REGULATION OF EXPRESSION OF THE ARYL HYDROCARBON RECEPTOR BY TCDD IN A DIOXIN-RESISTANCE MODEL

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Introduction

The dioxin congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has gained notoriety among the aromatic hydrocarbons since its potency as an environmental toxicant and its affinity for the aryl hydrocarbon receptor (AHR) surpass that of all other known chemicals. At unusually low doses, TCDD produces an extensive list of adverse effects including immune suppression, hepatotoxicity, severe anorexia-like wasting, carcinogenesis and death. Notwithstanding extensive studies, the cause of TCDD-induced death in animals remains unclear as do the primary target tissue(s) or organ(s) that are integral to acute lethality.

The AH receptor mediates TCDD toxicity as supported by several lines of evidence ranging from classical receptor theory and Mendelian genetics to contemporary gene knockouts[1-4]. The ability of TCDD to alter expression of a battery of genes including CYP1A1 is well established[5]. However, far less is known about what regulates expression of the AHR itself. Differences in regulation of expression of AHR levels may also have a profound impact on toxicological outcome. There exists a precedent for the chemical modulation of AHR level*s in vivo* by both receptor agonists and non-agonists. Phenobarbital[6], PCBs[7], *trans*-aminostilbene[8], staurosporine[9] and TCDD[10,11] have all been reported to influence AHR levels. Reports of the impact of TCDD on AHR levels *in vivo* are contradictory. Sloop and Lucier detected elevations in cytosolic AHR by radioligand binding following TCDD administration in rats[10]. Conversely, Pollenz *et al.* reported a consistent depression of AHR in whole-tissue lysates as measured by immunoblotting[11].

We are exploiting the greater than 1000-fold difference in sensitivity to TCDD lethality that exists between the "dioxin-sensitive" Long Evans (*Turku AB*) rat (LD_{50} ca. 10 μg/kg) and the "TCDD-resistant" Han/Wistar (*Kuopio*) rat (LD_{50}> 9600 μg/kg). These strains display similar TCDD binding affinities and many quantitatively identical biochemical and toxicological responses (including CYP1A1 induction) yet differ dramatically in LD_{50}[1,12]. Little is known about the mechanism underlying the differential sensitivity to TCDD in the rat at the molecular level. Recent molecular cloning of the AHRs in the strains revealed a mutation at an intron/exon junction generating splice variants of the AHR in H/W rats. This results in deletion of a 38 or 43 amino acid segment within the *trans*-activation domain and remains of unknown consequence[13].

The goals of our current study were twofold: i) to determine the effects of TCDD on AHR expression *in vivo* by using multiple and independent approaches to measure AHR levels following TCDD exposure and, ii) to investigate a potential mechanism for differential TCDD susceptibility in the rat model, namely, a differential pattern or degree of regulation of expression of the AHR by TCDD in the sensitive L-E versus the resistant H/W rats.

Experimental Approach

Female L-E, SD and H/W rats (10-12 weeks) were administered a single intragastric dose of 5 or 50 μg/kg TCDD and were allowed unlimited access to food and water until euthanasia by decapitation at 1, 4 or 10 days post TCDD (n=5/treatment group). Cytosol and nuclear extract were prepared from fresh tissue by differential centrifugation. Total RNA was isolated from frozen tissue by the phenol-guanidine isothiocyanate approach. RNA purity and integrity were monitored. AHR levels were measured by 3 independent approaches: radioligand binding (cytosols), immunoblotting (cytosols and nuclear extracts) and RT-PCR (for AHR mRNA levels).

Radioligand binding: Cytosols were incubated with 10 nM [^{3}H]TCDD for 1 hr at 4°C in the presence or absence of a 100-fold molar excess of tetrachlorodibenzofuran (TCDF) as the competitor and analyzed by velocity sedimentation on sucrose density gradients.

Semi-quantitative immunoblotting: Proteins from cytosols and nuclear extracts were separated by SDS-PAGE and immunoblotted for AHR and ARNT using polyclonal antibodies. Anti-AHR (a.a. 1-402 of the mouse Ah^{b-1} allele) was obtained from Biomol Research Laboratories. The anti-ARNT antibody (a.a. 399-777 of human ARNT) was generously provided by Dr. O. Hankinson. Fluorescent emissions from an alkaline phosphatase-catalyzed reaction were captured by a phosphorimager and signal intensities for the specific bands were normalized to a standard loaded onto each gel. Assays were performed in duplicate.

Semi-quantitative RT-PCR: Relative steady-state mRNA levels for the AHR, ARNT and CYP1A1 were measured by RT-PCR using ß-actin as the internal reference standard. mRNA was reverse transcribed to cDNA using M MLV viral reverse transcriptase primed by an oligo(dT) primer. All genes were amplified from the same pool of cDNA. Target and standard genes were PCR-amplified in separate tubes with simultaneous incorporation of [α-^{32}P]dCTP for radiodetection. PCR products were separated on a polyacrylamide gels and radioactive emissions captured and quantitated using the Storm Phosphorimager system. Steady state mRNA for each target mRNA was calculated as the signal ratio of target to internal standard. Assays were performed in triplicate from the same RNA isolate. Kinetic determinations were performed on all genes to validate the quantitative nature of the assay prior to analyses. The effect of TCDD was evaluated within each strain by comparing treatment groups to time-matched controls.

Results and Discussion

A summary of results for all endpoints at Day 1 and 10 is presented in the tables below. Values represent the ratio of treated/time-matched controls and arrows indicate the direction of change. Dashes represent no statistically significant change (P>0.05). The sensitivities of each rat strain relative to the TCDD dose is expressed as the ratio Dose/LD_{50}. Results for Day 4 are not shown

Low dose TCDD did not produce statistically significant changes in AHR levels after a single day of exposure. By Day 10, low dose TCDD produced a 1.5 to 3-fold increase in cytosolic AHR

levels in all rat strains as measured by each of the three approaches. Increases in AHR mRNA occurred earlier in SD rats and were statistically significant by Day 4 following treatment but returned to near control levels by Day 10. High dose TCDD (which exceeds the LD_{50} for L-E rats) consistently produced an initial drop in AHR protein and binding at Day 1 with no observable effect on AHR message levels. This depletion was followed by recovery to control levels (as depicted by no change vs. time-matched controls) in SD and H/W but not L-E rats. Recovery was accompanied by elevated AHR mRNA levels.

DAY 1

	5 µg/kg				50 µg/kg			
Strain	Dose / LD_{50}	binding	protein	mRNA	Dose / LD_{50}	binding	protein	mRNA
L-E	0.5	-	-	-	5	0.87 ↓	0.74 ↓	-
SD	0.1	-	-	-	1	0.67 ↓	0.38 ↓	-
H/W	0.0005	0.41 ↓	-	-	0.005	0.90 ↓	0.89 ↓	-

DAY 10

	5 µg/kg				50 µg/kg			
	Dose / LD_{50}	binding	protein	mRNA	Dose / LD_{50}	binding	protein	mRNA
L-E	0.5	1.95 ↑	3.03 ↑	2.60 ↑	5	0.76 ↓	0.79 ↓	-
SD	0.1	1.30 ↑	2.11 ↑	-	1	0.23 ↓	-	9.57 ↑
H/W	0.0005	1.57 ↑	3.22 ↑	2.28 ↑	0.005	-	-	2.95 ↑

Nuclear receptor levels, although detectable, approached experimental limits of detection at all time points and were not quantitated. ARNT protein and ARNT mRNA levels showed little response to TCDD. The only notable change was a gradual and modest decrease in ARNT protein in SD and L-E at the high dose. TCDD had no effect on steady-state ARNT mRNA levels. CYP1A1 mRNA levels were measured as a biomarker for the effectiveness of treatment. CYP1A1 induction was comparable in all rat strains; a dramatic elevation in CYP1A1 mRNA was produced by Day 1 and was maintained over the entire time course (data not shown).

The fact that the independent endpoints we used to measure AHR levels are in agreement indicates that regulation of the AHR by TCDD is genuine. Patterns produced by ligand binding and immunoblotting were similar although quantitatively greater when measured by immunoblotting. It remains unclear if this is a result of different ligand-binding ability of a newly-synthesized AHR-like protein or whether this is simply inherent to the different experimental approaches. TCDD-induced AHR regulation was influenced primarily by dose but also by duration of exposure and rat strain. Overt toxicity was observed in L-E rats at the 50 μg/kg which may explain different AHR regulatory patterns versus SD and H/W rats at this dose. Since patterns and degree of changes in TCDD-influenced AHR regulation in all strains were similar, it is not likely that differences in AHR expression following TCDD treatment account for the large strain differences to TCDD lethality in this rat model.

The mechanism of TCDD-induced AHR regulation remains unclear although concomitant elevations in AHR steady-state mRNA levels suggest a transcriptional mechanism, not to the exclusion of other possibilities. The mechanism of induction of CYP1A1 via dioxin-response elements (DREs) is well-understood[5] and serves as a benchmark for other regulatory phenomena. The observed changes in AHR mRNA levels were delayed and gradual in comparison with the dramatic, almost immediate induction of CYP1A1 by TCDD. This hints at different modes of regulation for these two genes.

Supported by the Medical Research Council of Canada (ABO); the Natural Sciences and Engineering Research Councilof Canada (MAF); the Academy of Finland, Research Council for Health (grant #43984) & the Finnish Research Program for Environmental Health [SYTTY] (grant # 42386) (RP). We thank Ms. Arja Tamminen and Ms. Minna Voutilainen for technical assistance

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