Disruption of CLOCK-BMAL1 Transcriptional Activity Is Responsible for Aryl Hydrocarbon Receptor–Mediated Regulation of Period1 Gene

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Received October 15, 2009; accepted January 21, 2010

The aryl hydrocarbon receptor (AhR) is a period-aryl hydrocarbon receptor nuclear transporter-simple minded domain transcription factor that shares structural similarity with circadian clock genes and readily interacts with components of the molecular clock. Activation of AhR by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters behavioral circadian rhythms and represses the Period1 (Per1) gene in murine hematopoietic stem and progenitor cells. Per1 expression is driven by circadian locomotor activity cycles kaput-brain muscle ARNT-like (CLOCK-BMAL1)–dependent activation of Eboxes in the Per1 promoter. We hypothesized that the effects of AhR activation on the circadian clock are mediated by disruption of CLOCK-BMAL1 function and subsequent Per1 gene suppression. Effects of AhR activation on rhythmic Per1 transcripts were examined in livers of mice after treatment with the AhR agonist, TCDD; the molecular mechanisms of Per1 repression by AhR were determined in hepatoma cells using TCDD and β-naphthoflavone as AhR activators. This study reports, for the first time, that AhR activation by TCDD alters the Per1 rhythm in the mouse liver and that Per1 gene suppression depends upon the presence of AhR. Furthermore, AhR interaction with BMAL1 attenuates CLOCK-BMAL1 activity and decreases CLOCK binding at Ebox1 and Ebox3 in the Per1 promoter. Taken together, these data suggest that AhR activation represses Per1 through disrupting CLOCK-BMAL1 activity, producing dysregulation of rhythmic Per1 gene expression. These data define alteration of the Per1 rhythm as novel signaling events downstream of AhR activation. Downregulation of Per1 could contribute to metabolic disease, cancer, and other detrimental effects resulting from exposure to certain environmental pollutants.

Key Words: Per1; gene regulation; Ebox; liver; AhR activation; BMAL1; circadian rhythm.

Regulation of circadian rhythms is critical for adaptation of organisms to ever-present environmental changes (e.g., light-dark cycle) and coordination of key physiological processes such as the sleep-wake cycle, hormone secretion, and liver metabolism (Bittman et al., 2003; Canaple et al., 2006; Cermakian and Boivin, 2003; Gekakis et al., 1998; Griffin et al., 1999; Ishikawa et al., 2002; Oishi et al., 2003). Coordination of systemic circadian rhythms in mammals is directed by a central clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore and Eichler, 1972; Stephan and Zucker, 1972). Although the SCN clock operates autonomously, it remains responsive to environmental signals, in particular the light-dark cycle, which acts to precisely regulate the clock phase (Boivin et al., 2003; Matsuo et al., 2003). The SCN transmits timing information to numerous peripheral clocks throughout the organism via neural and humoral signals, assuring the synchrony of multiple physiological processes (Asai et al., 2001; Mendoza et al., 2008; Mizuyachi et al., 2002). However, peripheral clocks may function independent of the SCN under certain circumstances. For example, restricted feeding can shift the rhythmicity of liver metabolism by 10 h within 2 days. Under these same conditions, the rhythmicity of the SCN remains phase locked to the light-dark cycle despite changes in food intake (Mendoza et al., 2008). Disruption of coordinated function between central and peripheral clocks is hypothesized to promote development of diseases (Mishima et al., 2005).

Circadian rhythms are generated by a molecular clock, which has an endogenous period of ~24 h. The Period1 (Per1) gene is a fundamental component of the circadian timing system. Regulation of Per1 is important both for endogenous rhythmicity and for coupling environmental information to the molecular clock (Hida et al., 2000; Kojima et al., 2003). The Per1 promoter includes five Eboxes, four cyclic adenosine monophosphate-response elements (CRE), and a single Sp1-binding site. CLOCK-BMAL1 and phosphorylated CRE-binding protein stimulate Per1 transcription by binding the Eboxes and CRE in the Per1 promoter, respectively. Eboxes are critical for circadian expression of Per1 (Hida et al., 2000). Although CRE elements are vital for light signaling to the central clock, it is not clear whether CRE elements are...
important for circadian regulation of physiological functions (Hida et al., 2000).

Dioxins and dioxin-like compounds are ubiquitous environmental toxicants that contribute to the development of diseases such as obesity, metabolic syndrome, diabetes, and multisite carcinogenesis (Bock, 1994; Mishima et al., 2005). At the molecular level, dioxins exert their effects through persistent activation of aryl hydrocarbon receptor (AhR; Schmidt and Bradfield, 1996). In the absence of ligand, AhR is bound to heat shock protein-90 (HSP-90) and other proteins to form a cytoplasmic complex. Upon ligand binding, the AhR-HSP-90-dioxin complex translocates into the nucleus, where HSP-90 is displaced by aryl hydrocarbon receptor nuclear translocator (ARNT). AhR and ARNT form a heterodimeric transcription factor that binds and activates specific dioxin-responsive element (DRE)-containing genes (Swanson, 2002). However, this well-described signaling pathway does not account for all the effects of dioxins. Cross talk between AhR-ARNT and other nuclear receptors, their coactivators, and/or corepressors likely also contribute to dioxin-induced disease (Lucier et al., 1991).

As members of the PAS domain family of transcription factors, AhR and the clock genes, CLOCK and BMAL1, share structural similarities; therefore, interactions between AhR signaling and the circadian clock warrants investigation. AhR expression exhibits diurnal changes in multiple tissues comparable with the diurnal variation in expression of the clock genes BMAL1 and Per1 (Mukai et al., 2008; Richardson et al., 1998). AhR-mediated responses stimulated by 2,3,7,8 tetrachloro-dibenzo-p-dioxin (TCDD) treatment, such as induction of Cyp1a1 expression, are enhanced in hepatic cells when Per1 is suppressed using small interfering ribonucleic acid (Qu et al., 2009). In turn, TCDD alters circadian rhythms and the expression of clock genes including Per1 and Per2 in murine hematopoietic stem and progenitor cells (Garrett and Gasiewicz, 2006). Our previous results demonstrate that levels of Per1 are increased in the AhR null mouse liver yet substantially decreased in the TCDD-treated wild-type mouse liver (Mukai et al., 2008). Thus, we tested the hypothesis that AhR directly inhibits Per1 expression through disrupting the activity of CLOCK-BMAL1 at the Per1 promoter. Our data demonstrate that activation of AhR may disrupt CLOCK-BMAL1 activity at Per1 promoter, thus resulting in the downregulation of Per1. These findings suggest that regulation of the circadian clock is likely a novel component in the mechanism by which dioxins may result in metabolic diseases, cancers, and other circadian-related diseases.

**MATERIALS AND METHODS**

**Cell lines and growth conditions.** The mouse Hepa-1c1c7 cell line, a subclone of the Hepa-1 hepatoma cell line, and the c12 cell line derived from Hepa-1c1c7 were purchased from American Type Culture Collection. Cells were cultured in Dulbecco’s modified Eagle’s medium reduced serum (DMEM-RS; HyClone) with 7.5% bovine growth serum (BGS; HyClone) and penicillin-streptomycin-amphotericin (MP Biomedicals) at 37°C in a humidified 5% CO2 atmosphere. Cultures were treated with β-naphthoflavone (β-NF; a gift from J. Flaws, University of Illinois at Urbana-Champaign) and TCDD (in dimethyl sulfoxide (DMSO) vehicle as indicated, and control cultures were treated with equivalent concentration of DMSO.

**Animals and tissue collection.** About 6- to 12-week-old wild-type male c57Bl/6 mice (Jackson Labs) were used in these experiments. Animal procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed three to four per cage in a light-tight chamber, provided with feed and water ad libitum and entrained for at least 2 weeks under controlled lighting (12:12-h light-dark, temperature (22°C), and humidity (39%). To investigate the effects of long-term AhR activation on the Per1 rhythm, as a marker of circadian clock function, mice were orally dosed with 1 μg/kg of body weight of TCDD or vehicle (corn oil) during their lights-on period. Five days after treatment, animals were released into constant darkness for 48 h prior to initiation of tissue collection. This experimental design allows the assessment of the state of the endogenous clock in the absence of environmental light-dark cues; release into constant darkness prior to tissue collection is a standard protocol for determining endogenous clock state (Hickok and Tischkau, 2010; Mukai et al., 2008). Animals were decapitated and livers obtained at 4-h intervals starting at CT0 (circadian time, where CT0 refers to the time of lights on in the previously entrained light-dark cycle; CT12 is the corresponding time of lights off). Tissues were snap frozen and stored at −80°C until use.

**Total RNA isolation, reverse transcription, and quantitative reverse transcription–PCR.** Total cellular RNA was isolated using TRI Reagent according to the manufacturer’s protocol (Sigma). Concentration, purity, and quality of the RNA were determined using Nanodrop ND-1000 UV Spectrophotometer at 260 and 280 nm and by gel electrophoresis. For detection of gene transcripts by reverse transcription–PCR, total RNA (1 μg) was used in a 20-μl reaction according to the manufacturer’s protocol (Promega). Following complementary DNA (cDNA) synthesis, 5 μl of diluted cDNA (1:5) was used in 20 μl of SYBR green PCR mix (Bio-Rad) containing 300nM Per1-specific primer pairs as previously published (Mukai et al., 2008), and 5 μl of diluted cDNA (1:5) was used in 20 μl of SYBR green PCR mix with 1X Quantitect Primer Assay for Cyp1a1 gene expression analysis (Qiagen). Amplification was performed using a Smart Cycler rapid thermal cycler (Cepheid) according to the following protocol: an initial 10-min denaturation step at 95°C, followed by 40 cycles of denaturation (95°C) for 30 s, annealing (primer-optimized temperature) for 30 s, and extension (72°C) for 30 s. Detection of the fluorescent product was carried out during each 72°C extension period, and emission data were quantified using threshold cycle values. Relative standard curves were created as previously described (Karman and Tischkau, 2006). Resulting standard curves were used to calculate the relative amounts of Per1 and Cyp1a1. Relative amount was converted to fold or percent change compared with control values (DMSO treated) unless otherwise stated. PCR product specificity from each primer pair was confirmed using melting curve analysis and subsequent agarose gel electrophoresis.

**Preparation of cell lysates, cytosol, and nuclear lysates and immunoblot analysis.** Following treatment, Hepa-1c1c7 and c12 cells were harvested by washing with cold PBS followed by lysis in Complete Lysis-M (Roche) on ice for 30 min. Liver sections were homogenized with a bead beater using cold glass beads with 1 ml of Complete Lysis-M and shaken twice for 30 s each. The supernatant was collected after centrifuging at 14,000 × g for 5 min. Cytosol and nuclear lysates were isolated using a modified Abcam corporation protocol. In brief, 500 μl of buffer A with protease inhibitor cocktail (Roche; 10nM 4-(2-Hydroxyethyl)pipеразине-1-ethanesulfinic acid sodium salt (HEPES), 1.5mM MgCl2, 10mM KCl, 0.5mM dithiothreitol (DTT), and 0.05% (w/v) NP-40, pH 7.9) was added to 100-mm plates on ice after washing with cold PBS; cells were scraped thoroughly and left on ice for 10 min. Lysates were centrifuged at 3000 × g for 10 min at 4°C. Supernatants were removed and kept as cytosol. Pellets were washed once with buffer A without Nonidet P40. Pellets were
resuspended in 60 µl buffer B (5 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, and 26% glycerol, pH 7.9), and 15 µl of 1.5 M NaCl was added to give 300 mM NaCl. Lysates were vortexed and centrifuged at 10,000 × g for 20 min at 4 ºC. Supernatants were collected and kept as nuclear lysates. Protein concentrations were determined with BCA kit (Thermo Scientific), and 20–60 µg of protein was heated to 95 ºC for 5 min in electrophoresis loading buffer. Electrophoresis was conducted at 90 V for 30 min in the stacking gel and 130 V for 60–90 min in the resolving gel. Proteins were transferred onto a nitrocellulose membrane at 260 mA for 160 min. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST; 20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h. The membrane was then incubated overnight at 4 ºC with specific primary antibodies, AhR (Biomol), CLOCK (Santa Cruz), BMAL1 (ABR), ARNT (ABR), α-tubulin (Sigma), or β-actin (Sigma), prepared in blocking buffer (Thermo Scientific). The membrane was washed for 30 min, followed by incubation with horseradish peroxidase (HRP)–conjugated secondary antibody (Jackson ImmunoResearch) for 1 h at room temperature. Protein was visualized using the Immun-Star HRP Chemiluminescence Kit (Bio-Rad), followed by phosphor imaging (FujiFilm). To control for equal loading, monoclonal anti-α-tubulin or anti-β-actin (nuclear lysates) antibody was used.

**Co-immunoprecipitation.** Hepa-1c1c7 cells were cultured in 100-mm plates to 90–100% confluence. Cells were treated with DMSO vehicle or 10 µM β-NF for 1.5 h. Cells were lysed in Complete Lysis-M. Lysates were centrifuged at 14,000 × g for 5 min and then quantitated with BCA kit. Soluble proteins (500 µg) were incubated on a rotating platform with 2 µg of goat anti-AhR antibody (Santa Cruz) or rabbit anti-BMAL1 antibody (ABR) for 4 ºC overnight, followed by incubation with 100 µl of immobilized protein A/G gel slurry (Pierce) at room temperature for 2 h with gentle mixing. Beads were collected by centrifugation and washed five times with 0.5 ml of immunoprecipitation (IP) buffer (25 mM Tris and 150 mM NaCl, pH 7.2). The complex-bound gel was washed with 0.5 ml of water and centrifuged for 3 min at 2500 × g, the supernatant was discarded, and electrophoresis loading buffer was added and boiled for 5 min at 95 ºC. The gel mixture was centrifuged and supernatants were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h. The membrane containing protein pulled down by anti-AhR antibody was incubated with rabbit anti-BMAL1 antibody, followed by three washes in TBST and then incubation with HRP-conjugated secondary antibody (Jackson ImmunoResearch) for 1 h at room temperature. The same blot was washed three times with TBST and then incubated with rabbit anti-AhR antibody (Biomol), followed by incubation with HRP-conjugated secondary antibody (Jackson ImmunoResearch) for 1 h at room temperature. Using the same procedure, a separate blot was prepared containing protein immunoprecipitated with the anti-BMAL1 antibody and probed with goat anti-CLOCK antibody (Santa Cruz). This blot was then washed three times with TBST and reprobed with rabbit anti-BMAL1 antibody.

**Luciferase assay.** The mPer1-luciferase reporter construct (mPer-luc) cloned into pG3L-basic vector, the mCLOCK ORF cloned into pSg5 vector, and mBMAL1 cloned into pCS2-MTK vector were gifts from Dr Paolo Sassone-Corsi at the University of California at Irvine (Travnickova-Bendova et al., 2002). The hAhR cloned in pcDNA3 vector was a gift from Dr Gary Perdew at Pennsylvania State University (Ramadoss and Perdew, 2005). Cotransfection experiments were performed with 12 cells. Cells were plated in 24-well plates in DMEM/RS medium, supplemented with 7.5% bovine growth serum. Transfection experiments were performed using TurboFect (Fermentas) according to the manufacturer’s protocol. Transfection mixtures generally contained mPer1-luc (10 ng), CLOCK-BMAL1 (250 ng), and/or AhR (250 ng), and the internal control β-galactosidase (β-gal) vector (10 ng). Empty vector was used to equalize the amount of plasmid DNA for each transfection. Transfected cells were cultured for an additional 24 h, washed once with cold PBS, and resuspended in passive lysis buffer (Promega). As a reporter gene, firefly luciferase activity was initiated by mixing an aliquot of lysates (20 µl) with Luciferase Assay Reagent II (Promega). Then, β-gal activity was detected as follows: lysis buffer (150 µl) and 150 µl 2× β-gal buffer was added to 50-µl lysates. Reactions were incubated at 37 ºC until yellow color appeared and then 500 µl of 1M sodium carbonate was added to stop the reaction; optical density was measured at 420 nm. Luciferase was normalized to the β-gal signal.

**Chromatin IP assay.** The chromatin IP (ChIP) protocol was performed with minor modifications as described previously (Weinmann and Fernham, 2002). Hepa-1c1c7 cells were grown in 150-cm2 flasks to 90–100% confluence (2 × 10⁵ cells). Cells were treated with DMSO vehicle or 10 µM β-NF for 1.5 h. Cross-linking was performed by adding formaldehyde directly to the culture medium to a final concentration of 1%, followed by 10-min incubation. The reaction was quenched using glycin at a final concentration of 0.125M. Cells were rinsed twice with cold PBS, scraped from the dishes, pelleted, and washed again with PBS containing 10 mM PMSF. Cell pellets were resuspended in cell lysis buffer (5 mM piperezine-1,4-bis(2-ethanesulfonic acid), pH 8.0; 85 mM KCl; 0.5% Nonidet P-40; 10 mM PMSF; 5 µg/ml leupeptin; and 5 µg/ml aprotinin) and incubated on ice for 10 min. Cells were then ground with a Dounce homogenizer 10–20 times on ice to aid in nuclei release. Nuclei were pelleted and resuspended in nuclei lysis buffer (50 mM Tris-Cl, pH 8.1; 10 mM EDTA; 1% SDS; 0.5 mM PMSF; 5 µg/ml leupeptin; and 5 µg/ml aprotinin) and incubated on ice for 10 min. Chromatin was sonicated to an average length of 600 bp with four 10-s pulses of 30 watts, maintaining samples on ice for 30 s between pulses. Sonicated chromatin was then pre-cleared for 60 min at 4 ºC with bovine serum albumin– and salmon sperm DNA–saturated protein A/G agarose (Thermo Scientific) in preparation for IP. The supernatant was divided equally among all samples and incubated overnight on a rotating platform at 4 ºC with 2 µg of rabbit anti-AhR (Biomol), rabbit anti-CLOCK (NOVUS), or rabbit anti-BMAL1 (ABR) or mouse anti-p300 (Millipore) antibody. A protein A/G gel slurry (50 µl) was added and incubated for 2 h at room temperature to allow for antibody binding. The gel was pelleted and washed twice with 1× dialysis buffer (50 mM Tris-Cl, pH 8.0; 2 mM EDTA; and 0.2% sarkosyl) and four times with IP wash buffer (100 mM Tris-Cl, pH 9.0; 500 mM LiCl; 1% Nonidet P-40; and 1% deoxycholic acid). Immune complexes were eluted from the beads with elution buffer (100 mM NaHCO3 and 1% SDS). Reverse of cross-links was carried out by heating the eluates at 65 ºC for 4–5 h. The eluates were then digested with proteinase K at 56 ºC for 2 h. DNA was extracted with phenol-chloroform, precipitated with ethanol, and dissolved in Tris-EDTA buffer. Samples were subjected to PCR amplification with promoter–specific primer.

## RESULTS

**Circadian Profiles of Per1 in Vehicle- and TCDD-Treated Mouse Liver during the Light-Dark Cycle**

To investigate the effects of long-term AhR activation on the endogenous circadian rhythm of Per1 transcripts, as an indicator of molecular clock function, we determined the
circadian profile of Per1 messenger RNA (mRNA) in the liver of vehicle-treated and TCDD-treated mice. Per1 was characterized by a rhythm with peak expression at CT12 in vehicle-treated controls ($p < 0.001$). After TCDD exposure, Per1 peak expression occurred at CT20 and peak transcript levels were attenuated by 33.3% ($p < 0.05$; Fig. 1A). As an indicator of AhR activation, we measured Cyp1a1, a primary target of the AhR signaling. Cyp1a1 was increased up to ~1000-fold relative to vehicle controls ($p < 0.001$; Fig. 1B).

**Activation of AhR Represses Per1 Gene Expression**

The effects of AhR activation on Per1 levels were examined in cell lines that express various levels of AhR to begin to elucidate potential mechanisms for the observed alteration of the liver’s molecular clock in response to TCDD *in vivo*. Western blot results showed that basal AhR levels were very high in Hepa-1c1c7 but very low in c12 cells (Fig. 2A). Thus, these lines were used for all subsequent experiments.

**FIG. 1.** Circadian profiles of Per1 transcripts in the mouse liver during the light-dark cycle. After 2 weeks of entrainment in 12:12-h light-dark, mice were orally dosed with 1 μg/kg body weight of vehicle (corn oil) or TCDD during their lights-on period. Five days later, animals were placed in constant darkness to allow expression of their endogenous circadian rhythm. After 48 h in constant darkness, vehicle- and TCDD-treated mice were sacrificed and livers were collected every 4 h. Quantitative PCR was used to measure Per1 (A) and Cyp1a1 (B) mRNA. Results are shown as mean fold change relative to levels at CT0 ± SEM. $n = 4–6$ per group, *$p < 0.05$ TCDD-treated peak versus vehicle-treated peak; **$p < 0.001$ peak versus vehicle control.

**FIG. 2.** Activation of AhR inhibits Per1 gene expression. (A) Proteins from Hepa-1c1c7 and c12 cell lines were prepared and 30 μg of total cell lysates for each cell line was subjected to Western blot for AhR using α-tubulin as a loading control. (B) One hour after the addition of vehicle (DMSO, 0.01%), increasing concentrations (0.1, 1, and 10μM) of β-NF, or 10nM of TCDD, total RNA was harvested in Hepa-1c1c7 and c12 cells. Quantitative PCR was used to measure Per1 mRNA. (C) RNA was harvested at the various times (0, 0.5, 1, 2, and 4 h) after the addition of DMSO vehicle media or 10μM β-NF in Hepa-1c1c7 and c12 cells. (D) c12 cells were transfected by FLAG-pcDNA3 control vector or pcDNA3-FLAG-AhR vector and incubated for 24 h, and then protein and RNA was extracted. Quantitative PCR was used to measure Per1 and Cyp1a1 mRNA. Western blot was used to detect transfected FLAG-tagged protein. (E) Hepa-1c1c7 and c12 cells were treated with 10μM of β-NF for 4 h. Cell lysates were collected and Per1 protein was analyzed by Western blot using 60 μg of total cell lysates. Quantitative PCR and Western blot was used to measure Per1 mRNA and protein levels, respectively. Results are shown as mean fold change relative to the vehicle or 0-h level ± SEM. $n = 3–4$ per group, *$p < 0.05$ and **$p < 0.01$ versus the vehicle or control. Results are a representative of three to four independent experiments.
To examine the effect of AhR activation on Per1 gene expression, Hepa-lc1c7 and c12 cells were treated with vehicle, increasing concentrations of the AhR ligand β-NF for 1 h, or 10nM of TCDD for 4 h (Fig. 2B). Quantitative PCR results revealed that 10μM β-NF and 10nM TCDD treatment resulted in a 38.5 and 36.0% decrease of Per1 mRNA expression relative to vehicle in Hepa-1c1c7 (p < 0.05), respectively (Fig. 2B). Cyp1a1 transcript levels were significantly increased in the same samples, indicating that the effects of β-NF and TCDD were associated with typical signs of AhR activation (data not shown). In contrast, exposure of AhR-deficient c12 cells to either β-NF or TCDD did not alter levels of Per1 mRNA (Fig. 2B) and caused only a minor increase in Cyp1a1 (data not shown).

Subsequently, we examined the time required for AhR-dependent suppression of the Per1 transcript. Hepa-1c1c7 and c12 cells were treated with DMSO vehicle as control or 10μM of β-NF for 0, 0.5, 1, 2, and 4 h. The Per1 transcript was not significantly changed in Hepa-1c1c7 cells after vehicle control treatment but was significantly decreased at 0.5, 1, and 2 h (p < 0.01) and 4 h (p < 0.05) by β-NF. In AhR-deficient c12 cells, the Per1 transcript was strongly elevated by media change and was not significantly inhibited after β-NF exposure (Fig. 2C). Cyp1a1 levels were dramatically elevated in a time-dependent
manner in β-NF-exposed Hepa-1c1c7 cells but only slightly elevated in c12 cells in the same samples (data not shown).

To show that AhR is activated upon transfection of AhR vector, and then inhibits the Per1 transcript without the addition of exogenous AhR agonist, c12 cells were transfected with a FLAG-tagged AhR vector. Western blot results showed that c12 expressed same level of FLAG after transfection with FLAG-pcDNA3 control or pcDNA3-FLAG-AhR vector (Fig. 2D). Transfected AhR vector significantly repressed FLAG-pcDNA3 control or pcDNA3-FLAG-AhR vector. Western blot results showed that c12 cells were transfected with a FLAG-tagged AhR vector, and then inhibits the Per1 transcript without the addition of exogenous AhR agonist, c12 cells were transfected with FLAG-pcDNA3 control or pcDNA3-FLAG-AhR vector. Western blot results showed that c12 cells were transfected with a FLAG-tagged AhR vector, and then inhibits the Per1 transcript without the addition of exogenous AhR agonist, c12 cells were transfected with a FLAG-tagged AhR vector, and then inhibits the Per1 transcript without the addition of exogenous AhR agonist, c12 cells were transfected with a FLAG-tagged AhR vector.

β-NF Results in Accumulation of Endogenous AhR in the Nucleus and Produces AhR Degradation

Previous studies have demonstrated that AhR accumulates in the nucleus of several cell lines within 60–90 min of TCDD exposure (Pollenz, 2002). The effect of β-NF on AhR protein levels was evaluated by Western blot analysis of total lysates. Treatment with 10μM of β-NF for 4 h in Hepa-1c1c7 cells resulted in a marked reduction of AhR (Figs. 3A and 3B). The nuclear fraction was analyzed by Western blot to investigate the effect of β-NF on AhR accumulation inside the nucleus. AhR protein became predominantly nuclear following 0.5 h of β-NF exposure (Fig. 3C), but nuclear protein decreased dramatically during continued 4 h of β-NF exposure. These results demonstrate a correlation between the effects of β-NF on AhR accumulation in the nucleus and time-dependent inhibition of Per1 expression.

β-NF Induces AhR-BMAL1 and Decreases CLOCK-BMAL1 Heterodimer Formation

The molecular mechanism for AhR-dependent Per1 repression was investigated. CLOCK-BMAL1 heterodimers bind to Eboxes in the Per1 promoter to induce Per1. Previous studies indicate that AhR interacts with BMAL1 in vitro (Hogenesch et al., 1997). To determine if AhR can interact with CLOCK-BMAL1 in hepatoma cells, co-IP was performed. Hepa-1c1c7 cells were exposed to vehicle or β-NF (10μM) for 1.5 h. Cell lysates were collected and immunoprecipitated by specific anti-AhR or anti-BMAL1 antibody, and then, associated proteins were detected by Western blot.

AhR Transfection Represses CLOCK-BMAL1–Induced Activation of Per1 Promoter

Because activated AhR can form complexes that include BMAL1, we explored whether this interaction affects CLOCK-BMAL1–regulated Per1 promoter activity. Cotransfection

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**FIG. 3.** β-NF induces AhR translocation and degradation. (A) Hepa-1c1c7 cells were treated with vehicle (DMSO, 0.01%) or increasing concentrations (0.1, 1, and 10μM) of β-NF for 4 h. (B) and (C) Hepa-1c1c7 cells were treated with 10μM of β-NF for various times (0, 0.5, 1, 2, and 4 h). Total cell lysates and nuclear lysates were prepared as detailed in the “Materials and Methods” section. Western blot was performed using 20 μg lysates of each sample to measure AhR protein. All blots were stained with α-tubulin or β-actin (nuclear lysates) antibody as a loading control. Results are a representative of three independent experiments.

As shown in Figure 4A top two blots, BMAL1 was present in protein complexes pulled down with the AhR-specific antibody after β-NF exposure. Furthermore, the bottom two blots in Figure 4A show that CLOCK immunoreactivity was decreased in protein complexes immunoprecipitated with the BMAL1 antibody after β-NF exposure. Western blot (Fig. 4B) indicated that the amounts of CLOCK and BMAL1 proteins were not changed after β-NF (10μM) exposure for 1.5 h in Hepa-1c1c7 cells. These results clearly demonstrate that β-NF promotes the formation of complexes that include both AhR and BMAL1 in Hepa-1c1c7 cells; furthermore, β-NF decreases the amount of CLOCK-BMAL1 complexes without altering the expression of CLOCK and BMAL1 proteins. AhR also interacts with BMAL1 in TCDD-treated mouse ovaries (data not shown).
experiments were used to test the hypothesis that transfection of AhR may repress CLOCK-BMAL1 activity and thus inhibit the Per1 promoter. AhR-deficient c12 cells were cotransfected with vectors containing Per1-luc, CLOCK-BMAL1, CLOCK, BMAL1, and/or AhR. As expected, luciferase assay results show that CLOCK and BMAL1 together induced 5.6-fold increase in transcriptional activity of Per1 (p < 0.001; Fig. 5). Cotransfection of AhR protein inhibited the CLOCK-BMAL1–induced increase in transcriptional activity by ~49% (p < 0.05), whereas the addition of AhR without CLOCK and BMAL1, or transfection of either CLOCK or BMAL1 alone did not alter the basal levels of Per1 (Fig. 5).

**Activation of AhR Displaces CLOCK from the Per1 Promoter and Results in Per1 Inhibition**

The transient transfection experiment reported above suggests the possibility that AhR protein may repress Per1 gene expression by blocking CLOCK-BMAL1 heterodimer binding to Eboxes. ChIP was used to analyze the interaction between CLOCK-BMAL1, AhR, and Per1 promoter to test this hypothesis. Hepa-1c1c7 cells were treated with the vehicle DMSO or 10 μM of β-NF for 1.5 h, and native chromatin was extracted from nuclear lysates. IPs were carried out using AhR, BMAL1, and CLOCK antibodies. Controls containing no antibody were included to verify the specificity of precipitation. Figure 6 demonstrates that β-NF treatment increased AhR binding to Ebox1, Ebox2, and Ebox3 in the Per1 promoter by 127.4- (p < 0.001), 1.5- (p < 0.05), and 1.6-fold (p < 0.05), respectively. β-NF treatment reduced CLOCK binding to Ebox1 (p < 0.001) and Ebox3 (p < 0.05) of the Per1 promoter by 50% but had no significant effect on CLOCK binding to
Ebox2. BMAL1 binding to Eboxes (Ebox1, Ebox2, and Ebox3) in the Per1 promoter was unaffected by β-NF exposure (data not shown). Cyp1a1 was used as a positive control; AhR binding to the DRE in the Cyp1a1 promoter was increased in the β-NF-treated group.

**DISCUSSION**

In the present study, we report that TCDD treatment markedly decreases the peak expression levels and causes an altered circadian expression pattern of Per1 transcripts in mouse liver (Fig. 1). Thus, TCDD alters Per1 gene expression in peripheral tissues that may lead to dysfunction of peripheral circadian rhythms. We used Hepa-1c1c7 hepatoma cells, which express high levels of AhR, and their c12 derivative, which expresses low levels of AhR, to test the hypothesis that the repression of Per1 observed in vivo after exposure to dioxin is dependent on AhR. The AhR agonist, β-NF, strongly inhibits Per1 mRNA and protein in Hepa-1c1c7 but not in c12 cells (Figs. 2B and 2E). These results suggest that activation of AhR strongly inhibits Per1 gene expression, which is a key component of the molecular circadian clockworks. These data represent the first evidence of this novel mechanism of action for the AhR.

The time required for Per1 repression after treatment with AhR agonists is consistent with the proposed model of AhR activation, nuclear translocation, and degradation of AhR. Upon ligand binding, AhR translocates into the nucleus and ultimately undergoes degradation (Pollenz, 2002). Serum can increase the Per1 transcript, as indicated by use of 7.5% BGS media as control in the time course for β-NF treatment. The Per1 transcript was moderately increased in control due to the presence of serum, but β-NF treatment significantly repressed the Per1 transcript at 0.5, 1, and 2 h after treatment; Per1 transcript levels recovered partly at 4 h in Hepa-1c1c7 cells (Fig. 2C). In the AhR-deficient c12 cells, Per1 was significantly elevated by 7.5% BGS media change and was not significantly inhibited after β-NF exposure (Fig. 2C). These results suggest that Per1 can be easily induced in AhR-deficient cells and that AhR is responsible for the inhibitory effects of β-NF. Evident at 0.5 h after β-NF treatment, continuous repression of the Per1 transcript was observed until 2 h of treatment, but the repression was attenuated at 4 h of treatment. Thus, the effects of β-NF on Per1 likely result from transient nuclear accumulation followed by degradation of nuclear AhR. AhR protein rapidly increased in nuclear extracts at 0.5 h of exposure but markedly declined at 4 h of exposure (Fig. 3C), consistent with a previous study that demonstrated similar changes in AhR after treatment of cultured cells with TCDD (Pollenz, 2002). These results provide strong evidence that β-NF represses Per1 through the AhR signaling pathway.

Previous co-IP studies have reported that AhR associates with BMAL1 in vitro, but yeast two-hybrid assays did not detect a direct interaction between AhR and BMAL1 in vivo (Hogenesch et al., 1997 1998). In the present study, co-IP experiments demonstrated the presence of BMAL1 in protein complexes pulled down with an AhR-specific antibody in Hepa-1c1c7 cells after β-NF treatment (Fig. 4). AhR co-immunoprecipitated BMAL1 in the mouse ovary (data not shown) and the TCDD treatment increases this interaction. There are several possible explanations for these data. First, BMAL1 co-immunoprecipitates with AhR in vitro, indicating that the two proteins have suitable domain structures to allow such interaction. Second, BMAL1 is similar in amino acid sequence to the known AhR dimerization partner, ARNT, suggesting that interaction might be possible. Third, the lack of interaction in the
yeast two-hybrid system may not accurately reflect interactions in the mammalian system.

The AhR principal target gene Cyp1a1 was dramatically induced after transfection with AhR vector in c12 cells. Per1 was also repressed after transfection with the AhR vector in c12 cells (Fig. 2D). Ectopic expression of AhR demonstrated that AhR can inhibit CLOCK-BMAL1 transcriptional activity in c12 cells. However, transfection of AhR alone did not change basal levels of Per1-luc (Fig. 5). These results suggest that ectopically expressed AhR can be activated and can repress the Per1 transcript in the absence of an exogenous ligand, which is in accordance with the report that ectopically expressed AhR activates and localizes to the nucleus in the absence of an exogenous ligand (Chang and Puga, 1998). Together with the co-IP data, these results suggest that AhR may repress Per1 through its interaction with BMAL1 and, consequently, may disrupt activity of CLOCK-BMAL1 and/or other coactivators.

Although the classical signaling pathway attributed to AhR consists of heterodimerization with ARNT and binding to DREs in dioxin-responsive genes, more recent studies highlight alternative strategies for AhR signaling. AhR reportedly binds to E2F-dependent gene promoters through displacement of the coactivator p300 to repress S phase-specific gene expression (Marlowe et al., 2004). Similarly, alternative dimerization partners for BMAL1 have been identified. For example, the coactivator CREB binding protein can interact with BMAL1 (Takahata et al., 2000), whereas p300 can interact with CLOCK to regulate Per1 transactivation (Etchegaray et al., 2003). In this report, β-NF treatment increased AhR binding to Eboxes of the Per1 promoter and decreased CLOCK binding to Ebox1 and Ebox3 (Fig. 6). However, β-NF treatment did not change p300 binding to Eboxes (data not shown). In addition, β-NF treatment decreased CLOCK-BMAL1 interactions. These results suggest at least four possible mechanisms for the repression of Per1 by AhR. First, AhR may displace CLOCK from the CLOCK-BMAL1 heterodimer, thereby preventing binding to Ebox1 and Ebox3. Second, AhR may bind with the CLOCK-BMAL1 heterodimer at Ebox2 and disrupt its activity, without necessarily removing CLOCK from Eboxes. Third, AhR may displace other coactivators (excluding p300) from Eboxes. Fourth, AhR may also recruit a corepressor to the Ebox. Future experiments will explore the interactions of AhR with other potential coactivators and/or corepressors.

Circadian rhythms are generated by molecular “clocks” present in virtually every cell. Peripheral clocks are synchronized to each other and to solar time by a “master clock” in the SCN (Asai et al., 2001; Mendoza et al., 2008; Mizuyachi et al., 2002). Shift work, which likely acts to disrupt the clock in human populations, predisposes the affected people to numerous health risks, including increased risk for breast and prostate cancers, increased risk for cardiovascular disease, and increased incidence of type 2 diabetes (Froy, 2007; Turek et al., 2005). Furthermore, clock disruption in mouse models has suggested a link between disruption of Per genes and cancer; Per gene–deficient mice are more susceptible to cancer, developing tumors earlier and more often than wild-type mice (Fu et al., 2002; Lee, 2005); Per1, Per2, and Per3 are downregulated in human breast cancer cells, and overexpression of Per1 in human cancer cells retards growth and increases sensitivity to ionizing radiation (Chen et al., 2005; Gery et al., 2006, 2007).

Long-term follow-up studies of dioxin exposure have revealed that dioxins can contribute to the development of cancers, metabolic disease, and other related diseases (Arisawa et al., 2005; Bertazzi et al., 1998; Bock, 1994; Henriksen et al., 1997; Larsen, 2006; Mishima et al., 2005; Roybal et al., 2007; Zambon et al., 2007). Although the mechanisms behind these metabolic and tumor-generating effects of dioxins have not been elucidated, circadian clock disruption in the liver by dioxins, as suggested by the current investigation, may be important. The current report clearly shows that AhR activation represses Per1 gene expression and alters circadian expression of Per1. These findings suggest heretofore unexplored mechanisms by which environmental pollutants may contribute to the development of metabolic disease and carcinogenesis through disrupting the circadian rhythm and repression of clock genes.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

National Institute of Environmental Health Sciences grant (ES012948); American Cancer Society grant to S.A.T.

ACKNOWLEDGMENTS

The authors express their appreciation to K. Bottum and S. Karmarkar for assistance with manuscript writing.

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