ABSTRACT:

The aryl hydrocarbon receptor (AhR) is targeted by ubiquitination for degradation by the proteasome shortly after its activation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In silico screening identified p-anilinoaniline (pAA) as a putative inhibitor of an E2 ligase that partners with an E3 ligase implicated in AhR ubiquitination. We investigated whether pAA could modify AhR-dependent activation of its target gene CYP1A1. pAA (1–200 μM) alone did not affect AhR content, or stimulate CYP1A1 mRNA accumulation in human mammary epithelial MCF10A cultures. However, pretreatment with ≥100 μM pAA suppressed TCDD-induced CYP1A1 activation and AhR degradation via its functioning as an AhR antagonist. At a lower concentration (25 μM), pAA cotreatment increased TCDD-induced CYP1A1 mRNA accumulation, without inhibiting AhR turnover or altering CYP1A1 mRNA half-life. Whereas TCDD alone did not affect MCF10A proliferation, 25 μM pAA was cytotactic and induced a G1 arrest that lasted ~7 h and induced an S phase arrest that peaked 5 to 8 h later. TCDD neither affected MCF10A cell cycle progression nor did it alter pAA effects on the cell cycle. The magnitude of CYP1A1 activation depended upon the time elapsed between pAA pretreatment and TCDD addition. Maximal AhR occupancy of the CYP1A1 promoter and accumulation of CYP1A1 heterogeneous nuclear RNA and mRNA occurred when pAA-pretreated cultures were exposed to TCDD in late G1 and early/mid S phase. TCDD-mediated induction of CYP2S1 was also cell cycle-dependent in MCF10A cultures. Similar studies with HepG2 cultures indicated that the cell cycle dependence of CYP1A1 induction is cell context-dependent.

Introduction

In many cell types the aryl hydrocarbon receptor (AhR) undergoes proteolysis after the binding of agonist. For example, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) binding reduces AhR half-life from 28 to ~3 h in murine hepatoma 1c1c7 cells (Ma and Baldwin, 2000). AhR degradation occurs as a consequence of its becoming polyubiquitinated, which targets it to the proteasome for proteolysis (Pollenz, 2002). Polyubiquitination is mediated by the sequential actions of three interacting but functionally distinct proteins: an E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzymes, and the E3 ubiquitin ligases (Pickart, 2001). There are approximately 50 known E2s and possibly hundreds of E3 proteins. The E3 proteins are responsible for substrate discrimination and the specificity of ubiquitination. A single E2 is able to associate with different E3s, and an individual E3 may associate with more than one E2 (Hershko and Ciechanover, 1998). Although the identities of the E2 and E3 proteins involved in ligand-induced AhR ubiquitination are not well characterized, some studies have implicated a role for the E3 ligase carboxyl terminus of Hsc70-interacting protein (CHIP) (Lees et al., 2001). CHIP contains a U-box domain, and it interacts with heat-shock protein 90 and hsc70 client proteins targeting them for degradation by the ubiquitin proteasome pathway (Connell et al., 2001). One of the E2-conjugating enzymes that partners with CHIP is UbcH5a (Jiang et al., 2001).

p-Anilinoaniline (pAA; also known as p-amidinophenylamine, N-phenyl-1,4-benzenediamine) is an aromatic amine that is used in hair coloring products. It is also a major metabolite of the azo dye metanil yellow (Srivastava et al., 1982). The latter is extensively used in the textile, paper, lacquer/stain industries (Mittal et al., 2008), and in India as a food-coloring agent (Khan et al., 1985). As a result of in silico screening, Banerjee (2006) identified pAA (identified as 05RB in the article) as a putative inhibitor of UbcH5a, an E2-conjugating enzyme that partners with the E3 ligase CHIP.

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; CHIP, chromatin immunoprecipitation; CHIP, carboxyl terminus of Hsc70-interacting protein; pAA, p-anilinoaniline; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; RT, reverse transcription; PCR, polymerase chain reaction; hnRNA, heterogeneous nuclear RNA; bp, base pair; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Ct, comparative cycle threshold; XRE, xenobiotic response element; EMSA, electrophoretic mobility shift assay; ActD, actinomycin D.
We initiated the current study with the intent of assessing pAA as a possible inhibitor of TCDD-induced AhR ubiquitination in the normal human mammary epithelial cell line MCF10A. We previously reported that TCDD induced AhR degradation and the expression of multiple AhR-responsive phase I and II metabolism-related genes (i.e., CYP1A1, CYP1A2, CYP1B1, and NQO1) in MCF10A cultures (Reiners et al., 1997; Gou et al., 2001; Jojakim et al., 2004). Unfortunately, MCF10A cultures ultimately proved to be an inappropriate model for the testing of our hypothesis because the line did not express UbcH5a. However, we found that pAA mediated a very reproducible time-dependent enhancement of CYP1A1 induction by TCDD that paralleled pAA-induced cell cycle arrest and release. In particular, our data indicate that TCDD-induced AhR occupancy of the CYP1A1 promoter and CYP1A1 transcriptional activation in MCF10A cells are maximal in late G1 and early/mid S phase cells.

Materials and Methods

Materials. TCDD was purchased from Midwest Research Institute (Kansas City, MO). TRIzol, trypsin/EDTA, epidermal growth factor, penicillin/streptomycin solution, horse serum, salmon sperm DNA, Taq DNA polymerase, phenol, Random Primers DNA Labeling System, PCRX Enhancer System, and PCRX Amplification mixture were purchased from Invitrogen (Carlsbad, CA). pAA, actinomycin D, dimethyl sulfoxide (DMSO), deoxyinosine triphosphates, deoxyribonucleoside 5′, phosphate inhibitor cocktail, protein A agarose, and ribonuclease A were obtained from Sigma-Aldrich (St. Louis, MO). RNAqueous-4PCR kit was obtained from Ambion Inc. (Austin, TX). TaqMan Reverse Transcription Reagent, TaqMan Gene Expression Assays, and SYBR Green PCR master mix were from Applied Biosystems (Foster City, CA).

Cell Culture. MCF10A human breast epithelial cell lines were obtained from the Cell Lines Resource, Karmanos Cancer Institute (Detroit, MI) and maintained as adherent cultures in supplemented Dulbecco’s modified Eagle medium/Ham’s F-12 medium as described by Guo et al. (2001). HepG2 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium supplemented with nonessential amino acids, 100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen), and 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA). Cultures were maintained at 37°C in a humidified atmosphere consisting of 95% air and 5% CO2. Cells were seeded at densities that assured exponential growth for at least 5 days. Cultures were treated on the second day of plating with either DMSO or different concentrations of pAA dissolved in DMSO. Solvent never exceeded 0.1%. For estimates of cell number and viability, cultures were trypsinized, washed with phosphate-buffered saline (PBS), and suspended in PBS containing trypan blue, and counted with a hemocytometer. Viability was scored as the ability to exclude trypan blue.

Cell Cycle Analyses. The procedure used for the determination of cell cycle phase by fluorescence-activated cell sorting has been described in detail (Reiners et al., 1999).

Western Blot Analyses. The conditions used for the preparation of cell extracts, separation of proteins on SDS-polyacrylamide gels, and transfer of separated proteins onto nitrocellulose membranes have been described in detail (Gou et al., 2001). Nonspecific antibody binding to transferred proteins was blocked by preincubating membranes in PBS-T (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween-20) supplemented with 5% nonfat dry milk proteins. After washing with PBS-T, membranes were incubated with the appropriate horseradish peroxidase secondary antibody for 1.5 h at room temperature. Antibody detection was performed with an enhanced chemiluminescence reaction kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and recorded on X-ray film.

RNA Preparation and Northern Blot Analyses. The conditions used for RNA isolation, resolution of RNAs on agarose/formaldehyde gels, and transfer to nitrocellulose membranes have been described by Reiners et al. (1997). The probes and hybridization conditions used for the detection of human 7S, CYP1A1, and CYP1A2 RNAs have been described in detail (Reiners et al., 1997; Jojakim et al., 2004). Northern blot data were normalized by calculating CYP1A1 or CYP1A2 mRNA to 7S RNA signal strengths.

Real-Time Reverse Transcription-Polymerase Chain Reaction of CYP1A1 Heterogeneous Nuclear RNA. Total RNA was isolated from MCF10A cultures using the RNAqueous-4PCR kit according to the supplier’s specifications. RNA was treated with DNsase I according to the instructions provided by the supplier to remove trace amounts of DNA, and it was quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA was synthesized from isolated RNA using TaqMan Reverse Transcriptation Reagents, as described by the supplier. For quantification of CYP1A1 heterogeneous nuclear (hnRNA), PCR amplification used the previously described forward primer 5′- TTGGTATCCACGGCTCCAAGA-3′ and the reverse primer 5′-GGAGGCGACAAAAATGTTCCTTT-3′. These primers amplify a 120-base pair sequence that corresponds to a region spanning the first exon-intron (GenBank accession number AF253322). For glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA quantification, we used the GAPDH forward PCR primer 5′-AAGAAACCTGCTCAAGATGATC-3′ and the reverse PCR primer 5′-GCCAGGATGCCCCTTGA-3′. This amplifies a region from 894 to 927 bp downstream of the transcript start site. PCR data were analyzed using the comparative cycle threshold (Ct) method. Relative quantification was based on real-time expression of CYP1A1 normalized against GAPDH. Relative expression of the target gene was calculated as 2−ΔΔCt, where ΔCt was obtained by subtracting ΔCt of untreated cells from the ΔCt of treated cells. The ΔCt was calculated by subtracting the average Ct value for GAPDH from the average Ct value of CYP1A1. All PCRs were performed in triplicate (technical replicates).

Real-Time Reverse Transcription-PCR of CYP1A1 and CYP2S1 mRNAs. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed using the Omniscript RT kit (Qiagen) and random primers (Invitrogen) according to the manufacturers’ instructions. Human CYP1A1 and CYP2S1 mRNA contents were quantified as described previously (Ouniecz-Dmochowski et al., 2007), using TaqMan Gene Expression Assays Hs01531520_m1 and Hs00258076_m1, respectively, in multiplex reactions with TaqMan 18S rRNA Endogenous Control (primer limited) to quantify endogenous 18S rRNA (Applied Biosystems). Cycle threshold values were used to normalize CYP1A1 or CYP2S1 levels to 18S RNA levels as described above.

Chromatin Immunoprecipitation Assay. Culture medium was adjusted to 1% formaldehyde and incubated for 10 min at room temperature to cross-link protein-DNA complexes. Cultures were subsequently rinsed with ice-cold PBS and incubated for 5 min at room temperature with a 125 mM glycine/PBS solution. Cells were then washed with PBS, covered with PBS supplemented with 1× (v/v) protease inhibitor cocktail (Sigma-Aldrich), and detached by mechanical scraping. The cell suspensions were pelleted by centrifugation, quick frozen, and stored at −80°C until further processing. The cell pellets were subsequently mixed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) and sonicated using conditions that yielded DNA lengths of 200 to 1000 bp. The sonicated chromatin was diluted 1:10 with chromatin immunoprecipitation (ChIP) buffer [0.1% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.0), and 167 mM NaCl] supplemented with protease inhibitors. Chromatin was precleared with salmon sperm DNA/protein A agarose (60 μl of a 50% slurry), 5 μg of salmon sperm DNA, and 1 μg of rabbit IgG with gentle agitation for 2 h at 4°C. Agarose beads were pelleted by centrifugation at 700g for 1 min. The supernatant fluid was transferred to a new tube, and a sample was put aside for determination of input DNA. One microgram of either AhR antibody or rabbit IgG was added to the remaining supernatant fluid. After overnight incubation at 4°C on a rotating platform, salmon sperm DNA/protein A agarose (40 μl of a 50% slurry) was added, and an additional incubation was performed for 1.5 h at 4°C before pelleting the agarose beads by centrifugation. The agarose beads were sequentially washed for 10 min each in 1 ml of low salt wash buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS], 1 ml of high salt wash buffer [20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS], 1 ml of LiCl wash buffer [10 mM Tris-HCl (pH 8.0), 0.25 M LiCl, 1 mM EDTA, 1% Igepal, 1% sodium deoxycholate], and 2 × 1 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Protein-DNA complexes were eluted from the beads by incubation with 250 μl of NaHCO3 (0.1 M) and 1% SDS for 30 min. Cross-linking was reversed by incubating the supernatant overnight at 65°C in 200 mM NaCl. DNA was subsequently purified by standard procedures.

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AhR-DNA interactions were monitored using forward (5′-ACCGGCCAC- 
CCTTCGACAGTTCC-3′) and reverse (5′-TGCCAGGCTTTGCTG- 
GAGAACG-3′) primers to amplify a region from −980 to −1125 of the 
CYP1A1 promoter (Matthews et al., 2005). This region contains two functional 
exonotopic response elements (XREs) and yields a PCR product of 146 bp. The 
PCR mixture contained input or ChIP-isolated DNA, 1× PCR amplification 
buffer, 1× PCRx enhancer solution, 0.2 mM deoxynucleoside triphosphate 
mix, 2.5 mM MgSO4, 0.4 µM each of forward and reverse primers, and 0.5 U 
of Taq DNA polymerase. The PCR used the following conditions and cycles: 
95°C for 3 min, 35 cycles of 95°C for 45s, 58°C for 45s, and 70°C for 1 min, 
and then 70°C for 5 min. PCR products were separated on agarose gels and 
stained with ethidium bromide.

Electrophoretic Mobility Shift Assay. The conditions used for TCDD-
mediated AhR transformation in rat liver extracts, electrophoretic mobility 
shift assay (EMSA), and the sequence of the double-stranded radiolabeled 
oligonucleotide containing a consensus XRE have been published previously 
(Joiakim et al., 2004).

Image and Statistical Analyses. EMSA, Western blot, and Northern blot 
films were scanned at 800 to 1000 dpi and saved as .tif images. Band 
intensities in the images were quantified with ImageJ software (National 
Institutes of Health, Bethesda, MD). Data were analyzed by one-way analysis 
of variance followed by Tukey’s multiple comparison test, using GraphPad 
Institutes of Health, Bethesda, MD). Data were analyzed by one-way analysis 
of variance followed by Tukey’s multiple comparison test, using GraphPad 
Prism version 4.02 for Windows (GraphPad Software Inc., San Diego, CA). 
P < 0.05 was considered statistically significant.

Results

Effects of pAA on TCDD Induction of CYP1A1. Exposure of 
MCF10A cultures to 10 nM TCDD induced the accumulation of 
CYP1A1 mRNA, whereas exposure to 1 to 200 µM pAA had no 
effect (Fig. 1A). However, depending on the concentration used, pAA 
pretreatment could either suppress or enhance dioxin-induced 
CYP1A1 mRNA accumulation (Fig. 1, A and B). In particular, 
pretreatment with concentrations of pAA ≥75 µM suppressed 
TCDD-induced CYP1A1 mRNA accumulation, whereas, concentrations 
of pAA ≥10 µM and ≤50 µM enhanced CYP1A1 mRNA accumulation. Kinetic analyses indicated that induced steady-state 
CYP1A1 mRNA levels were fairly constant after 5 to 18 h of TCDD 
treatment (Fig. 1C). In contrast, TCDD-induced CYP1A1 mRNA 
accumulation was notably bell-shaped in pAA-pretreated cultures, 
with maximal accumulation (e.g., ~3–4-fold greater than dioxin 
alone) occurring 7.5 to 12 h after TCDD addition (Fig. 1C).

CYP1A2, like CYP1A1, is transcriptionally activated by TCDD in 
MCF10A cultures (Joiakim et al., 2004). pAA alone had no effect on 
CYP1A2 mRNA content in MCF10A cultures (A. Joiakim, unpub-
lished data). However, pretreatment with 25 µM pAA enhanced 
dioxin-induced CYP1A2 mRNA contents by ~2-fold (Fig. 1D). Note 
that maximal CYP1A1 and CYP1A2 mRNA accumulations occurred 
within a similar time period (compare Fig. 1, C and D).

The accumulation of CYP1A1 mRNA after TCDD treatment could 
reflect either enhanced CYP1A1 transcription or a stabilization of 
CYP1A1 mRNA. To examine the former, we made cDNA to total 
RNA and used PCR to amplify hnRNA sequences that encompassed 
the exon 1-intron 1 boundary of CYP1A1 (Fig. 2A). Analysis of 
hnRNA has been used as a surrogate assay for monitoring the tran-
scriptional activation of CYP1A1 (Ellerink and Reiners, 1996). Exposure 
to either DMSO or 25 µM pAA did not alter CYP1A1 hnRNA 
content over an 8-h time period, whereas TCDD treatment resulted in 
an ~20-fold increase (Fig. 2B). Pretreatment with 25 µM pAA, 
followed by TCDD addition, increased CYP1A1 hnRNA content 
~2-fold above what accumulated after 6 h of just TCDD exposure 
(Fig. 2C).

To assess the effects of pAA on CYP1A1 mRNA stability, we 
pretreated cultures with TCDD ± pAA for 8 h before adding 5 µg/ml 
TCDD.
actinomycin D (ActD), a concentration that completely inhibits CYP1A1 transcription (Chen et al., 1995; Ciolino et al., 1999). Total RNA was subsequently collected at 2-h intervals for measurement of CYP1A1 mRNA (Fig. 3A). Decreases in mRNA content after ActD treatment became very apparent with passing time. Figure 3B depicts CYP1A1 mRNA half-life analyses based on data generated in four independent experiments. pAA pretreatment had no statistically significant effect on the rate of CYP1A1 mRNA turnover (Fig. 3B).

**pAA Modulation of TCDD-Induced AhR Degradation.** A substantial reduction in AhR content occurred in MCF10A cultures within 6 h of dioxin addition (Fig. 4A). Pretreatment with pAA suppressed TCDD-induced AhR turnover in a concentration-dependent manner (Fig. 4A). A strong inhibition of TCDD-induced AhR loss was observed in cultures cotreated with 100 μM pAA (p < 0.05 for n = 4 independent experiments; Fig. 4A), whereas 10 μM pAA was without effect (Fig. 4A). Kinetic analyses of intermediate concentrations suggested that 50 μM pAA partially blocked TCDD-induced AhR degradation (p < 0.05 for n = 3 independent experiments; Fig. 4B). Although 25 μM pAA appeared to exhibit a small protective trend, the AhR contents of TCDD and TCDD + pAA-treated cultures were not significantly different (Fig. 4C).

Although the effect of pretreatment with 25 to 50 μM pAA on AhR content was small, we wondered whether it might be sufficient to mediate the enhanced accumulation of CYP1A1 mRNA after TCDD exposure. As an approach to the issue, we intended to knock down the putative E2 target of pAA (i.e., UbcH5a). Although we could easily detect Ubch5a in human embryonic kidney 293 cells by Western blotting, we were unable to detect it in MCF10A cultures (data not presented). Hence, it seemed unlikely that the effects of pAA on AhR content and CYP1A1 induction were related to pAA effects on the E2 UbcH5a.

**AhR Agonist and Antagonist Activities of pAA.** The ability of pAA to suppress both TCDD-induced AhR proteolysis and CYP1A1 transcription at 100 μM is consistent with it functioning as an AhR antagonist. We used an EMSA to examine this issue. Incubation of rat liver cytosol with 10 nM TCDD effectively transformed the AhR into a species capable of binding to a radiolabeled oligo containing a xenobiotic-responsive element (Fig. 5A). In contrast, AhR transformation did not occur after incubation of cytosol with 1 to 200 μM pAA (Fig. 5A). However, cotreatment of rat liver cytosol with pAA suppressed the formation of TCDD-induced AhR-DNA complexes, in a concentration-dependent fashion (Fig. 5B). This suppression was observed at concentrations of pAA ≥ 50 μM. Hence, the EMSA assay suggests that pAA has no AhR agonist activity but can function as an AhR antagonist once a critical concentration is reached.

**pAA Effects on CYP1A1 and CYP2S1 Induction Are Cell Cycle-Dependent.** We previously reported that pAA is cytostatic to MCF10A cultures in a concentration-dependent fashion over a range of 10 to 50 μM and causes an almost complete suppression of proliferation at 50 μM (Elliott and Reiners, 2008). Cell cycle analyses indicated that 25 μM pAA induced simultaneous G1 and S phase blocks in MCF10A cultures (Fig. 6A). During the first 7 h of pAA treatment, the proportion of cells in S phase held fairly constant, whereas the proportion of G1 cells increased due to the progression of G2/M cells. Thereafter, G1 cells began to transition into S phase where they accumulated for the next 8 h. Arrested S phase cells began to transition into G2/M ~ 15 h after pAA addition (Fig. 6A). Notably, the onset of G1 cells transitioning into S phase, and the period of S phase cell accumulation, roughly correlate with...
when pAA cotreatment enhanced TCDD activation of CYP1A1 (compare Figs. 1C and 6A). This temporal relationship is significant because we previously reported that CYP1A1 induction may be cell cycle-regulated (Santini et al., 2001).

To examine the relationship between AhR function and cell cycle phase in detail, we quantified TCDD-mediated transcriptional activation of CYP1A1 at different times after pAA treatment. Before initiating this study, we determined whether TCDD had any effects on MCF10A cell cycle progression. A concentration of TCDD sufficient to activate CYP1A1 transcription neither altered cell cycle phase distributions over a 24-h treatment period when used singularly (Supplemental Fig. 1) nor altered the development and resolution of pAA-induced G1 and S phase arrest. The latter studies were performed in two ways. In one protocol, TCDD was added 1.5 h after pAA addition, and cell cycle analyses were performed throughout a 24-h period (Supplemental Fig. 1). In the second protocol, we treated cultures with TCDD at various times after pAA addition and harvested cultures 3 h after dioxin addition (Fig. 6B). This latter protocol facilitated very detailed analyses of TCDD effects on specific phases of the cell cycle. Overall, TCDD had no detectable effects on MCF10A cell cycle progression.

Cultures treated similarly to those in Fig. 6B were analyzed for CYP1A1 mRNA (Fig. 6C) and hnRNA (Fig. 6D) after the TCDD addition. In these studies, we harvested cultures within 3 h of TCDD addition to restrict the effects of dioxin to defined stages of the cell cycle. The length of time elapsed between pAA and dioxin additions influenced the extent to which CYP1A1 mRNA and hnRNA accumulated. CYP1A1 mRNA contents were maximally increased when dioxin was added to cultures in late G1 or when transitioning from G1 to S (i.e., time of harvest = 13 h), and in early/middle S phase (i.e., time of harvest = 15 h; Fig. 6C). Late S phase cells (i.e., time of harvest = 18 h) exhibited less CYP1A1 mRNA, and CYP1A1 mRNA content continued to drop as S phase cells transitioned into G2/M (Fig. 6C). The kinetics of CYP1A1 hnRNA accumulation and decline paralleled CYP1A1 mRNA content (compare Fig. 6, C and D). ChIP assays with AhR antibodies did not detect AhR occupancy of the CYP1A1 promoter in nontreated cultures, or cultures treated with only pAA for 1.5, 11, or 18 h (Fig. 6E). In pAA-pretreated cultures, AhR occupancy of the CYP1A1 promoter after TCDD addition qualitatively correlated with CYP1A1 hnRNA levels (compare Fig. 6, D and E). In particular, AhR occupancy of the CYP1A1 promoter was markedly less in cultures treated with TCDD 1.5 h after pAA addition, relative to cultures treated with TCDD 11 and 18 h after pAA addition.

CYP2S1 is transcriptionally activated by TCDD through an AhR-dependent mechanism (Saarikoski et al., 2005; Rivera et al., 2007). Thomas et al. (2006) previously reported that CYP2S1 mRNA accumulates in MCF10A cultures after the addition of dioxin. In asynchronous MCF10A cultures, we observed no accumulation of CYP2S1 mRNA within 3 h of TCDD addition (Fig. 7A). However, this time period was sufficient for CYP2S1 mRNA accumulation if the cultures were pretreated with pAA (Fig. 7A). Like CYP1A1, optimal accumulations of CYP2S1 mRNA occurred when cultures were treated with TCDD in late G1, during the G1/S transition and early/middle S phase (5–12 h after pAA addition; compare Fig. 7, A and B).

pAA Effects on CYP1A1 Induction in HepG2 Cultures. We examined the effects of pAA in HepG2 cells to determine whether its effects on CYP1A1 induction were cell-type specific. pAA suppressed the proliferation of HepG2 in a concentration-dependent fashion (Fig. 8A) that mirrored the effects observed in MCF10A cells (Elliott and Reiners, 2008). Concentrations of pAA ≥25 μM were

**Fig. 4.** Effects of pAA on TCDD-induced AhR degradation. A, MCF10A cultures were treated with 100 μM pAA for 7.5 h before harvest, or pretreated with 1 to 100 μM pAA for 1.5 h before the addition of 10 nM TCDD. Cultures were harvested 6 h after TCDD addition for analyses of AhR and actin content by Western blot. Similar results were obtained in two additional experiments. B, cultures were treated with nothing, 10 nM TCDD, or pretreated with 50 μM pAA for 1.5 h before the addition of TCDD. Cultures were harvested 1 to 6 h after the addition of TCDD for analyses of AhR and actin proteins. C, cultures were treated with 10 nM TCDD, DMSO, or 25 μM pAA for 1.5 h before TCDD addition, and subsequently harvested 2 to 10 h after TCDD addition for analyses of AhR and GAPDH by Western blot. The lower panel represents means ± S.D. of four independent experiments using 25 μM pAA pretreatment. Relative quantification was achieved by first calculating AhR/GAPDH ratios, and then normalizing to the ratio of nontreated controls, which was set as 100%. The AhR contents of the two treatment groups were not statistically different from one another at any of the time points. Western blots used 25 μg of protein lysate.

**Fig. 5.** AhR antagonist properties of pAA. A, rat liver extract was incubated with 10 nM TCDD or different concentrations of pAA before the addition of a radiolabeled oligo, containing a consensus XRE, and subsequent EMSA. B, rat liver extract was coincubated with 10 nM TCDD and 1 to 200 μM pAA before the addition of a radiolabeled oligo, containing a consensus XRE, and subsequent EMSA. Parallel reaction mixtures containing a 50-fold excess of unlabeled oligo were used to control for nonspecific AhR-DNA interactions. Similar results were obtained in a second independent study.
very cytostatic, with antiproliferative effects that persisted for at least 72 h. The cytostatic effects of 25 μM pAA reflected the induction of an early G1, and S phase arrest, with arrested G1 phase cells transitioning into S phase 8 to 16 h after pAA addition (Fig. 8B). Unlike MCF10A cultures, pAA-treated cultures remained arrested in S phase 28 h after treatment (Fig. 8B). Treatment of asynchronous HepG2 cultures with TCDD increased CYP1A1 mRNA content 11- to 19-fold within 3 h after dioxin addition (Fig. 8C). Pretreatment with pAA increased TCDD-induced CYP1A1 mRNA accumulation above that achieved with just TCDD (Fig. 8C). However, unlike what we observed with MCF10A cultures, the effects of pAA pretreatment on TCDD-mediated CYP1A1 induction were not cell cycle-dependent. Instead, pAA enhanced CYP1A1 mRNA accumulation irrespective of the cell cycle stage at which TCDD was added (Fig. 8C).

Although CYP2S1 mRNA was detected in the nontreated asynchronous HepG2 cultures reported in Fig. 8, we observed no induction of CYP2S1 in these cultures after a 3-h treatment with TCDD, or combined pAA and TCDD cotreatment (J. J. Reiners and Z. Duniec-Dmuchowski, unpublished data).

**Discussion**

We initiated the current study to determine whether pAA was a modulator of agonist-activated AhR degradation. Unexpectedly, the culture model we used proved to be inappropriate because MCF10A cells failed to express the E2 ligase (i.e., UbcH5a) putatively targeted by pAA. Nevertheless, cotreatment with ≥50 μM pAA suppressed TCDD-induced AhR degradation in a concentration-dependent manner. Based on EMSA results, this activity most likely reflects the ability of pAA to function as an AhR antagonist and suppressor of AhR activation at such concentrations.

A second pAA-related activity became apparent at concentrations that ranged from 10 to 50 μM. In particular, treatment of MCF10A cultures with these concentrations before TCDD addition elevated CYP1A1 mRNA contents above that occurring with dioxin alone. We previously reported that this concentration range of pAA induces a graded cytostatic response in MCF10A cultures (Elliott and Reiners, 2008). The studies reported in Fig. 6A indicate that dioxin induction of AhR occupancy of the CYP1A1 promoter in highly enriched phases of the cell cycle. The studies depicted in Figs. 6, B to E, indicate that dioxin induction of AhR occupancy of the CYP1A1 promoter and CYP1A1 transcription are optimal in late G1 MCF10A cultures, and as cells transition from G1 to S phase, and during early/middle S phase. This cell cycle phase dependence provides an explanation for the bell-shaped kinetics of CYP1A1 mRNA accumulation observed in Fig. 1C. In particular, the times at which
AA potentiated TCDD-induced CYP1A1 mRNA accumulation in MCF10A cultures corresponded to the period at which cultures contained the highest percentage of late stage G1 and S phase cells. Our observation of a cell cycle dependence for AhR agonist induction of CYP1A1 is consistent with two other independent studies. In particular, we previously used centrifugal elutriation of asynchronous cycling U937 cell cultures to isolate populations in defined stages of the cell cycle (Santini et al., 2001). Subsequent treatment of these populations with TCDD showed a gradient of CYP1A1 mRNA accumulation, with maximal accumulation occurring in late G1 and early S phase cells. Jiao et al. (2007) used several approaches to generate MCF-7 cultures enriched in G0/G1, S, or G2/M phase cells. Maximal accumulation of CYP1A1 mRNA occurred in S phase-enriched cultures after short-term exposure to the AhR agonist benzo[a]pyrene. In addition to CYP1A1, the ligand-activated AhR regulates the transcription of numerous genes that have XREs in their promoters (Sun et al., 2004). In the current study, we observed that the induction of CYP2S1 by TCDD in MCF10A cultures was also cell cycle-dependent. Likewise, Jiao et al. (2007) reported maximal accumulations of CYP1A2 and CYP1B1 mRNA in S phase MCF-7 cultures after benzo[a]pyrene treatment. Hence, agonist induction of multiple AhR-responsive genes appears to be cell cycle-dependent in MCF10A and MCF-7 cultures. However, given that similar effects were not observed in HepG2 cultures, it appears that the cell cycle dependence of CYP1A1 induction may be cell-type specific. We do not know the basis for this cell context dependence, but it also extends to other aspects of CYP1A1 regulation in the three cell lines. Whereas pretreatment of MCF10A (Guo et al., 2001) and MCF-7 (Moore et al., 1993) cultures with 12-O-tetradecanoylphorbol-13-acetate initially suppresses TCDD-mediated induction of CYP1A1, a similar pretreatment enhances CYP1A1 transcription in HepG2 cultures (Chen and Tukey, 1996; Morgan et al., 1998).

Two timekeepers appear to regulate TCDD-mediated transcriptional activation of CYP1A1. The first entails the cell cycle and the

**FIG. 7.** TCDD-induced CYP2S1 transcription in MCF10A cultures is cell cycle-dependent. A, MCF10A cultures were left untreated or pretreated with 25 μM pAA for different lengths of time before the addition of 10 nM TCDD. At parallel times, some untreated cultures were treated with DMSO or 10 nM TCDD. All cultures were harvested 3 h after the additions of either DMSO or TCDD for isolation of RNAs and real-time PCR analyses of CYP2S1 mRNA and 18S ribosomal RNA. Relative CYP2S1 content was determined as described under Materials and Methods, using the no-treatment group value as 1. Data represent means ± S.D. of analyses involving three independent culture dishes per time point and treatment group, * greater than the no-treatment, DMSO-treated, and TCDD-only treated groups, p < 0.05. B, MCF10A cultures were exposed to 25 μM pAA and subsequently harvested 2 to 20 h later for analyses of the percentages of cells in G1, S, and G2/M. Each column represents analyses of 2 × 10⁴ cells.

**FIG. 8.** Effects of pAA on CYP1A1 mRNA accumulation in HepG2 cultures. A, HepG2 cultures were treated with different pAA concentrations, for different lengths of time, before being harvested for assessment of cell number (closed symbols) or viability (open symbols, absence of trypan blue staining). Data represent means ± S.D. of analyses performed on three independent plates per time and treatment group. Error bars are hidden in many cases by symbols. Treatments are noted in the figure. B, HepG2 cultures were exposed to 25 μM pAA and subsequently harvested 8 to 28 h later for analyses of the percentages of cells in G1, S, and G2/M. Each column represents analyses of 2 × 10⁴ cells. C, HepG2 cultures were treated as described in Fig. 7A and processed for real-time PCR analyses of CYP1A1 and 18S ribosomal RNA. Data represent means ± S.D. of analyses involving three independent culture dishes per time point and treatment group, * greater than the no-treatment, DMSO-treated, and TCDD-treated groups, p < 0.05.
second circadian rhythms. Regarding the latter, TCDD-mediated induction of CYP1A1 in the liver and mammary glands of mice is 23- to 40-fold greater at night than during daytime (Qu et al., 2007, 2010). This diurnal rhythm in CYP1A1 responsiveness to TCDD is inversely related to the expression of the protein Period 1 (Metz et al., 2006: Qu et al., 2010), a key protein regulator of the circadian clock (Reppert and Weaver, 2002). The occurrence of this cyclical rhythm for CYP1A1 responsiveness in a quiescent tissue (such as the liver) emphasizes the independence of the two timekeepers in some situations. However, several studies have demonstrated that circadian rhythms gate the expression/activation of cell cycle-regulated genes in proliferating tissues (Matsuo et al., 2003; Yang et al., 2009). For example, in normal human oral mucosa the maximal accumulation of Period 1 occurs in G1 before the expression of cyclin E, and the onset of S phase occurs after appreciable Period 1 loss (Bjarnason et al., 2001). A similar pattern is also observed in human skin (Bjarnason et al., 2001). Although speculative, it is conceivable that cell cycle- and circadian rhythm-dependent regulation of TCDD-induced CYP1A1 transcription may be linked in vivo, in some proliferating tissues.

Protocols that facilitate manipulation of cell density indicate that cell-cell contact also influences AhR activation and the transcription of several AhR-responsive genes (Cho et al., 2004; Ikuta et al., 2004; and references within). In particular, the expression of both CYP1A1 and CYP1B1 in cultured adherent cells, in the absence of any exogenous AhR ligand, is inversely related to cell density (Cho et al., 2004; Ikuta et al., 2004). In some instances, the level of CYP1A1 or CYP1B1 expression induced by culturing at low density or suspension culturing approximate what is achieved after treatment of near confluent, adherent cultures with AhR agonists (Cho et al., 2004; Ikuta et al., 2004; and references within). Studies with cultured keratinocytes (Ikuta et al., 2004) and fibroblasts (Cho et al., 2004) indicate that cell contact/density-mediated regulation of the AhR and CYP1A1/CYP1B1 is cell cycle phase-independent.

Agonist activation of the AhR can have diverse effects on the cell cycle. For example, contact inhibited, quiescent rat liver epithelial WB-F344 cells reenter the cell cycle and proliferate after exposure to TCDD and several polychlorinated biphenyl and polycyclic aromatic hydrocarbon AhR agonists (Chramostová et al., 2004; Vondrácek et al., 2005). In contrast, TCDD induces a profound G1 or S phase arrest in a variety of cultured cell types (reviewed by Marlowe and Puga, 2005; Puga et al., 2009; Barhoover et al., 2010). In many cases, this arrest has been shown to be AhR-dependent (Marlowe and Puga, 2005). The MCF10A cells used in the current study appear to represent a third case. In particular, concentrations of dioxin sufficient to induce CYP1A1 transcription had no notable effect on MCF10A cell cycle progression. Taken together, these data indicate that dioxin effects on the cell cycle are cell context-dependent. However, it should be noted that even for a specific cell type, additional factors radically influence the effects of dioxin on the cell cycle. For example, whereas dioxin suppresses compensatory liver regeneration induced by partial hepatectomy, it enhances hepatocyte proliferation induced by the hepatomitogen 1,4-bis[3,5-dichloropyridyl]benzene (Mitchell et al., 2010).

In summary, the current study demonstrates that dioxin-mediated transcriptional activation of CYP1A1 is optimal in late G1 and early/ middle S phase MCF10A cells. Similar observations have been made in other cell lines (Santini et al., 2001; Jiao et al., 2007). These findings raise the broader issue of whether other genes associated with metabolic transformation are cell cycle-regulated. A recent report by Sugatani et al. (2010) demonstrated that UGT1A1 and CYP2B6 content in HepG2 cells inversely correlated with the activation state of cyclin-dependent kinase 2, a cell cycle-regulated protein.


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Supplemental Figure 1. Absence of TCDD effect on pAA induced G1 and S phase arrest. Cultures of MCF10A cells were treated with nothing, DMSO alone, 25 µM pAA alone, 10 nM TCDD alone, or with pAA for 1.5 h prior to the addition of TCDD. Cultures were harvested at various times after TCDD addition for FACS analyses of cell cycle distribution. Data represent analyses of 20,000 cells. Similar results were obtained in two additional experiments.