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Sustained Increase in Intracellular Free Calcium and Activation of Cyclooxygenase-2 Expression in Mouse Hepatoma Cells Treated with Dioxin

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ABSTRACT. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a non-genotoxic environmental pollutant that causes multiple adverse effects in experimental animals and in humans. We show here that TCDD treatment of mouse hepatoma cells causes a rapid mobilization of intracellular calcium both in wild type Hepa-1 cells and in its *c2* variant, a cell line that has highly reduced levels of functional aromatic hydrocarbon (Ah) receptor (AHR). In wild type cells, but not in the *c2* variant, TCDD treatment leads to a sustained elevation of cytosolic free calcium. TCDD also induces elevated levels of cyclooxygenase-2 (COX-2) mRNA in wild type and in *c37*, a CYP1A1-deficient cell line, but not in *c2* cells. Induction of *Cox-2* is in fact dependent on the presence of a functional Ah receptor, since it can be blocked by antisense oligonucleotides to Ah receptor mRNA. Most likely as a consequence of Cox-2 induction, we find a significant increase in the level of 12-hydroxyheptadecatrienoic acid (12-HHT) secreted from TCDD-treated Hepa-1 cells. In addition, we observe elevated levels of 6-keto prostaglandin $F_{1\alpha}$ in *c2* cells and high levels of secreted prostaglandin $F_{2\alpha}$ in *c2*, *c37* and *c4*, the variant cell line lacking aromatic hydrocarbon nuclear translocator protein. These data suggest that Cox-2 activation by TCDD leads to the release of prostaglandins, eicosanoids and other mediators which may have an important role in the biological and toxic effects of TCDD. BIOCHEM PHARMACOL **54**;12:1287–1296, 1997. © 1997 Elsevier Science Inc.

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The dioxins comprise a group of non-genotoxic HAHs† found in the environment that cause their toxic effects by mechanisms that are largely unknown. One of the best characterized dioxin congeners is TCDD, a developmental teratogen that causes hydronephrosis, cleft palate, and other craniofacial abnormalities in exposed rodents [1–4]. In adult mice, TCDD also causes immunosuppression, thymic involution and apoptosis of immature thymocytes [5, 6]. In two-stage carcinogenesis tests, TCDD is one of the most potent tumor promoters ever tested, causing an elevated incidence of hepatic carcinoma, and pulmonary and skin tumors [7–11]. In humans, TCDD and other chlorinated phenols cause chloracne, a long-lasting skin disease characterized by the hyperkeratinization of follicular sebocytes [12–14]. Epidemiologic evidence indicates that TCDD is a human carcinogen; recent long-term studies have established a strong association between TCDD exposure and certain types of cancers [15–17]. In addition, dose-dependent TCDD exposure correlates with death from ischemic heart disease in humans [17].

The molecular mechanisms by which TCDD exerts its toxic effects have not been elucidated. It has been known for some time that TCDD binds to AHR, a cytosolic receptor responsible for the induction of the cytochrome P450 CYP1A1 gene and of several other genes that form the [Ah] gene battery [18]. Gene induction is dependent on the heterodimerization of AHR with ARNT and binding of the complex to response elements in the regulatory regions of [Ah] battery genes (reviewed in [19]). Based largely on genetic evidence in congenic mouse strains, it is generally accepted that the AHR, and possibly cytochrome P450 metabolites, play an important causative role in TCDD toxicity.

Recent work from our laboratory [20] and others [21–23] has shown that TCDD causes a rapid increase in Ca^{2+} influx rates from extracellular sources, followed by induction of the immediate-early *fos* and *jun* proto-oncogenes

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† *Abbreviations:* AHR, aromatic hydrocarbon receptor; AhRE, aromatic hydrocarbon receptor response element (also termed XRE and DRE); ARNT, the aromatic hydrocarbon receptor nuclear translocator; DMSO, dimethylsufoxide; Gly-PI, glycerophosphoinositol; HAH, halogenated aromatic hydrocarbons; 5-HETE, 5-Hydroxyeicosa-6E,8Z,11Z,14Z-tetraenoic acid; 12-HETE, 12-Hydroxyeicosa-5Z,8Z,10E,14Z-tetraenoic; 15-HETE, 15-hydroxyeicosa-5Z,8Z,11Z,13E-tetraenoic; 12-HHT, 12(*S*)-hydroxyheptadeca-5Z,8E,10E-trienoic acid; IP, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; IP_4, inositol tetrakisphosphate; α-MEM, modified Eagle's α-medium; PG, prostaglandin; PLA_2, phospholipase A_2; PLC, phospholipase C; RT-PCR, reverse transcriptase-polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin).

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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 [24–26] as well as protein kinases [27–31], the *Ras* protooncogene [32, 33] and cyclin-dependent kinases [34]. This work has provided support for the hypothesis that the toxic effects of TCDD result from the disruption of signal transduction processes critical for mobilization of second messengers and subsequent interference with the normal transcriptional controls that regulate tissue-specific gene expression. Specifically, it has been proposed that TCDD toxicity results from the effects of arachidonic acid metabolites catalyzed by TCDD-induced cytochrome P450 enzymes [22, 35, 36]. In agreement with this hypothesis, several arachidonate metabolites have been shown to activate *fos* and *jun*, possibly as a result of an elevation of the prooxidant status of the cell [37–39]. To investigate whether a similar mechanism of immediate-early gene activation is operative in TCDD-treated cells, we have analyzed arachidonate metabolism, calcium release and cyclooxygenase gene expression in mouse hepatoma cells treated with TCDD. We find that TCDD causes the rapid mobilization of intracellular calcium and induces elevated levels of COX-2 mRNA. Most likely as a consequence of *Cox-2* induction, TCDD also appears to affect the secreted levels of various arachidonate metabolites.

MATERIALS AND METHODS

Cell Lines and Growth Conditions

The wild-type mouse hepatoma cell line Hepa-1c1c7 [40] and its mutant derivatives deficient in CYP1A1 induction [41, 42] were routinely grown as monolayer cultures in α-MEM containing 5% fetal calf serum. The mutant cell lines used in these studies were *c2*, a derivative that has less than 5% of the wild-type levels of Ah receptor [41, 43–45]; *c4*, a derivative defective in the ARNT gene [41, 45, 46]; and *c37*, a Cyp1a1 metabolism-deficient line with two missense mutations in the *Cyp1a1* gene [41, 47]. Cell viability was determined by fluorescence imaging, using 1 μM propidium iodide, a membrane impermeant, DNA intercalating probe. Excitation and emission wavelengths were 495 nm and 600 nm, respectively.

Assay for Protein Thiols and Carbonyls, and Lipid Peroxidation

Fluorescence detection of thiol-bimane in isocratic HPLC assays were used to quantitate glutathione and cysteine in cell homogenates treated with 25 mM ethylmorpholine (pH 8.4) and 5 mM monobromobimane, as described previously, [48, 49]. Similarly, spectrophotometric assays using dinitro phenylhydrazine were used for quantitation of protein carbonyls [48]. Lipid peroxidation decomposition products were determined as thiobarbiturate-reactive compounds, as described [50]. Protein determinations were performed using the bicinchoninic acid method (Pierce Chemical Co.), following the procedures suggested by the manufacturer.

Cellular Imaging for Cytosolic Ca^{2+} Determination

Cells were grown on 25 × 0.15 mm glass microscope cover slips in Petri dishes. Cover slips were mounted in a chemotaxis chamber, washed with 1 ml MEM and placed on the stage of a Nikon Diaphot TMD inverted fluorescence microscope. Data were processed with Image-1 processing and analysis software (Universal Imaging Corp.).

Intracellular Ca^{2+} was determined by fluorescence cellular imaging, using FURA-2 acetoxy methyl ester [51] as described previously [48]. Briefly, cell were loaded for 10 min at 37° with 1 μM FURA-2; under these conditions, sufficient dye is taken up and desterified to report intracellular Ca^{2+}. Cells were evaluated before and after the addition of 5 nM TCDD in DMSO, using excitation wavelengths of 340 nm and 380 nm, coupled to an emission wavelength of 510 nm. Control experiments using and equivalent volume of solvent (final concentration 0.05 to 0.1% DMSO) showed no effect on calcium mobilization. Background images obtained in the absence of FURA-2 were subtracted from the images obtained in the presence of the probe. The resultant 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+}. This method of *in vitro* calibration generates reliable relative values for intracellular Ca^{2+} concentrations; however, due to differences between *in vitro* and *in vivo* conditions that may affect the absolute fluorescence, the method does not return quantitative determinations of intracellular concentrations [52]. Data were continuously collected for periods ranging between 60 min to 2 hr and stored in optical disks. Mean fluorescence ratios determined at the two excitation wavelengths were calculated from pixel intensities integrated over 1-min periods. The results presented are expressed as the mean ± SD for 7–11 randomly chosen cells and are shown at the mid-point of the integration interval. The estimated range of the ordinate was from 100 nM to 600 nM Ca^{2+}. See ref. 48 for a complete analysis of the ratioing methodology used in this work. All experiments were repeated 3 or 4 times with essentially the same results.

Analysis of Arachidonic Acid Metabolites

Cells were labelled by incubation with 1 μCi/ml [^{3}H]-arachidonic acid (sp. act. 223 Ci/mmol, Amersham Life Science) in complete α-MEM for 24 hr. Thereafter, cells were washed three times in PBS and after the last wash, PBS containing DMSO vehicle or 10 nM TCDD was placed in each dish. Individual dishes of cells were incubated at 37° for an additional 30 min, 1 hr, 2 hr, or 4 hr. The medium was removed and metabolites were extracted with 2 vol. of ethyl acetate (acidified with formic acid),

dried under nitrogen and dissolved in a 70:30 mixture of water:acetonitrile in 2.5 mM phosphoric acid (solvent A). The 30-min and 1-hr points showed insufficient [^{3}H]-dpm for analysis; cyclooxygenase and lipooxygenase products were measured as previously described [53] in the 2-hr and 4-hr points by reverse phase HPLC on a C_{18} column eluted with a gradient of 44:56 solvent A:solvent B (60:40 methanol:acetonitrile in 2.5 mM phosphoric acid) to 100% solvent B. [^{3}H]-metabolites were monitored with an in-line continuous flow β-counter and identified by comparison with elution characteristics and retention times of purified standards. This method separates each probable arachidonate metabolite with the exception of the cysteinyl leukotrienes (i.e., LTC_4, LTD_4 and LTE_4). To normalize for differential recovery between experiments, the values reported correspond to the integrated dpm for each identified compound relative to the total dpm eluted. The 2-hr and 4-hr points for each cell line showed essentially the same elution profiles. All experiments were repeated twice. The values shown are the means ± SEM of the 2-hr and 4-hr points.

Analysis of Inositol Phosphate Formation

Formation of inositol phosphates was measured as described by others [54]. Confluent cultures grown in 6 mm dishes were labeled to equilibrium with 2 μCi/ml *myo*-[^{3}H]-inositol (sp. act. 17 Ci/mmol; Amersham Corporation) for 20 hr in α-MEM containing 0.1% fetal calf serum. Labeling medium was removed and the cells were stimulated with DMSO vehicle or with 10 nM TCDD in α-MEM containing 0.1% fetal calf serum and 20 mM LiCl. At various time intervals, inositol phosphate formation was stopped by removal of the medium and the cells were washed with PBS. Inositols were extracted with 0.5 mL of 10 mM formic acid, pH 3 for 30 min, diluted with 2 mL of 5 mM NH_4OH (final pH 8–9) and applied to a 0.5 mL column of Bio-Rad anion exchange resin AG1-X8 (formate form, 200–400 mesh). Successive elution with 4 mL of, 1) water, 2) 40 mM, 3) 200 mM 4) 600 mM, and 5) 2 M ammonium formate/formic acid, pH 5, eluted free inositol, gly-PI, and IP, IP_2, and IP_3 + IP_4, respectively.

RNA Quantitation

Culture cells were mock-treated with DMSO vehicle or treated with various concentrations of TCDD for different lengths of time. For experiments with cells grown in the presence of antisense oligonucleotides to Ah receptor mRNA, cells were grown for 3 doublings (approximately 50 hr) in the presence of two 21-mer phosphorothioated oligonucleotides complementary to the −33 to −13 and −12 to +9, respectively, coordinates of the *Ahr* mRNA [44], with +1 being the translation initiation site. As control, cells were grown in the presence of a phosphorothioated oligonucleotide of the same base composition as the antisense ones but with a scrambled sequence. In preliminary experiments, cell viability and effects on Ah receptor-dependent transcription were determined as a function of oligonucleotide dose; we found that 5 nM was sufficient to block CYP1A1 expression and that oligonucleotide concentrations as high as 5 μM were tolerated by the cells without any apparent loss of viability. All experiments thereafter were done at the 5 nM concentration.

Total RNA was extracted by the acid guanidinium thiocyanate method [55]. Relative amounts of mRNA were determined by RT-PCR using RTth reverse transcriptase/DNA polymerase (Perkin Elmer/Cetus) following the manufacturer's specifications, except that 2 μCi of [^{32}P]dCTP (sp. act. 3000 Ci/mmol; Amersham Corp.) were included in each PCR to label the amplified products. Reverse transcriptase reactions contained 250 ng of the appropriate RNA and were run for 30 min at 70°, using 5 μM of a random octamer primer. COX-1, COX-2 and CYP1A1 cDNA's were coamplified with β-actin cDNA, used to normalize the results. The primers used for amplification were the following: mouse *Cyp1a1* (GenBank Accession Number K02588): forward primer, (1970)-GTGTCTGGT TACTTTGACAAGTGG-(1993); reverse primer, (2164)-AACATGGACATGCAAGGACA-(2149); amplification product, 198 bp. Mouse β-actin (GenBank Accession Number M12481): forward primer, (64)-GGTCAGAAG GACTCCTATGTGG-(85); reverse primer, (166)-TG TCGTCCCAGTTGGTAACA-(147); amplification product, 103 bp. Mouse *Cox-1* (GenBank Accession Number M34141): forward primer, (2537)-CAAAAGAACCCAGT GTCC-(2554); reverse primer, (2669)-ATGAGTCCATCT GTTCCC-(2652); amplification product, 133 bp. Mouse *Cox-2* (GenBank Accession Number M88242): forward primer, (2952)-CACAGTATGATGTAACAG TCC-(2972); reverse primer, (3175)-AACACAGCT ACGAAAACC-(3158); amplification product, 224 bp. The numbers in parenthesis refer to the coordinates in the published cDNA sequences.

PCR reactions were run for 26, 28, 30, 32 and 34 cycles to determine the plateau levels for each amplified product. Annealing was for 30 sec at 54°, followed by 30 sec synthesis at 72°, and 30 sec denaturation at 94°. Amplified DNA was resolved in 6% acrylamide gels and quantitated by exposure to X-ray film and densitometry of the autoradiographs.

Statistics

Statistical analyses were performed using SigmaStat Statistical Analysis software (Jandel Corporation). Differences between group mean values were determined by a one-way ANOVA, followed by Student–Newman–Keuls test for a pairwise comparison of means.

RESULTS

TCDD Alters Arachidonic Acid Metabolism

TCDD treatment of tissue culture cells may be expected to induce the expression of several cytochromes P450, includ-

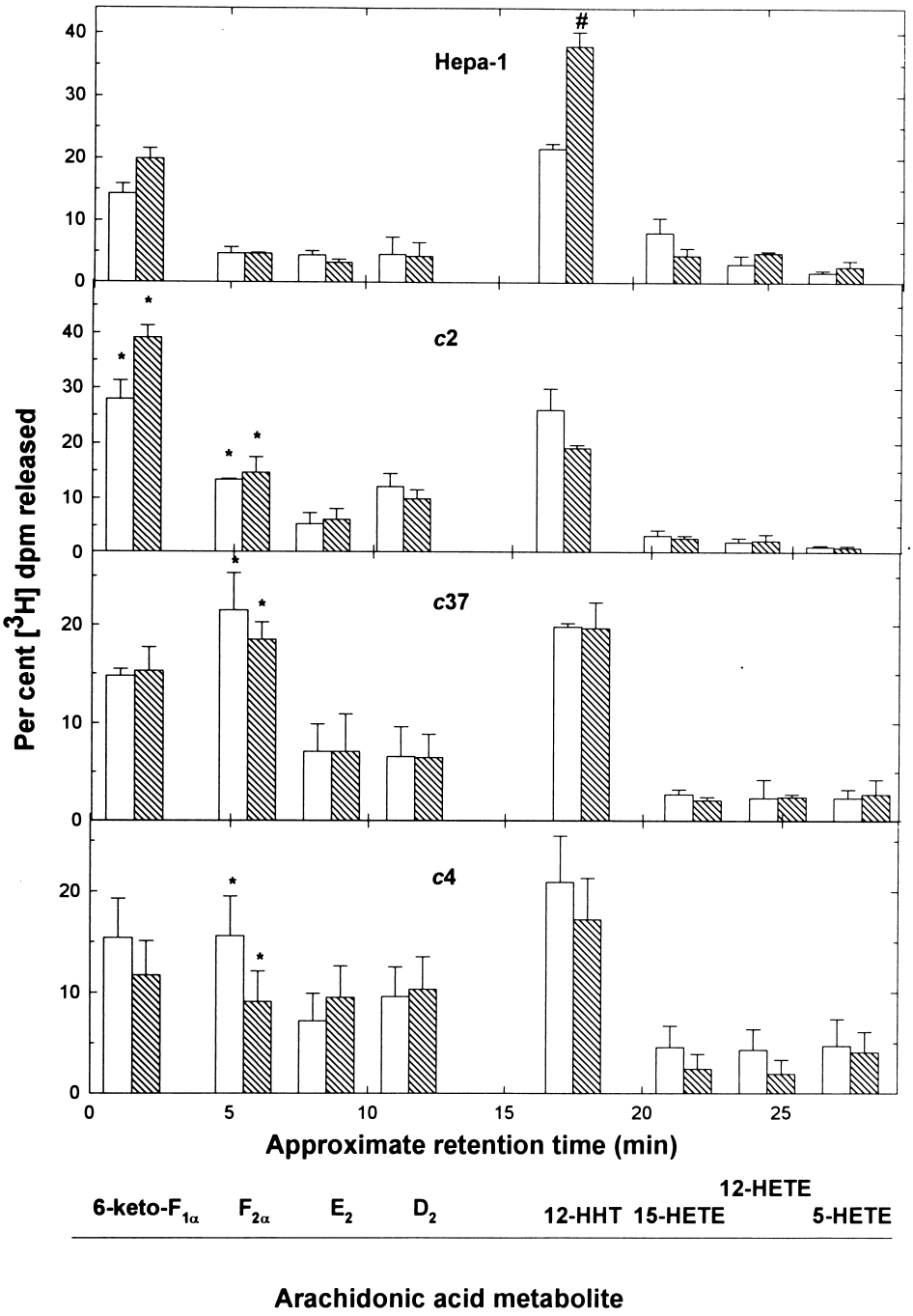

FIG. 1. HPLC analysis of arachidonic acid metabolites in Hepa-1 cells and its derivatives. Tritium dpm from the indicated [^{3}H]-labeled arachidonic acid metabolites were determined with an in-line continuous flow β-counter. Experimental conditions are described in the "Materials and Methods". The values shown are the mean ± SEM of four determinations in two independent experiments expressed as percent of the total radioactivity recovered in identifiable compounds. The abscissa indicates Approximate retention time since the four HPLC profiles for each cell line extract were not ansolutely identical. Significantly higher ($P < 0.05$) levels of PGs in *c2*, *c37*, and *c4* cells than in Hepa-1 cells are denoted by an asterisk (*). In Hepa-1 cells, but not in the others, TCDD treatment led to a significant increase ($P < 0.05$) in 12-HHT levels, denoted by the number symbol (#). *Open bars*: untreated; *hatched bars*: TCDD-treated.

ing the CYP1A1 and CYP1A2 enzymes [56–58] and others [59, 60], that possess arachidonic acid epoxygenase activity. Furthermore, TCDD may also directly affect the cyclooxygenase and lipooxygenase pathways of arachidonic acid metabolism. To evaluate TCDD-mediated alterations in arachidonic acid metabolism by the latter two pathways, and the possible role of CYP1A1 metabolism, the Ah receptor and ARNT, we analyzed the distribution of cyclooxygenase and lipooxygenase metabolites in the wild type Hepa-1 cell line and in its *c37*, *c2* and *c4* derivatives. Following TCDD or mock treatment, arachidonate metabolites were analyzed by HPLC. Results from these experiments are shown in Fig. 1. In wild type Hepa-1 cells, TCDD caused a significant increase in 12-HHT and no apparent effect on 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGD_2, PGE_2, 5-, 12-, or 15-HETE. An increase in 12-HHT could be expected if TCDD were to cause the elevation of prostaglandin endoperoxide H synthase (cyclooxygenase) activity. Hence, these data suggest that dioxin induces cyclooxygenase activity, but not the activity of 5-, 12-, or 15-lipoxygenases.

In CYP1A1-deficient cells (*c37*), ARNT-less cells (*c4*) and cells with reduced Ah receptor levels (*c2*) TCDD did not increase the levels of any of the PGs analyzed; however, the mutations in these cells affected the amounts of PG

released into the medium. Thus, all three variant cell lines had significantly higher levels of $PGF_{2\alpha}$ released, and *c2* showed, in addition, a much elevated level of 6-keto-$PGF_{1\alpha}$ (Fig. 1). These results suggest that the Ah receptor, ARNT and a functional CYP1A1 enzyme each participate in the metabolism of arachidonic acid.

TCDD Alters Intracellular Calcium Homeostasis

Stimulation of eicosanoid metabolism might result from a TCDD-dependent increase in intracellular calcium levels, since membrane phospholipid arachidonic acid is mobilized by activation of PLA_2, and several PLA_2s are calcium- and calmodulin-regulated enzymes [61–63]. In fact, we and others have shown that TCDD increases calcium influx rates into Hepa-1 cells [20–23] and TCDD has also been shown to increase intracellular calcium mobilization in chick embryo cardiac myocytes, decreasing β-adrenergic responses and impairing ventricular contractility [21, 22]. To examine the potential involvement of calcium and the Ah receptor in mediating alterations in eicosanoid metabolism, Hepa-1 wild-type cells and Ah receptor-deficient (*c2*) cells were examined for alterations in intracellular calcium levels following TCDD treatment. TCDD addition produced a rapid, nearly immediate increase in intracellular calcium mobilization, as determined by FURA-2 ratioing (Fig. 2, top panel). This effect was independent of the Ah receptor, because *c2* cells, containing less that 5% the normal Ah receptor levels, also responded. Apparently, calcium levels return to normal after this initial spike, since, within 10 min of addition, the FURA-2 ratio returned to baseline values (Fig. 2, top panel). However, when cells were maintained in 5 nM TCDD for 16–24 hr, a major difference emerged between wild type and *c2* cells. Prolonged exposure to TCDD caused wild type Hepa-1 cells to reset their cytosolic calcium to a level such that the FURA-2 ratio became twice as high as that of the untreated cells, a phenomenon that did not take place in *c2* cells (Fig. 2, bottom panel). In addition, TCDD-pretreated wild type cells, but not *c2* cells, responded rapidly to a second TCDD challenge (Fig. 2, bottom panel).

These Ah receptor-dependent changes in calcium levels in TCDD-treated cells could be responsible for activation of PLA_2 and mobilization of arachidonic acid from membrane depots. We found, however, no evidence for activation of PLC and concomitant stimulation of the inositol-lipid signaling pathway. Wild-type Hepa-1 cells and *c2* cells were prelabeled with *myo*-[^{3}H]-Inositol, stimulated with DMSO vehicle or with 10 nM TCDD and analyzed for inositol phosphate formation. We observed no significant differences in the relative amounts of *myo*-[^{3}H]-Inositol found in Gly-PI, IP, IP_2, or IP_4 in either wild-type or AHR-deficient cells, treated or not with TCDD (Fig. 3), suggesting that the persistent increase in cytosolic free calcium in wild-type cells did not result from PLC activation and a sustained opening of IP_3-sensitive intracellular calcium channels.

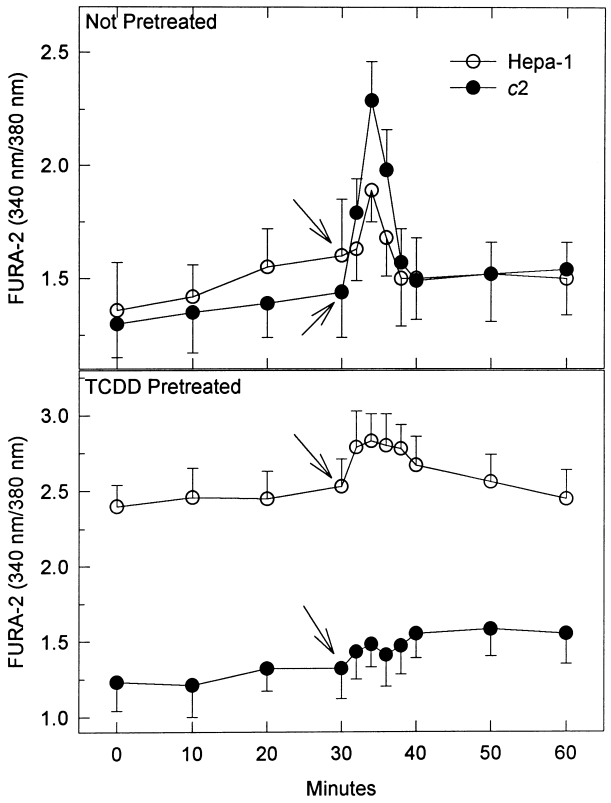

FIG. 2. Calcium mobilization in wild-type Hepa-1 and *c2* cells treated with TCDD. In the upper panel cells were treated with 5 nM TCDD, as indicated by the arrow, while under observation. The responses of Hepa-1 and *c2* cells were not significantly different ($P = 0.23$, two-tailed ANOVA). In the lower panel, cells were treated with 5 nM TCDD for 24 hr prior to observation. In the latter case, the effect of a second addition of TCDD, indicated by the arrow, was also determined. The responses of Hepa-1 and *c2* cells were significantly different ($P = 0.048$, two-tailed ANOVA). The results shown are the mean ratio ± SD of fluorescence determined at the two excitation wavelengths for 7–11 randomly chosen cells. The estimated range of the ordinate was from 100 nM to 600 nM Ca^{2+}.

We also found no significant differences in lipid or protein oxidative damage between wild-type cells untreated or treated with either 1 nM or 10 nM TCDD. We observed no changes in intracellular GSH, cysteine or protein thiols, nor did we detect any substantial increase in plasma membrane protein carbonyls or in thiobarbituric acid-reactive species, indicative of lipid peroxidation. TCDD also had no detectable toxic effects on the viability of Hepa-1 cells, even after 24 hr exposure (negative data not shown). These results lead us to conclude that TCDD does not cause a major disturbance of the redox state of the cells.

TCDD Induces Cox-2

To determine whether the effect of TCDD and the Ah receptor on PG distribution involved gene activation downstream from PLA_2, we examined mRNA levels of genes coding for key enzymes in PG biosynthesis. The rate-limiting enzyme for the production of PGs from ara-

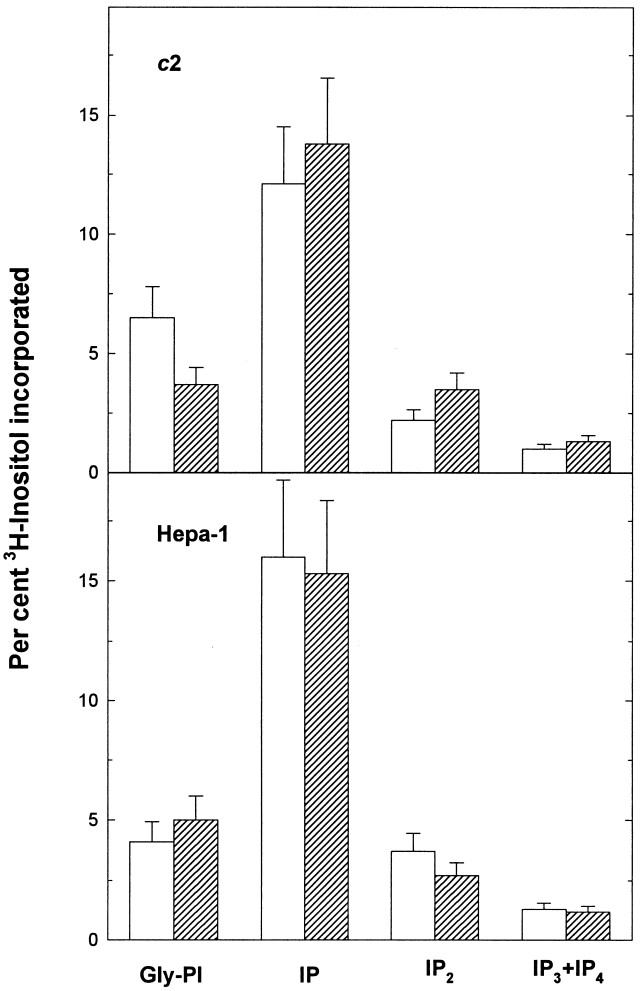

FIG. 3. Distribution of inositol and phosphoinositols in wild type Hepa-1 and c2 cells treated with TCDD. The values shown are the mean ± SEM of two experiments. Phosphoinositols were determined as indicated in the "Materials and Methods" section.

chidonic acid is the cyclooxygenase PGG/H endoperoxide synthase. This enzyme is bifunctional, converting arachidonic acid into PGG_2 and PGG_2 into PGH_2. Recently, two isoforms of this enzyme have been described and cloned from human, sheep and mouse cells: COX-1 (PGG/HS-1), constitutively expressed and showing little change with different treatments, and COX-2 (PGG/HS-2), inducible by phorbol esters and interleukin-1, and behaving as an immediate-early response gene [64–67]. Total RNA from Hepa-1 cells treated with vehicle or with 10 nM TCDD for increasing lengths of time was used for RT-PCR amplification of COX-1 and COX-2 mRNAs. As controls, CYP1A1 and β-actin mRNAs were also coamplified. Within 2 hr of treatment, we detected a significant increase in accumulated levels of COX-2 mRNA, which were even higher at 4 hr and decreased by 8 hr; in contrast, the levels of COX-1 mRNA were unaffected by treatment. Of the two controls, the levels of CYP1A1 mRNA were increased within 1 hr of treatment and showed a large increase up to at least 8 hr, the end of our experiments, while β-actin mRNA levels were unchanged by treatment (Fig. 4A). The relative induction levels, as determined by densitometry of the autoradiograms, were 20- to 30-fold for *Cox-2* and 50- to 70-fold for *Cyp1a1*.

Stimulation of COX-2 mRNA accumulation by TCDD did not require the presence of a functional CYP1A1 enzyme, but did require the Ah receptor. When we analyzed total RNA from *c37* and *c2* cells for TCDD-dependent changes in COX-2 mRNA levels, we found large increases, comparable to those in Hepa-1 cells, in *c37* cells, but not in *c2* cells (Fig. 4B). A small increase was detected in *c2* cells, most likely due to residual levels of Ah receptor in these cells. To verify this conclusion and to confirm the requirement of the Ah receptor for COX-2 mRNA accumulation, we grew Hepa-1 cells for three population doublings in the presence of a phosphorothioated antisense oligonucleotide to *Ahr* mRNA or of a control oligonucleotide. Thereafter, cells were treated with TCDD or with vehicle. The antisense oligonucleotide, but not the control, blocked the TCDD-dependent induction of both *Cyp1a1* and *Cox-2* (Fig. 4D), confirming that AHR is required for the activation of *Cox-2* by TCDD. Neither antisense or control oligonucleotides affected the accumulation of β-actin mRNA (not shown). In both Hepa-1 and *c37* cells, maximal activation of *Cox-2* was obtained at doses of TCDD between 0.01 and 0.1 nM (Fig. 4C).

DISCUSSION

The results that we described in this article are consistent with the view that TCDD mobilizes intracellular calcium. TCDD also causes the elevation of COX-2 mRNA levels and, based on the observation that secreted levels of at least one cyclooxygenase product, 12-HHT, are significantly elevated in TCDD-treated wild-type Hepa-1 cells, the results also support the conclusion that elevation of COX-2 mRNA by TCDD is accompanied by a concomitant increase in PGG/H endoperoxide synthase (cyclooxygenase), the rate-limiting cyclooxygenase for the production of PGs from arachidonic acid. In comparison, 5-, 12- and 15-lipooxygenases are unaffected by TCDD. In the absence of functional AHR, ARNT or CYP1A1, the secreted levels of other PGs, particularly 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$, become also markedly elevated independently of TCDD, suggesting that these three members of the drug-metabolizing cascade are somehow involved in the arachidonate cascade. Conversely, since 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ accumulate in *c2* cells having depressed levels of AHR, it is plausible that these arachidonate metabolites may play a role in endogenous AHR bioactivation processes.

TCDD also causes an immediate increase in intracellular free calcium levels, both in wild-type cells and in cells lacking significant levels of AHR. We and others have previously shown that TCDD also causes a rapid increase in Ca^{2+} influx rates from the medium, and that this increase is independent of the presence of a functional AHR [21–23, 68]. We now find that AHR is responsible for a sustained

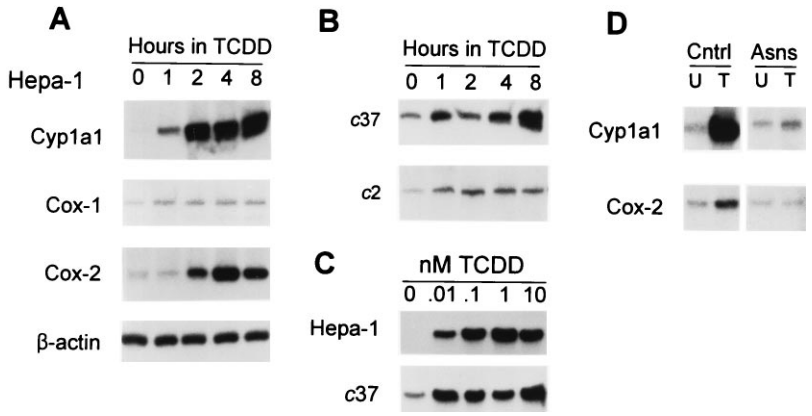

FIG. 4. COX-2 mRNA levels in TCDD-treated Hepa-1 cells and its derivatives. (A) Time-course of accumulation in TCDD-treated Hepa-1 cells. Total RNA samples were obtained at the indicated times after placing the cells in medium containing 10 nM TCDD and analyzed by RT-PCR for CYP1A1, COX-1, COX-2 and β-actin. (B) Time course of COX-2 mRNA accumulation in *c37* and *c2* cells. The experimental design was the same as in (A), except that different cell lines (*c37* and *c2*) were used. (C) TCDD-dose response. Hepa-1 and *c37* cells were exposed to the indicated doses of TCDD for 6 hr prior to RNA extraction and RT-PCR amplification of COX-2 mRNA. (D) Effect of anti-*Ahr* mRNA antisense oligonucleotides on COX-2 mRNA accumulation. Hepa-1 cells were grown for three population doublings in the presence of phosphorothioated antisense (*Asns*) or control (*Cntrl*) oligonucleotides. After a 6-hr treatment with 10 nM TCDD (*T*) or with DMSO vehicle (*U*), RNA was extracted and COX-2 mRNA was amplified by RT-PCR. All experimental procedures are described in the "Materials and Methods".

effect of TCDD on intracellular free calcium levels. In wild-type, but not in *c2* cells, extended exposure to 5 nM TCDD leads to a resetting of cytosolic calcium to a higher level high than in the absence of treatment. Since no changes in inositol metabolism were noted in TCDD-treated cells, we conclude that, in Hepa-1 cells, TCDD does not cause significant sustained activation of PLC. This suggests that the new levels of intracellular free calcium result from influx of extracellular calcium, possibly through Ca^{2+}-sensitive calcium channels, or from an impairment of the ability to re-sequester calcium after it has been released.

Cyclooxygenases are reported to have low turnover numbers and very short enzymatic half-lives (<10 min); their *de novo* synthesis is believed to be the main form of control of prostanoid production [67], although the precise mechanisms of their regulation have not yet been elucidated. Rapid and transient induction of *Cox-2* is believed to have an important role in signal transduction pathways and has been observed in cells treated with cytokines and tumor promoters [64–67]. In an earlier report, we described that TCDD stimulated by several-fold the accumulation of COX-2 but not COX-1 mRNA in Hepa-1 cells [69]. Similar results have been reported by Kraemer et al. in canine kidney cells [70]. In these cells, stimulation of *Cox-2* takes place at the transcriptional level, although AhRE motifs in the regulatory domains of the *Cox-2* gene are not sufficient to explain the transcriptional induction [70]. Our results show that the Ah receptor is an essential element of the induction process, since TCDD has no effect on COX-2 mRNA levels in *c2* cells, or in wild type cells grown in the presence of anti-*Ahr* mRNA antisense oligonucleotides. If we examine the results of Kraemer et al. in the context of our own, we would conclude that, in addition to a functional AHR, it is likely that ARNT would also be required for *Cox-2* induction, since ARNT is part of the transcriptional activity of the Ah receptor complex. On the other hand, a functional CYP1A1 enzyme is not needed for *Cox-2* induction, since COX-2 is also induced in the CYP1A1-deficient *c37* cell line. In these cells, there is no appreciable effect of TCDD on any of the arachidonate metabolites measured, suggesting that the various enzymes that metabolize PGG/H_2 are not affected by TCDD exposure. A notable exception is the absence in *c37* cells of the TCDD-dependent increase in 12-HHT observed in wild-type Hepa-1 cells. 12-HHT has been shown to result from the activity of thromboxane synthase [71] and COX-1, COX-2 and selenium-dependent glutathione peroxidases [72] on PGH_2. Our results suggest that the enzymatic activity of CYP1A1, absent in *c37* cells, may yet be another contributing factor to the biosynthesis of 12-HHT.

In addition to COX-2, TCDD also activates the arachidonic acid epoxygenase activity of CYP1A1, CYP1A2 and other cytochromes P450 in liver microsomes [56–60, 73, 74] and in heart, increasing intracellular calcium in myocytes and decreasing contractility [21, 22]. Many of the arachidonate metabolites that TCDD-induced cyclooxygenase and epoxygenase activities can form are powerful mediators of inflammation, immune function and vasoconstriction [75–78], as well as activators of protooncogene expression [37–39]. The picture that emerges is one in which TCDD, on the one hand, causes a dramatic and sustained increase in intracellular free calcium and, on the other, activates a number of prostaglandin mediators. Calcium increases, we speculate, might lead to activation of calcium-dependent phosphatases and hence to profound changes in the phosphorylation state of critical signal transduction effectors. Activation of prostaglandin and eicosanoid mediators, in turn, would have important consequences not only for tumor promotion and immune function, but also for some of the less well understood toxic

effects of TCDD, such as vasoconstriction and endothelial and vascular smooth muscle cell injury.

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