Dioxin '97, Indianapolis, Indiana, USA

CYP1B1 and CYP1A1 exhibit different dose-response relationships in the TCDD-treated female Sprague-Dawley rat liver.

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Introduction

In chronic rodent bioassays, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) is a potent carcinogen in multiple species, at multiple sites and in both sexes[1]. The ubiquitous presence of TCDD and dioxin-like compounds in the food chain and the environment, and their long half life in humans, results in a chronic and persistent exposure to human populations. As a consequence, there is concern that exposure to TCDD poses a threat to human health.

Most, if not all, of the effects of dioxin are believed to be mediated via a ligand activated nuclear receptor known as the aromatic (aryl) hydrocarbon receptor (AhR). Binding of ligand (TCDD and dioxin-like compounds) and subsequent activation of the AhR results in transcriptional activation and/or altered expression of dioxin responsive genes[2]. It is believed that the development of adverse effects associated with exposure to TCDD are fundamentally due to the changes in the expression of these dioxin-responsive genes[3]. This mode of action of TCDD is conserved between humans and rodents and previous studies indicate that humans are at least as responsive as rodents to TCDD exposure[4,5]. Consideration of the conservation of mode of action, and review of the tumorigenicity of TCDD in rodents and in human populations exposed to TCDD[6], have recently led to the classification of this compound as a known human carcinogen[7].

Risk assessment for human exposure to TCDD may be aided by the use of mathematical models of dose-dependent changes in TCDD regulated endpoints. The CYP1 family of cytochromes P450 are the most well characterized TCDD-responsive genes, and current dose response models for TCDD have been constructed for the expression of CYP1A1 and CYP1A2 in the Sprague-Dawley rat liver[8]. CYP1B1 is a recently identified TCDD inducible cytochrome P450 that is the first member of a new 1B subfamily of the CYP1 cytochromes P450[9,10]. CYP1B1 in humans is responsible for the metabolism of 17β-estradiol to form the potentially genotoxic catechol estrogen; 4-hydroxyestradiol[11]. The formation of the catechol estrogen, 4-hydroxyestradiol is believed to be involved in the development of estrogen-induced kidney tumors in hamsters[12] and in the development of leiomyomas in humans[13]. In rats, TCDD induction of the development of altered hepatic foci and changes in cell proliferation are dependent upon ovarian hormones, presumably estrogen [14]. One hypothesis for this hormone dependence is the metabolic activation of estrogen to catechol estrogens. Therefore CYP1B1 represent a dioxin inducible gene that is plausibly linked to tumor formation. The quantitative analysis of CYP1B1 expression may therefore aid in our understanding of the mechanism of carcinogenesis of TCDD and in the risk assessment for chronic human exposure to TCDD and dioxin-like compounds. The aim of the current study was to quantitate the dose dependent changes in expression of CYP1B1 in the Sprague-Dawley rat liver following chronic exposure to TCDD.

Experimental Methods

Animals and treatment
For analysis of cytochrome P450 expression we used archived liver samples from a previously published chronic study of the dose-dependent effect of TCDD on tumor promotion in rats[15]. In this study 10 week old female Sprague-Dawley rats were initiated with 175mg diethylnitrosamine/kg and from 12 weeks of age onwards they were treated bi-weekly with 50, 150, 500, and 1750ng TCDD (in corn oil) for 30 weeks to approximate daily doses of 3.57, 10.7, 35.7 and 125 ng TCDD/kg body weight/day. Control animals received vehicle alone. One week after the last treatment, animals were killed by CO_2 asphyxiation. Representative sections of liver were removed, minced and aliquots frozen in liquid nitrogen.

RT-PCR analysis
Quantitative RT-PCR was carried out using a competitive titration assay using a heterologous recombinant internal standard RNA[16]. Each internal standard amplicon consists of target RNA-specific forward and reverse primers (Table 1) separated by a heterologous spacer molecule derived by amplification using primers specific to the human GSTM1 gene. Liver total RNA (100ng) was reverse transcribed in the presence of increasing copies of each recombinant internal standard RNA followed by amplification by PCR. A digitized image of the stained PCR products was quantitated using the NIH Image analysis package. The log of the ratio of the density of the internal standard amplicon and target amplicon was plotted against the log of the number of molecules of internal standard in each reaction and a fitted by linear regression. The number of molecules of target amplicon was determined by interpolation at log ratio=0 (i.e. where the ratio of target RNA and internal standard molecules is equal.).

Table 1. Target RNA RT-PCR primers and amplicon lengths.

Target RNA	Forward primer (5'-3)	Reverse Primer (5'-3)	Length
CYP1B1	accgcaacttcagcaacttc	gtgttggcagtggtggcatg	427 bp
CYP1A1	ccatgaccaggaactatggg	tctggtgagcatccaggaca	341 bp
β-Actin	cagcctctatgccaacacagt	tagagccaccaatccacacag	247 bp

Results and Discussion

Our previous studies of CYP1B1 expression in rodent liver by RNA hybridization failed to observe any expression of CYP1B1 in control animals. For this reason we developed a quantitative RT-PCR assay for the analysis of CYP1B1 expression to increase sensitivity of detection and to allow for the measurement of absolute values of CYP1B1 RNA in controls. In this study we used this assay for the analysis of CYP1B1 and CYP1A1 in a Sprague-Dawley rats following chronic exposure to TCDD.

Expression of CYP1B1 was very low in control animals and levels of CYP1A1 RNA were approximately 1500X greater than corresponding levels of CYP1B1 in these controls (Table 2). Following exposure to TCDD at various doses for 30 weeks there was a dose dependent increase in the levels of both CYP1B1 and CYP1A1 RNA levels in the livers of female rats (Figure 1A). Increases in CYP1B1 and CYP1A1 RNA levels were maximal at the highest dose of 125 ng TCDD/kg/day. At the highest dose CYP1B1 and CYP1A1 were induced approximately 4885 and 175 fold, respectively (Table 2). By comparison, the range of RNA levels for the housekeeping gene, β-actin, were within 2.6 fold across all exposure groups (Figure 1A).

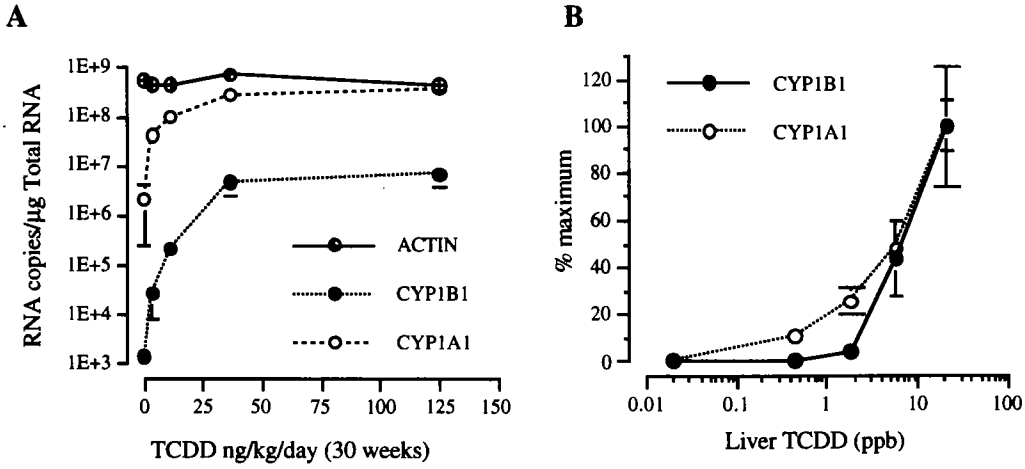
Table 2. Dose dependent changes in the expression of CYP1B1 and CYP1A1.

CYP1B1 and CYP1A1 RNA levels were measured by quantitative RT-PCR in the livers of Sprague-Dawley rats treated for 30 weeks with various doses of TCDD. RNA levels are expressed as the average fold induction relative to control for three animals at each dose. TCDD levels were measured by GC/MS and expressed relative to liver wet weight. CYP1A1/CYP1B1 represents the ratio of the average CYP1A1 and CYP1B1 RNA copies/ug total RNA in each dose group.

Oral Dose (ng/kg/day)	Liver TCDD (ppb)	CYP1B1 (Fold)	CYP1A1 (Fold)	CYP1A1/ CYP1B1
0	0.02 (n=1)	1	1	1498
3.5	0.45 ± 0.04	18	19	1901
10.7	1.80 ± 0.35	159	44	411
35.7	5.68 ± 0.55	3213	131	69
125	21.17 ± 0.60	4885	175	58

Figure 1. Dose dependent increase in CYP1B1 and CYP1A1 expression.

(A) RNA levels for CYP1B1, CYP1A1 and β-actin were measured by quantitative RT-PCR and plotted relative to administered dose of TCDD. (B) RNA levels were then normalized against the actin mRNA level for each animal and plotted as percent of the maximum average value relative to liver TCDD burden (wet weight). Error bars represent the standard deviation for three animals.



The most appropriate dose-measurement for dose response relationships following TCDD exposure is the target organ burden of TCDD. We have previously shown that in this study there was a linear increase in liver TCDD burden with increasing oral dose of TCDD[15,17]. The average liver burdens of TCDD at each oral dose are shown in Table 2. These tissue burdens reflect an equilibrium between the liver and adipose tissue as a result of the chronic dosing regimen used.

When analyzed relative to liver TCDD burden it was observed that the liver burden required for half maximal induction of P450 expression (ED50) for CYP1A1 and CYP1B1 was similar (5 ppb) and this is consistent with previously published studies on the expression of CYP1A1[17]. However, the doses required for 10% maximal and 1% maximal induction (ED10 and ED01 respectively) of CYP1B1 were significantly higher than that of CYP1A1 indicating that in the lower region of the dose response curve, CYP1B1 would appear to be a less sensitive response than CYP1A1 (Figure 1B). CYP1B1 and CYP1A1 were both induced approximately 20 fold relative to control levels at the lowest dose used of 3.5ng/kg (0.45ppb) (Table 2).

In a chronic 2 -year rodent bioassay of TCDD in Sprague-Dawley rats, animals with TCDD liver burdens of 5 ppb and 24 ppb had significantly higher incidences of hepatocellular adenomas compared with controls[18,19]. The data in this paper show that at similar liver burdens of TCDD, CYP1B1 and CYP1A1 are at least half maximally induced. The NOAEL for TCDD in this bioassay was 1 ng/kg/day, corresponding to a liver burden of 0.54 ppb TCDD. This body burden is similar to the dose required for 10% and 0.3 % maximal induction of CYP1A1 and respectively. The role of CYP1B1 in metabolism of estrogen to genotoxic intermediates and the dose-response for CYP1B1 expression suggest that CYP1B1 may be involved in the mechanism of carcinogenesis of TCDD.

Summary

We used a quantitative RT-PCR assay to determine the dose response relationships for CYP1B1, a recently identified TCDD-inducible cytochrome P450. CYP1B1 was expressed at levels 1500x lower than CYP1A1 in control animals and was more highly inducible than CYP1A1 (relative to control levels) at equivalent doses. Expression of CYP1B1 and CYP1A1 showed a dose-dependent increase in the liver of female Sprague Dawley rats treated for 30 weeks with TCDD. CYP1B1 and CYP1A1 exhibited different dose-response relationships, with CYP1B1 showing lower expression than CYP1A1 (relative to the maximal level) at liver burdens below the respective ED50s (both 5 ppb TCDD liver burden).

Acknwoledgements

The authors would like to thank Dr. Scott Masten for critical review of this manuscript.

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