Estimating the Total TEQ in Human Blood from Naturally-Occurring vs. Anthropogenic Dioxins: A Dietary Study

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Introduction

Numerous naturally-occurring compounds in the human diet can bind to the aryl hydrocarbon, or *dioxin* receptor (AhR) and activate the AhR signaling pathway[1]. These compounds include certain indole carbinols and their derivatives, heterocyclic aromatic amines, flavonoids, carotinoids, vitamin A derivatives (retinoids), and tryptophan metabolites. Several researchers have suggested that the daily dietary intake of these "endodioxins", in terms of a TCDD-equivalency (TEQ) is likely to be far greater than that associated with daily background intake of anthropogenic dioxins[2-5]. For example, Connor and Finley[3] estimated that the daily TEQ dose of indolo[3,2-*b*]carbazole (ICZ), an indole carbinol formed from compounds in cruciferous vegetables, is likely to be over 10,000-fold greater than the daily dietary TEQ dose associated with the background intake of PCDD/Fs and PCBs. Further, when TEQ doses were expressed as accumulated body burdens or area-under-the-curve doses, which accounted for different biological half-lives, ICZ still constituted >95% of the total (internal) TEQ dose.

There is little information available concerning comparative TEQ body burdens of dietary endodioxins vs. anthropogenic dioxins; however, some data has been provided by studies using cellular bioassays, and the results are consistent with the analysis of Connor and Finley[3]. Specifically, Schecter et al.[6] utilized the CALUX® bioassay to measure total TEQ in human blood samples from nine individuals and a pooled sample of 100 different patients. The CALUX® bioassay uses a luciferase reporter gene to detect AhR agonists in a manner that does not discriminate between anthropogenic vs. naturally-occurring dioxins, and thus is capable of measuring all Ah-active compounds present in a sample. Typically, CALUX® analysis is coupled with sample cleanup procedures that remove endodioxins, and this system will provide TEQ estimates for PCDD/Fs and PCBs in blood that are consistent with GC/MS derived estimates of TEQ[7]. However, Schecter et al.[6] processed the blood samples only using crude solvent extractions and the resulting CALUX® TEQs ranged from 97 to 823 ppt. The PCDD/F and PCB TEQs in the same samples, based on GC/MS analysis, ranged from 0.04-0.52 ppt and therefore comprised only

a very small fraction of the total TEQ. These findings suggest that the vast majority of the biological TEQ in human blood is due to endogenous ligands, dietary endodioxins, and/or other anthropogenic compounds with AhR activity (e.g., polychlorinated napthalenes, polybrominated biphenyls, polybrominated diphenyl ethers).

The purpose of this study was to provide preliminary data for evaluating whether dietary endodioxins may in fact be significant contributors to the non-PCDD/F and PCB fraction of the blood TEQ. This was accomplished by measuring the total bioassay (CALUX®) TEQ in the blood of several volunteers under various dietary regimens. Specifically, blood samples were collected from volunteers who maintained a baseline diet, which was relatively free of vegetables, followed by a diet enriched in endodioxin-containing vegetables. The background blood levels of PCDD/Fs and PCBs were measured for each volunteer at the beginning of the study in order to establish a baseline TEQ for each participant. To provide a measure of study sensitivity, CALUX® analysis was also performed on blood samples from volunteers who took an off-the-shelf indole-3-carbinole (I3C) supplement. I3C is the main dietary ICZ precursor and could be expected to increase the levels of this endodioxin in blood.

Methods

Ten volunteers were recruited without preference as to race, sex, or age and were enrolled in the study only after giving full informed consent. The study protocol was reviewed and approved by an external Institutional Review Board (IRB) (Essex, Lebanon, NJ). Volunteers were asked to maintain a specified (vegetable free or vegetable rich) diet over the course of two 4-day periods. Meals were to be consumed at approximately 8 a.m., 1 p.m., and 7 p.m.

The *baseline* diet comprised a diet that was absent, to the greatest extent possible, of endodioxin-containing fruits, vegetables, and herbs. A vegetable-free diet described by Lampe et al.[8] was adopted for this purpose, modified to include a moderate amount of low-fat meat and dairy products. The *endodioxin-rich* diet was open to the foods normally consumed by each participant, with some foods specifically recommended and others prohibited. This diet contained specified amounts of cruciferous vegetables (e.g., cabbage, broccoli, Brussels sprouts, and cauliflower), which contain known endodioxins or their precursors.

Two of the ten volunteers participating in the study continued the endodioxin diet for an additional two-day period, while also taking an I3C extract (Life Extensions™). The participants took three 200 mg tablets per day, in accordance with the recommendations on the container label.
Blood samples were collected by a registered nurse at multiple time points during the dietary intervention. One 100 mL sample was taken before commencing the baseline diet, which was used for GC/MS analysis of the PCDD/Fs and PCBs. During the four-day baseline diet, samples were collected in the afternoon (3-4 p.m.) of days 3 and 4 (hereafter referred to as samples B1 and B2). During the four-day endodioxin-rich diet, samples were collected in the afternoon on day 2, in the morning (10-11 a.m.) on day 3, and in the afternoon on day 4 (hereafter referred to as samples E1, E2, and E3). For the two individuals taking oral I3C supplements, additional blood samples were collected on the following two days (samples ES1 and ES2).

Bioassay analyses of the whole blood samples were carried out using the CALUX® reporter gene bioassay derived from the mouse hepatoma H1L6.1c3 cell line stably transfected with the dioxin-inducible pGudLuc6.1 reporter plasmid[9]. A 0.5 mL volume of each whole blood sample was extracted three times (at room temperature) with an equivalent volume of *tert*-butyl methyl ether, and organic layers decanted following vortexing and subsequent centrifugation (10 minutes at 2,800 *g*). The organic phases recovered from each were combined and the *tert*-butyl methyl ether was evaporated under a stream of nitrogen gas. Samples were resuspended in 200 μL hexane, and multiple dilutions were evaporated into DMSO for use in the bioassays.

Cells were dosed with 2-3 dilutions of each extracted sample, in 96-well plates. After a 4-hour dosing period, the induction of luciferase activity was quantified using the luciferase assay kit from Promega. The 4-hour exposure time was found to yield the highest activity levels for the blood samples, as compared with longer (24 hour) incubation periods, where metabolism of endodioxins may have been a factor. A TCDD standard curve was constructed for every 96-well culture plate, using at least 10 dose levels of TCDD (1, 1.9, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, and 1,000 ppt).

PCDD, PCDF, and PCB concentrations in the blood samples were quantified using high-resolution GC/MS. This work was performed by AXYS Analytical, Ltd. (Sidney, B.C.)[10], as described in U.S.EPA Methods 8290[11] and 1668A[12].

Results and Discussion

Analytical TEQs for the PCDD/Fs and PCBs were calculated using CALUX®-based TEFs[13, 14] as well as the consensus WHO TEFs[15] (Table 1). Use of the CALUX® TEFs is arguably more appropriate for purposes of comparing the analytical TEQ to the bioassay TEQ. These TEQs were calculated with analytical data expressed on a whole blood basis, since the bioassay TEQs are also derived on a whole blood basis. The mean analytical TEQ values calculated in this manner for the group of ten participants ranged from 0.022–0.119 ppt (Table 1). As can be seen in Table 1, the analytical TEQs calculated with the WHO TEFs are very similar to the TEQs calculated using the CALUX® TEFs (the WHO-based values tend to be about 20% higher on average).

Table 1. Whole blood TEQ concentrations of PCDD/F and PCB compounds by class, calculated using WHO TEFs and CALUX®-based TEFs.

Compounds	Mean Analytical TEQs (S.D.); whole blood, ppt [Range]; n=10	
	With WHO TEFs	With CALUX® TEFs
PCDD/Fs	0.040 (0.030) [0.017-0.121]	0.041 (0.027) [0.020-0.112]
PCBs[a]	0.013 (0.009) [0.004-0.031]	0.0035 (0.002) [0.001-0.008]
Sum PCBs/PCDD/Fs[b]	0.053 (0.037) [0.026-0.149]	0.045 (0.029) [0.022-0.119]

[a] Analytical TEQs include PCBs 77, 81, 105, 114, 118, 123, 126, 156/157, 167, 169, and 189.
[b] Mean of summed PCDD/F and PCB TEQs from 10 individuals.

The TEQs resulting from the CALUX® bioassay of 54 blood samples ranged from 13.4 to 218 ppt (whole blood basis) (Table 2). In participants eating a vegetable-deprived (baseline) diet, the bioassay TEQs ranged from 13–66 ppt; in participants eating an endodioxin-enriched diet, the bioassay TEQs were 18–133 ppt; and in participants eating an endodioxin-enriched diet and taking I3C supplements (oral, 3 × 200 mg daily), the bioassay TEQs were 62–218 ppt.

The results of this study confirm the findings of Schecter et al.[6] and are consistent with the analysis by Connor and Finley[3]. For each participant, every blood sample contained a total TEQ (the CALUX® TEQ) that was 2 – 4 orders of magnitude greater than the corresponding analytical TEQ (based on PCDD/Fs and PCBs). This was true regardless of dietary regimen, time of sampling (morning or afternoon), gender, age, or regional location. The PCDD/Fs and PCBs did not comprise more than 0.5% of the total (bioassay) TEQ in any sample. Even in blood samples drawn during the vegetable-deprived diet, which would be expected to contain the lowest endodioxin content, the total (CALUX) TEQs (13–66 ppt, whole blood) were hundreds to thousands of times higher than the analytical TEQs (0.02–0.15 ppt, whole blood basis). Thus, without the deliberate consumption of endodioxin-containing food, there was a considerable 'background' blood TEQ, which does not appear to be related to the blood levels of anthropogenic dioxins. Furthermore, the two participants taking the I3C supplement while maintaining the endodioxin diet were found to have the highest overall CALUX® TEQ (62, 95, 99, and 218 ppt). Hence, these data suggest that dietary endodioxins do influence and contribute to the non-PCDD/PCDF/PCB fraction of the bioassay TEQ.

Table 2. CALUX® TEQs (whole blood basis) measured throughout the period of dietary intervention.

Group	Mean TEQ (S.D.) [range] (all in ppt, whole blood basis) *Sample code (time of day)*		
	B1 (p.m.)	*B2 (p.m.)*	
Baseline Diet	28.7 (15.1) [13.4–66.3] (n=10)	34.6 (13.4) [20.0–64.5] (n=10)	
	E1 (p.m.)	*E2 (a.m.)*	*E3 (p.m.)*
Endodioxin Diet	42.9 (22.9)* [21.9–89.9] (n=10)	40.5 (24.6)* [20.0–104] (n=10)	79.3 (38.5)* [18.0–133] (n=10)
	ES1 (p.m.)	*ES2 (p.m.)*	
Endodioxin Diet + I3C Supplement	97.3 (2.6)[a] [95, 99] (n=2)	140 (111)[a] [62, 218] (n=2)	

Notes:
* Statistically different ($p<0.05$) than TEQs measured on days B1 and B2 (averaged), as determined using Student's T and Wilcoxon rank-sum paired difference tests.
[a]Sample sizes (n=2) in these groups is not sufficient for a paired differences test. TEQ values on these days were consistently higher than those measured on previous days.

The results of this study clearly indicate that PCDD/Fs and PCBs comprise a very small fraction of the total TEQ in human blood. Presumably, the only substances that could be responsible for the vast majority of the blood TEQ are dietary endodioxins, endogenous ligands, and/or other anthropogenic compounds (besides PCDD/Fs and PCBs). Other anthropogenic compounds are probably not significant contributors; it is commonly believed that PCDD/Fs and PCBs comprise most of the anthropogenic TEQ in the diet. Although the current results do not permit an analysis of the relative contribution of dietary endodioxins vs. endogenous ligands, the fact that a diet high in vegetables caused significant increases in the blood TEQ suggests that dietary endodioxins make a significant contribution to the total blood TEQ. The results of this study would therefore appear to conflict with assertions that low level PCDD/F and PCB exposure poses a substantial health risk. Additional research would permit a more informed understanding of the anthropogenic vs. endodioxin contributions to TEQ.

Acknowledgments

The authors are grateful to Michael Denison, Stephen Safe, Chester Clarke, Mark Roberts, Lesa Aylward, and Robert Budinsky for thoughtful comments on the study protocol. Financial support for this study was provided by the American Chemistry Council (ACC).

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