***p*-Anilinoaniline Enhancement of Dioxin-Induced *CYP1A1* Transcription and Aryl Hydrocarbon Receptor Occupancy of *CYP1A1* Promoter: Role of the Cell Cycle^S**

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Received August 26, 2011; accepted February 16, 2012

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 (*p*AA) as a putative inhibitor of an E2 ligase that partners with an E3 ligase implicated in AhR ubiquitination. We investigated whether *p*AA could modify AhR-dependent activation of its target gene *CYP1A1*. *p*AA (1–200 μ M) alone did not affect AhR content, or stimulate *CYP1A1* mRNA accumulation in human mammary epithelial MCF10A cultures. However, pretreatment with ≥ 100 μ M *p*AA suppressed TCDD-induced *CYP1A1* activation and AhR degradation via its functioning as an AhR antagonist. At a lower concentration (25 μ M), *p*AA 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 μ M *p*AA was cytostatic 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 *p*AA effects on the cell cycle. The magnitude of *CYP1A1* activation depended upon the time elapsed between *p*AA pretreatment and TCDD addition. Maximal AhR occupancy of the *CYP1A1* promoter and accumulation of *CYP1A1* heterogeneous nuclear RNA and mRNA occurred when *p*AA-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., 2003; Morales and Perdew, 2007). 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 (*p*AA; also known as *p*-aminodiphenylamine, *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 (Khanna et al., 1985). As a result of in silico screening, Banerjee (2006) identified *p*AA (identified as 05RB in the article) as a putative inhibitor of UbcH5a, an E2-conjugating enzyme that partners with the E3 ligase CHIP.

This work was supported in part by the National Institutes of Health National Heart, Lung, and Blood Institute [Grant HL050710].

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

<http://dx.doi.org/10.1124/dmd.111.042549>.

^S The online version of this article (available at <http://dmd.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CHIP, chromatin immunoprecipitation; CHIP, carboxyl terminus of Hsc70-interacting protein; *p*AA, *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; Joiakim 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), deoxynucleoside triphosphates, deoxyribonuclease I, protease 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 cells were obtained from the Cell Lines Resource, Karmanos Cancer Institute (Detroit, MI) and maintained as attached 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), 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 (Guo 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; Joiakim et al., 2004). Northern blot data were normalized by calculating *CYP1A1* or *CYP1A2* mRNA to 7S RNA signal strength ratios.

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 DNase 1 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 Transcription Reagents, as described by the supplier. For quantification of *CYP1A1* heterogeneous nuclear (hnRNA), PCR amplification used the *CYP1A1* forward primer 5'-TTGTGATCCCAGGCTCCAAGA-3' and the reverse primer 5'-GGAGGCACCAAAATGTTCTTT-3'. These primers amplify a 120-base pairs (bp) 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'-AGAAAAACCTGCCAAATATGATGAC-3' and the reverse PCR primer 5'-GCCAGGATGCCCTTGA-3'. This amplifies a region 849 to 927 bp downstream of the transcription start site. PCR data were analyzed using the comparative cycle threshold (Ct) method. Relative quantification was based on relative expression of *CYP1A1* normalized against GAPDH. Relative expression of the target gene was calculated as $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ was obtained by subtracting ΔC_t of untreated cells from the ΔC_t of treated cells. The ΔC_t 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 (Duniec-Dmuchowski et al., 2007), using TaqMan Gene Expression Assays Hs00153120_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 [0.01% 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.

AhR-DNA interactions were monitored using forward (5'-ACCGCCAC-CCTTCGACAGTTC-3') and reverse (5'-TGCCAGGCGTTGCGT-GAGAAG-3') primers to amplify a region from -980 to -1125 of the *CYP1A1* promoter (Matthews et al., 2005). This region contains two functional xenobiotic response elements (XREs) and yields a PCR product of 146 bp. The PCR mixture contained input or ChIP-isolated DNA, 1× PCRx 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 Prism version 4.02 for Windows (GraphPad Software Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Effects of *p*A 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 *p*A had no effect (Fig. 1A). However, depending on the concentration used, *p*A pretreatment could either suppress or enhance dioxin-induced *CYP1A1* mRNA accumulation (Fig. 1, A and B). In particular, pretreatment with concentrations of *p*A ≥ 75 μM suppressed

TCDD-induced *CYP1A1* mRNA accumulation, whereas, concentrations of *p*A ≥ 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 *p*A-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). *p*A alone had no effect on *CYP1A2* mRNA content in MCF10A cultures (A. Joiakim, unpublished data). However, pretreatment with 25 μM *p*A 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 transcriptional activation of *CYP1A1* (Elferink and Reiners, 1996). Exposure to either DMSO or 25 μM *p*A 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 *p*A, 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 *p*A on *CYP1A1* mRNA stability, we pretreated cultures with TCDD ± *p*A for 8 h before adding 5 μg/ml

Fig. 1. Effects of *p*A on *CYP1A1* mRNA accumulation. A, MCF10A cultures were treated with 1 to 200 μM *p*A for 6.5 h before harvest, or pretreated with *p*A for 1.5 h before the addition of 10 nM TCDD, and harvested 5 h later for analyses of *CYP1A1* and 7S RNA. B, cultures were treated with nothing, 10 nM TCDD, or pretreated with 1 to 100 μM *p*A for 1.5 h before the addition of TCDD. Cultures were harvested 8 h after the addition of TCDD for analyses of *CYP1A1* and 7S RNA. At any specific *p*A concentration, data are presented for two independent RNA preparations. Data in the lower panel represent means ± S.D. of three to five independent experiments in which RNAs were isolated either 5 or 8 h after TCDD addition. *, different from the *CYP1A1*/7S ratio obtained with 1 μM *p*A, $p < 0.05$. C, cultures were treated with 10 nM TCDD or pretreated with 25 μM *p*A for 1.5 h before the addition of TCDD. Cultures were subsequently harvested 3 to 18 h after TCDD addition for analyses of *CYP1A1* and 7S RNA. Data in the lower panel represent means ± S.D. of three to four independent experiments in which the *CYP1A1*/7S ratio for the TCDD only sample, at each time point, was set as one. *, greater than ratio measured at 3 h, $p < 0.05$. D, cultures were treated as in C but analyzed for *CYP1A2* and 7S RNA. Similar results were obtained in a second experiment.

FIG. 2. Effect of *pAA* on *CYP1A1* transcription. A, schematic representation of the region of *CYP1A1* hnRNA analyzed by real-time PCR. B and C, MCF10A cultures were left untreated, or treated with DMSO, 10 nM TCDD, 25 μ M *pAA*, or 10 nM TCDD + 25 μ M *pAA* for varying lengths of time before being harvested and processed for analyses of *CYP1A1* hnRNA and GAPDH mRNA. Treatment groups are defined in the figure. Data in B represent means $\pm$ S.D. of three technical replicates per treatment for a single experiment. Data in C represent means $\pm$ S.D. of three biological replicates for a single experiment. Similar results were obtained in two additional experiments. In cotreatment protocols, *pAA* was added 1.5 h before TCDD. Harvest times are relative to TCDD addition. *, greater than the corresponding TCDD group, $p < 0.05$.

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 μ M *pAA* ($p < 0.05$ for $n = 4$ independent experiments; Fig. 4A), whereas 10 μ M *pAA* was without effect (Fig. 4A). Kinetic analyses of intermediate concentrations suggested that 50 μ M *pAA* partially blocked TCDD-induced AhR degradation ($p < 0.05$ for $n = 3$ independent experiments; Fig. 4B). Although 25 μ M *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 μ M *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 μ M 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 μ M *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 μ M. 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 μ M and causes an almost complete suppression of proliferation at 50 μ M (Elliott and Reiners, 2008). Cell cycle analyses indicated that 25 μ M *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

FIG. 3. Effect of *pAA* on *CYP1A1* mRNA stability. MCF10A cultures were exposed to 10 nM TCDD or 10 nM TCDD + 25 μ M *pAA* for 8 h to induce *CYP1A1* transcription. ActD was then added to 5 μ g/ml, without medium change, to inhibit transcription. Cultures were harvested at various times after ActD addition. Treatments are noted in the figure. A, Northern blot analysis of *CYP1A1* and 7S RNAs. One of four representative experiments is shown. B, semilog graph of normalized *CYP1A1* mRNA contents. mRNA amount is expressed as a percentage of treatment at T = 0 (set as 100%). Data represent means $\pm$ S.D. of four independent experiments. Analysis of variance analyses indicated that the curves were not significantly different.

FIG. 6. TCDD-induced *CYP1A1* transcription in MCF10A cultures is cell cycle-dependent. A, MCF10A cultures were exposed to 25 μ M pAA and subsequently harvested 1.5 to 21 h later for analyses of the percentages of cells in G₁, S, and G₂/M. B, cultures were treated with 25 μ M pAA and, at varied times thereafter, were exposed to 10 nM TCDD for an additional 3 h before processing for cell cycle analyses. Each column in A and B represents analyses of 2×10^4 cells. C, cultures were treated as in B and harvested for Northern blot analyses of *CYP1A1* and 7S RNAs. The lower panel represents quantification of the Northern blot data. D, real-time RT-PCR analysis of *CYP1A1* hnRNA in the RNA preparations used in C. The data presented in A to D are from a single experiment. A second experiment that encompassed the same types of analyses as presented in B and C yielded very similar results. E, MCF10A cultures were exposed to 25 μ M pAA for 1.5, 11, or 18 h before the addition of 10 nM TCDD. Cultures were subsequently harvested 0.5, 2, or 3 h after dioxin addition for ChIP analyses of AhR occupancy of the *CYP1A1* promoter.

very cytostatic, with antiproliferative effects that persisted for at least 72 h. The cytostatic effects of 25 μ M 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 of 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 μ M 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 μ M. 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 25 μ M pAA rapidly initiated simultaneous G₁ and S phase arrest in MCF10A cultures. Arrested G₁ cells begin to transition into S phase ~ 7 to 10 h after pAA treatment and continued to accumulate in S phase for at least an additional 8 h. Thereafter, S phase cells transitioned into G₂. By analyzing the effects of adding TCDD at different times after pAA treatment, we were able to assess the responsiveness of *CYP1A1* 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 into 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

FIG. 7. TCDD-induced *CYP2S1* transcription in MCF10A cultures is cell cycle-dependent. A, MCF10A cultures were left untreated or pretreated with 25 μ M 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 set as 1. Data represent means $\pm$ 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 μ M 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 2×10^4 cells.

pAA 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* induc-

tion 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. 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 $\pm$ 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 μ M 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 2×10^4 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 $\pm$ 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-related 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 cultures reenter the cell cycle and proliferate after exposure to TCDD and several polychlorinated biphenyl and polycyclic aromatic hydrocarbon AhR agonists (Chramostová et al., 2004; Vondráček 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[2-(3,5-dichloropyridyloxy)] 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. Knowledge

of the cell cycle dependence of drug-metabolizing enzymes may be useful in the design of combinational therapeutic protocols in which one of the therapeutics is a cell cycle modulator.

Authorship Contributions

Participated in research design: Elliott, Joiakim, Kocarek, and Reiners Jr.
Conducted experiments: Elliott, Joiakim, Mathieu, Duniec-Dmuchowski, and Reiners Jr.
Contributed new reagents or analytic tools: Elliott, Joiakim, and Kocarek.
Performed data analysis: Elliott, Joiakim, Mathieu, Duniec-Dmuchowski, and Reiners Jr.
Wrote or contributed to the writing of the manuscript: Elliott and Reiners Jr.

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