Dioxin Alters Calcium Homeostasis and the Regulation of Arachidonate Metabolism in Mouse Hepatoma Cells

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1. Introduction

Non-genotoxic environmental pollutants such as dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD) cause their toxic effects by mechanisms that are yet to be fully elucidated. TCDD is a powerful teratogen during embryonic development, causing hydronephrosis, cleft palate, and other craniofacial abnormalities in exposed rodents [1-4]. In humans, TCDD and other chlorinated phenols have been shown to cause chloracne, a long-lasting skin disease characterized by the hyperkeratinization of follicular sebocytes [5-7]. TCDD also causes thymic involution and apoptosis of immature thymocytes [8,9]. In two-stage carcinogenesis tests, TCDD is among the most potent tumor promoters ever tested in experimental animals, causing an elevated incidence of hepatic carcinoma and pulmonary and skin tumors in rodents [10-14]. In humans, there is insufficient evidence to determine whether TCDD, and dioxins in general, are carcinogenic, although recent long-term epidemiological studies have established a link between high levels of TCDD exposure and certain types of cancers [15,16].

The molecular basis of the biological effects of TCDD is largely unknown. TCDD binds to a cytosolic receptor, the aromatic hydrocarbon (Ah) receptor, and is responsible for the induction of the cytochrome P450 *CYP1A1* gene and of several other genes that form the Ah gene battery [17]. Based on genetic evidence in congenic mouse strains, it is generally accepted that the Ah receptor, and possibly, cytochrome P450 metabolites, play an important causative role in TCDD toxicity. Recent work from our laboratory [18] and others [19,20] has shown that TCDD causes a rapid increase in Ca^{2+} influx, followed by induction of the immediate-early FOS and JUN proto-oncogenes and a concomitant increase in transcription factor AP-1. Work in other laboratories has demonstrated that TCDD activates the tissue-specific expression of many other genes, including TGF-α, TGF-β, plasminogen activator inhibitor-2, IL-1β, and others [21-23]. This work has provided support for the hypothesis that TCDD causes its biological effects by altering the transcriptional controls that regulate tissue-specific gene expression. We have begun to investigate this hypothesis by analyzing the effects of TCDD on cellular processes critical in signal transduction for the mobilization of second messengers.

2. Methods

Intracellular Ca^{2+} was determined by fluorescence cellular imaging, using the fluorescence

ratioing dye, Fura-2, as described in detail previously [24]. Cells were evaluated for intracellular Ca^{2+} before and after addition of 5 nM TCDD. The wavelengths of excitation were 340 nm and 380 nm, coupled to an emission wavelength of 510 nm. The image at 340 nm was divided by that at 380 nm, and the resultant ratioed fluorescence image was calibrated against a standard curve of EGTA-buffered Ca^{2+}. The results are expressed as the mean ratio of fluorescence determined at the two excitation wavelengths (± SD) for 7-11 randomly chosen cells. The estimated range of the abscissa was from 100 nM to 600 nM Ca^{2+}. This calibration method generates reliable relative values for intracellular Ca^{2+} concentrations, but does not provide absolute quantitative determinations [25].

Arachidonic acid metabolites were labelled by preincubation of cells with [^{3}H]-arachidonic acid at 1μCi/ml in complete medium for 24 h. Cells were washed, treated with 10 nM TCDD for 4 h in phosphate-buffered saline, and metabolites were extracted from the buffer and resolved by reverse phase high performance liquid chromatography (HPLC) as described [26], adding a known amount of prostaglandin (PG) B_2 prior to extraction as an internal standard.

To determine relative amounts of mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) we used Tth reverse transcriptase/DNApolymerase from Perkin Elmer Cetus, following the manufacturer's specifications, except that 2 μCi of[^{32}P]dCTP were included for PCR. . Cells were mock-treated or treated with 10 nM TCDD for 6 h. Cox-1, Cox-2 and CYP1A1 mRNAs were coamplified with β-actin mRNA, used to normalize the results obtained for the other mRNAs. Each reverse transcriptase reaction, containing 250 ng of Hepa-1 total RNA was run for 30 min at 70°C, using 5 μM of a random oligonucleotide octamer as primer. PCR was for 30 cycles of 30 sec each at 54°C for annealing, 72°C for synthesis, and 94°C for denaturation. Amplified DNA was resolved in agarose gels and quantitated by densitometry.

Results

It has been proposed that TCDD toxicity could result from the effects of arachidonic acid metabolites catalyzed by TCDD-induced cytochrome P450 enzymes [20,27,28]. This idea is supported by the observation that several cytochromes P450, including the TCDD-inducible CYP1A1 and CYP1A2 enzymes [29-31] and others [32,33], possess arachidonic acid epoxygenase activity. We argued that epoxygenase activity might generate arachidonate metabolites that in turn would elevate the prooxidant status of the cell, and, as a result, cause the activation of *fos* and *jun*. Other prooxidants have been shown to activate *fos* and *jun* by this pathway [34-36].

Stimulation of eicosanoid metabolism might result from an increase in intracellular calcium levels, since membrane phospholipid arachidonic acid is mobilized by activation of phospholipase A_2, a calcium- and calmodulin-sensitive enzyme [37,38]. In order to examine the potential involvement of calcium and the Ah receptor in mediating alterations in eicosanoid metabolism, Hepa-1 wild-type (*wt*) cells [39] and Ah receptor-deficient (*c2*) [40] cells were examined for alterations in intracellular calcium levels following TCDD treatment. The results shown in Fig. 1 indicate that TCDD produces a rapid increase in intracellular calcium mobilization in both cell lines, with the greater effect in the cell line lacking the Ah receptor. Calcium levels returned to baseline values within 10 min, but such a transient elevation in calcium levels would be sufficient to activate phospholipase A_2 and mobilize arachidonic acid from membrane depots.

To evaluate TCDD-mediated alterations in arachidonic acid metabolism, and a possible

role of the Ah receptor, we determined the distribution of several arachidonate metabolites in *wt* and *c2* cells, and in a CYP1A1-defective cell line (*c37*) [41]. The results of these experiments are shown in Table 1. In wild type Hepa-1 cells, TCDD caused a small, but noticeable increase in PGs $F_{2\alpha}$ and D_2, a large increase in 12-HHT, and no effect on PGE_2, 12-HETE, 15-HETE or 5-HETE, suggesting that dioxin may affect the activity of PG synthases, but not that of lipoxygenases. These effects on the cyclooxygenase pathway appear likely to be due to activation of CYP1A1, since they are not observed in CYP1A1-deficient cells (*c*37) nor in the receptorless cell line (*c*2). The most surprising finding, however, is the large effect that the Ah receptor and CYP1A1 metabolism appear to have on the accumulation of PGs. All three measured PGs were elevated by a factor of 6-fold in the absence of TCDD, and even higher for $PGF_{2\alpha}$ and PGE_2 in cells treated with TCDD. PG levels in CYP1A1-deficient cells were also elevated, although their levels were intermediate between those found in wild type and in *c*2 cells, suggesting that both the Ah receptor and CYP1A1 activity were involved in the effects observed.

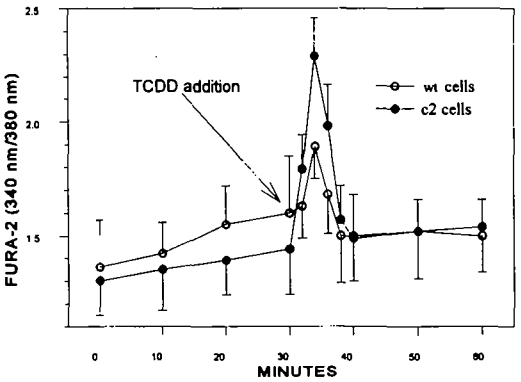

Fig. 1. Intracellular Calcium mobilization after TCDD treatment.

Table I. The Effect of TCCD on the Distribution of Arachidonate Metabolites in Hepa-1 Cells and in Mutant Cell Line Derivatives (Values are percentages of total ^3H cpm recovered).

	Hepa-1		*c*2		*c*37	
Compound	-TCDD	+TCDD	-TCDD	+TCDD	-TCDD	+TCDD
$PGF_{2\alpha}$	3.0	4.3	20.3	40.5	15.2	7.8
PGE_2	1.0	1.1	7.3	12.2	6.0	4.2
PGD_2	0.3	0.9	1.8	2.8	2.2	1.5
12-HHT	4.0	9.7	16.5	6.0	5.2	3.6
15-HETE	1.1	1.3	1.2	1.4	0.3	0.8
12-HETE	0.8	1.0	0.8	0.8	0.8	0.5
5-HETE	0.4	0.5	0.4	0.4	0.3	0.4

To determine whether the effect of dioxin and the Ah receptor on PG distribution involves gene induction processes, we have begun to examine the mRNA levels of genes coding for key enzymes in PG biosynthesis. We have started by measuring mRNA accumulation for PGG/H endoperoxide synthase, the rate-limiting enzyme for the production of PGs from arachidonic acid. The enzyme is bifunctional, converting arachidonic acid into PGG_2, and PGG_2 into PGH_2. Recently, two isoforms of this enzyme have been described in mouse cells; Cox-1 (PGG/HS-1), constitutively expressed and showing little change with diferent treatments, and Cox-2 (PGG/HS-2), inducible by phorbol esters and interleukin-1,

and behaving as an immediate-early gene [42-45]. We prepared total RNA from Hepa-1 cells treated with 10 nM TCDD for 6 h and measured Cox-1, Cox-2, and Cyp1a1 mRNA levels by RT-PCR. In all reactions, we coamplified β-actin mRNA, to use as an internal control to normalize the measurements.

Table2. mRNA Induction by TCDD	
mRNA	Fold-induction
Cox-1	1.3 ± 0.2
Cox-2	5.8 ± 2.1
Cyp1a1	12.2 ± 3.0

The data shown in Table 2 represents an average of three separate experiments to quantitate mRNA. We find that Cox-1mRNA accumulation is not affected by TCDD, whereas Cox-2 and Cyp1a1mRNAs are elevated by TCDD treatment, suggesting that the effects of TCDD on prostaglandin distribution result from induction of Cox-2.

3. Conclusions

Cyclooxygenases are reported to have low turnover numbers and very short enzymatic half-lives (< 10 min). *De novo* synthesis of the enzyme is assumed to be the main form of control of prostanoid production [45], although the precise mechanisms of Cox regulation have yet been to be described. Rapid and transient induction of Cox-2 has been observed in cells treated with cytokines and tumor promoters, and it is believed to have an important role in signal transduction pathways [42-45]. We show here that TCDD mobilizes intracellular calcium and causes induction of Cox-2 mRNA expression, increasing certain prostaglandins and augmenting the prooxidant status of the cell. These data suggest that increased calcium levels may lead to the release of PGs, eicosanoids, and other mediators, which may have an important role in the biological effects of TCDD.

4. Acknowledgments

This work was supported by NIH Grants R01 ES006273 (A. P.), R01 ES006677 , R01 006562 and Center for Indoor Air Research CAR-94-08 (G.D.L.), and NIH P30 ES006096 (A.P., G.D.L., and H.G.S.).

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