine with ?-glucuronidase, yielded hydrolyzed metabolites (>75%). More than 82 % of the hydrolyzed aqueous layer could be further hydrolyzed with aryl sulfatase (data not shown)—indicative of Phase II biotransformation. Glucuronide ether and sulfate ester conjugates have been observed in other dioxin metabolism studies.[10-12]

TCDD and its metabolites were quantitated in the fecal extracts of both study groups. Standard [^{14}C]-TCDD had an Rf equal to 0.70-0.79, and metabolites had Rf's <0.55. Cumulative extractable metabolites amounted to 3.2 % of the dose in C57BL/6N feces and 2.3 % of the dose in KO feces (Table 2). Metabolites were characterized as being hydroxylated, because they reacted quantitatively with diazomethane to yield methylated derivatives which had significantly higher Rf's on TLC (data not shown). Cumulative non-extractable fecal ^{14}C amounted to 4.6 % and 1.9% of the dose for C57BL/6N and KO mice, respectively (Table 2), and was an indication that further metabolism had occurred, so that covalent bonds had formed with fecal lipids and/or proteins.

The overall level of metabolism of TCDD was determined for both study groups as the sum of ^{14}C in 0-96h urine, non-extractable feces and metabolites in extractable feces. A higher level of overall metabolism was observed for the parental strain (C57BL/6N) of mice than the CYP1A2 knockout mice, i.e. 11.14 % vs 5.92 % of the dose, respectively (Table 2). The difference is probably due to rapid redistribution of TCDD into lipophilic tissues for storage, which made the TCDD unavailable to hepatic metabolizing enzymes.

In conclusion, the data presented in this study contradicts the hypothesis that hepatic sequestration of TCDD by CYP1A2 makes TCDD unavailable for metabolism that would readily occur in its absence. Indeed, the data demonstrated that slightly more overall metabolism occurred in a control mouse strain when compared to CYP1A2 (-/-) KO mice following a single oral dose, presumably due to low hepatic retention and high fat storage in the KO mice. The data confirmed that TCDD has an inherently slow metabolism in mammals, perhaps via the inducible CYP1A1[13] and CYP1B1[14] isozymes and/or non-P450 dependant mechanisms.[15]

Table 1. ^{14}C recovery (as percent of administered dose) and tissue concentrations of [^{14}C] TCDD in male C57BL/6N parental strain and CYP1A2 (-/-) knockout mice following an oral administration of 156 µg/kg body weight in 0.1 ml of peanut oil (mean ± std). Tissue concentrations assume all tissue ^{14}C is that of parent TCDD. (n.d. is not determined).

	C57BL/6N		CYP1A2 KO	
Excreta/Tissue	% of Dose	Conc (mmol/g)	% of Dose	Conc. (mmol/g)
Urine				
0-24h	1.67 ± 0.80	—	0.99 ± 0.60	—
24-48h	0.67 ± 0.28	—	0.54 ± 0.28	—
48-72h	0.45 ± 0.092	—	0.45 ± 0.09	—
72-96h	0.57 ± 0.23	—	0.44 ± 0.24	—
Feces				
0-24h	10.07 ± 4.49	—	8.82 ± 4.62	—
24-48h	2.26 ± 0.62	—	1.86 ± 0.47	—
48-72h	2.21 ± 0.38	—	1.01 ± 0.24	—
72-96h	2.36 ± 0.27	—	0.95 ± 0.10	—
Adipose (epid.)	10.98 ± 2.27	0.92	14.24 ± 3.49	1.48
Carcass	23.22 ± 2.48	0.19	55.40 ± 6.30	0.46
GI Tract	4.95 ± 0.62	n.d.	7.76 ± 1.58	0.92
Heart	0.028 ± 0.011	n.d.	0.18 ± 0.14	0.40
Kidney	0.60 ± 0.24	n.d.	1.19 ± 0.24	0.93
Liver	41.34 ± 3.00	3.76	4.22 ± 1.02	0.85
Lung	0.14 ± 0.11	n.d.	0.15 ± 0.063	0.42
Muscle	n.d.	0.10	n.d.	0.23
Skin	n.d.	0.47	n.d	0.71
Spleen	0.067 ± 0.027	n.d.	0.09 ± 0.032	0.54
Testes	0.26 ±0.18	n.d.	0.69 ± 0.39	0.77
Thymus	0.023 ± 0.016	0.16	0.22 ± 0.10	0.46
Total	**101.73 ± 3.33**	**99.58 ± 7.23**		

Table 2. The percent of a TCDD dose excreted as metabolites in male C57BL/6N parental strain and CYP1A2 (-/-) KO mice. Non-extractable fecal ^{14}C was assumed to represent metabolites of TCDD covalently bound to lipids and/or proteins in the feces.

	% of Dose	
	C57BL/6N (n=9)	CYP1A2 KO (n=11)
0-96h Urine	3.36	2.42
0-96h Feces (extractable)	3.16	2.24
0-96h Feces (non-extractable)	4.62	1.26
Total metabolism	**11.14**	**5.92**

Acknowledgements

The technical assistance of Barbara Magelky and Colleen Pfaff was greatly appreciated.

Disclaimer

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