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Disruption of period gene expression alters the inductive effects of dioxin on the AhR signaling pathway in the mouse liver

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Abstract

The aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (ARNT) are transcription factors that express Per-Arnt-Sim (PAS) DNA-binding motifs and mediate the metabolism of drugs and environmental toxins in the liver. Because these transcription factors interact with other PAS genes in molecular feedback loops forming the mammalian circadian clockworks, we determined whether targeted disruption or siRNA inhibition of *Per1* and *Per2* expression alters toxin-mediated regulation of the AhR signaling pathway in the mouse liver and Hepa1c1c7 hepatoma cells *in vitro*. Treatment with the prototypical AhR ligand, 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), had inductive effects on the primary targets of AhR signaling, *Cyp1A1* and *Cyp1B1*, in the liver of all animals, but genotype-based differences were evident such that the toxin-mediated induction of *Cyp1A1* expression was significantly greater (2-fold) in mice with targeted disruption of *Per1* (*Per1^{ldc}* and *Per1^{ldc}/Per2^{ldc}*). *In vitro* experiments yielded similar results demonstrating that siRNA inhibition of *Per1* significantly increases the TCDD-induced expression of *Cyp1A1* and *Cyp1B1* in Hepa1c1c7 cells. *Per2* inhibition in siRNA-infected Hepa1c1c7 cells had the opposite effect and significantly decreased both the induction of these *p450* genes as well as *AhR* and *Arnt* expression in response to TCDD treatment. These findings suggest that *Per1* may play a distinctive role in modulating AhR-regulated responses to TCDD in the liver.

Keywords

Clock genes; Period1; Period2; PAS genes; Circadian rhythms; Hepa1c1c7; Xenobiotics; Polycyclic aromatic hydrocarbons; Aryl hydrocarbon receptor; AhR nuclear translocator; Cytochrome p450 enzymes; siRNA

Introduction

In vertebrate species, the liver is the primary center for detoxifying ingested xenobiotics such as drugs and environmental contaminants. Among xenobiotics metabolized by the liver, polycyclic aromatic hydrocarbons (PAHs) are especially toxic. PAHs are ubiquitous environmental toxins found in grilled foods, vehicle exhaust, asphalt, hazardous waste sites and smoke from cigarettes, residential wood burning and waste incinerators. The oxidation of PAHs and other xenobiotics in the liver often results in the generation of reactive metabolites that can produce hepatotoxicity and other deleterious effects including immunosuppression, teratogenesis, and carcinogenesis.

The aryl hydrocarbon receptor (*AhR*) and AhR nuclear translocator (*Arnt*) are critical components of the signaling pathway responsible for the mutagenic effects of PAHs in the liver. Activation of this signaling pathway occurs when PAHs enter the cell and bind with AhR, which induces the dissociation of its complex with 90 kD heat shock proteins (*Hsp90*) and the aryl hydrocarbon receptor interacting protein (*Aip*) (Mimura and Fujii-Kuriyama, 2003). PAH binding also triggers nuclear translocation of AhR and its association with ARNT. In the nucleus, AhR:ARNT heterodimers induce transcriptional activation of xenobiotic metabolizing enzymes by binding to xenobiotic response elements (XRE) in their promoter regions. The primary targets of PAH-induced AhR signaling are cytochrome *p450* enzymes of the A and B subfamily, including *Cyp1a1*, *Cyp1a2* and *Cyp1b1*. Induction of cytochrome *p450*s triggers oxidation of PAHs and unless rendered less reactive or suitable for excretion through conjugation by phase II detoxifying enzymes, the resulting metabolites can form DNA adducts leading to mutations and increased cancer risk.

AhR and *Arnt* are members of a structurally related family of transcription factors that characteristically contain multi-functional Per-Arnt-Sim (PAS) domains in the N-terminal region. Similar to AhR and ARNT function in regulating xenobiotic responses, other PAS proteins form heterodimeric pairs consisting of a sensor protein complexed with a general binding partner and through these interactions mediate biological responses to environmental conditions. For example, the PAS genes, circadian locomotor output cycles kaput (*Clock*), brain, muscle ARNT-like protein 1 (*Bmal1*), Period 1 (*Per1*) and *Per2*, form interacting transcription-translation feedback loops that comprise the circadian timekeeping mechanism in mammals. PER1 and PER2 partner with the protein products of the Cryptochrome (*Cry*) genes, and following a delay, these heterodimeric complexes are translocated to the nucleus (Kume et al., 1999; Yagita et al., 2000). CRY proteins then act as potent inhibitors of *Clock* and *Bmal1* transcription (Griffin et al., 1999). In turn, rhythmic increases in *Bmal1* transcription and the formation of CLOCK:BMAL1 heterodimers drive the rhythmic transcription of *Per* and *Cry* genes at the beginning of each day via the activation of E-box elements (Gekakis et al., 1998; Hogenesch et al., 1998; Jin et al., 1999) so as to reset the clock and start the cycle anew. CLOCK:BMAL1 complexes also mediate the regulation of clock-controlled outputs that provide for the rhythmic programming of downstream processes.

The multi-functional properties of the PAS domain in mediating ligand and DNA binding as well as interactions between PAS and non-PAS proteins have important implications for intercommunication between different PAS protein-regulated pathways through a variety of mechanisms including competition for binding partners (Woods and Whitelaw, 2002), functional interference (Moffett et al., 1997), direct interaction (Hogenesch et al., 1998), and transcriptional regulation (Chilov et al., 2001). Recent studies suggest that PAS genes in the circadian clockworks may interact with the AhR signaling pathway and modulate its function in the regulation of toxin metabolism. The results of these studies indicate that: 1) *Drosophila* PER forms dimers with AhR and ARNT via the PAS domain, and this interaction with PER interferes with the DNA binding activity of AhR: ARNT heterodimers (Lindebro et

al., 1995); 2) BMAL1 associates with AhR (Hogenesch et al., 1997); and 3) *Per1* is involved in modulating responses of the AhR signaling pathway to its prototypical AhR agonist, 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) (Qu et al., 2007). To further examine clock gene function in AhR-regulated toxin metabolism, experiments were conducted to determine whether the TCDD-mediated regulation of cytochrome *p450s* and other components of the AhR signaling pathway is altered in mice with targeted disruption of *Per1* and/or *Per2* and in Hepa1c1c7 hepatocyte cultures infected with small interfering RNA (siRNA) constructs against these genes. Our results demonstrate that TCDD-induced *p450* expression is potentiated in the liver of mice with targeted disruption of *Per1* and in *Per1* siRNA-infected hepatocytes *in vitro*.

Methods

Animals

Experimental subjects were female wild type (WT) 129/sv mice ($N=38$) purchased from Charles River (Wilmington, MA) and *Per1^{ldc}*, *Per2^{ldc}*, and *Per1^{ldc}/Per2^{ldc}* mutant mice ($N=18$ for each genotype) derived from breeding pairs generously provided by Dr. David Weaver (University of Massachusetts Medical School, Worcester, MA). Establishment, characterization and behavioral analysis of these transgenic mice have been described previously (Bae et al., 2001). Animals were maintained in the vivarium at Texas A&M University System Health Science Center under a standard 12 h light:12 h dark cycle (LD 12:12; lights-on at 0600 h) with access to food and water *ad libitum*. Procedures used in this study were approved by the University Laboratory Animal Care Committee at Texas A&M University.

Experiment 1: Effects of targeted disruption of *Per1*, *Per2*, and *Per1/Per2* on TCDD-induced responses of the AhR Signaling pathway in the mouse liver *in vivo*

Responses of the AhR signaling pathway were examined in 8-week-old female mice treated with TCDD (provided by Dr. Stephen Safe, Texas A&M University School of Veterinary Medicine, College Station, TX) at a dose of 10 µg/kg body weight. TCDD treatment was based on previous studies showing that hepatic *Cyp1A1* expression in mice is significantly induced 24 h after a single injection of TCDD at 5 µg/kg or higher (Narasimhan et al., 1994). In the current study, animals received an intraperitoneal injection of vehicle (corn oil) or TCDD about 6 h after lights-on in the LD 12:12 photoperiod (1200 h; Zeitgeber Time [ZT] 6). Twenty-four hours after the treatment, animals were euthanized by cervical dislocation at 1200 h and liver tissues were collected in RNA Stabilization Reagent (RNAlater, QIAGEN, Valencia, CA) for subsequent extraction of total RNA. For each tissue sample, approximately 30 mg of liver tissue was homogenized and processed for extraction of total cellular RNA using the RNeasy Mini Kit (QIAGEN). The final RNA pellet was subjected to on-column DNase digestion (QIAGEN), suspended in 100 µl RNase-free water, and then stored at -80 °C.

Experiment 2: Effects of siRNA inhibition of *Per1* or *Per2* expression on TCDD-induced responses of the AhR signaling pathway in Hepa1c1c7 cells

Murine Hepa1c1c7 hepatoma cells (provided by Dr. Yanan Tian, Texas A&M University School of Veterinary Medicine, College Station, TX) were cultured on 6-well-plates in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine (Invitrogen) and 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% CO₂. Hairpin siRNA-encoding oligonucleotide sequences targeting the mouse *Per1* or *Per2* genes and a negative control siRNA were designed by Applied Biosystems/Ambion (Austin, TX) using the Cenix algorithm and pSilencer insert design tool. The complementary strand sequences of these siRNA oligonucleotides are listed in Table 1. BLAST searches were performed on all oligonucleotides to avoid effects on nonspecific

targets. Oligonucleotides encoding these sequences were synthesized at Integrated DNA Technologies (Coralville, IA) and cloned into the pSilencer 5.1-U6 Retro Vector (Applied Biosystems/Ambion). Retrovirus production was accomplished using HEK-293T cells (with permission from Dr. Gary Nolan, Stanford University, Stanford, CA) transfected with vectors containing hairpin siRNA or scrambled control-encoding oligonucleotides (siCON). Transductions were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. Twenty-four hours after transduction, the medium was replaced and HEK-293T cells were incubated in normal DMEM and supplements for 48 h. Virus-conditioned medium from HEK-293T cultures was collected, filtered (0.45 μ m pore) to remove dissociated cells, frozen in liquid nitrogen, and stored at -80°C . At 50–70% confluency, Hepa1c1c7 cells were successively incubated for 16 and 24 h at 32°C with fresh virus-containing medium supplemented with 4 $\mu\text{g/ml}$ polybrene (Sigma-Aldrich, St. Louis, MO). Stably infected cells were selected by treatment with 4 $\mu\text{g/ml}$ puromycin (Sigma-Aldrich) for 2–3 days. Puromycin selection was performed in parallel on mock-infected cultures of Hepa1c1c7 cells. The infection efficiency of retroviral constructs was assessed by light microscopy and ranged from 60% to 90% in Hepa1c1c7 cells. Cultures of siCON- and siRNA-infected Hepa1c1c7 cells were established on 6-well plates and treated at confluence with vehicle ($N=3$; dimethyl sulfoxide [DMSO], Sigma-Aldrich) or 10 nM TCDD ($N=3$) for 24 h. After treatment, cells were removed by trypsinization at 37°C for 5 min and total RNA was extracted using RNeasy mini kit (QIAGEN). Effects of siRNA constructs on *Per1* and *Per2* expression were determined by quantitative PCR (qt-PCR) analysis of mRNA abundance and by Western blot analysis of protein levels.

Western blot analysis

siRNA inhibition of PER1 and PER2 protein levels in Hepa1c1c7 cells was assessed by Western blot analysis using the XCell SureLock™ Mini-cell and Novex Western Transfer Apparatus (Invitrogen). Total cellular protein was extracted from individual cultures of siCON- and siRNA-infected Hepa1c1c7 cells using mammalian protein extraction reagent (M-PER; Pierce Biotechnology, Rockford, IL) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and a serine protease inhibitor, phenylmethylsulphonyl-fluoride (PMSF; Sigma-Aldrich). Sample protein content was determined using the bicinchoninic acid method (BCA Protein Assay Reagent Kit; Pierce Biotechnology). The samples were boiled in 1% sodium dodecyl sulfate (SDS) sample buffer and loaded at 25 μg protein per lane onto 7.5% Tris–Tricine gels. Following separation at 25 mA for about 2 h, proteins were transferred onto 0.45 μm nitrocellulose membranes (Invitrogen) and blocked at room temperature for 1 h with 5% non-fat dried milk in Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl)-Tween (0.1%). With interceding rinses in TBS-Tween, membranes were probed overnight at 4°C with rabbit anti-PER1 (2 $\mu\text{g/ml}$; Alpha Diagnostic International, San Antonio, Texas), chicken anti-PER2 (1 $\mu\text{g/ml}$; Millipore, Temecula, CA) or monoclonal mouse anti- β -actin (1:5000; Sigma-Aldrich) followed by a 1-hour incubation at 22°C with HRP-conjugated donkey anti-rabbit IgG for PER1, rabbit anti-chicken IgY for PER2 or goat anti-mouse IgG for β -actin (all at 1:10,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Immunoreactive signal for PER1, PER2 or β -actin was generated using enhanced chemiluminescence (ECL) reagent (Pierce Biotechnology) and detected on film (Biomax; Kodak, Rochester, NY). Optical density measurements for size-appropriate bands were obtained with ImageJ software (National Institute of Health, USA). To control for differences in protein content between samples, densitometric measurements for PER1 and PER2 were normalized to the values for β -actin in each sample.

Quantitative real-time PCR analysis

Relative quantification of mRNA abundance for genes in the AhR signaling pathway and for siRNA inhibition of *Per* gene expression was performed using SYBR-Green real-time PCR

technology (Applied Biosystems, Inc. [ABI], Foster City, CA) as described previously (Metz et al., 2006). Total RNA (1 µg) was reverse transcribed using Superscript II (Invitrogen) and random hexamers. For each sample, the cDNA equivalent to 1.25 ng total RNA per 12.5 µl reaction was amplified in an ABI 7500 Fast Real-time PCR System using 9600 emulation modes. To control for differences in sample RNA content, cyclophilin A (CypA) or β -actin was amplified from the same samples. Primer sequences listed in Table 2 were designed for PCR amplification of target and control genes using PrimerExpress software (ABI).

The comparative C_T method was used to calculate the relative mRNA abundance for a given target gene. Using this method, the amount of target gene mRNA in each sample was normalized first to corresponding *CypA* or β -actin mRNA levels, and then relative to a calibrator consisting of pooled cDNA from multiple samples that was analyzed on each reaction plate.

Statistical analysis

The raw data were first examined using two-way analyses of variance (ANOVAs) with treatment (vehicle vs. TCDD) and genotype (WT, *Per1^{ldc}*, *Per2^{ldc}*, and *Per1^{ldc}/Per2^{ldc}*) in Experiment 1 or siRNA construct (siCON, siPer1, or siPer2) in Experiment 2 as two independent variables. If significant main effects of treatment were identified, planned comparisons using independent *t*-tests were applied to compare gene expression between vehicle and TCDD treatment groups within the same genotype (Experiment 1) or among cells infected with the same siRNA construct (Experiment 2). Differences in expression of a given gene were evaluated separately within the control and TCDD groups using one-way ANOVA and, if required, Fisher's least significant difference (LSD) post hoc analyses to determine whether genotype (Experiment 1) or siRNA inhibition of *Per1* or *Per2* (Experiment 2) had a significant effect on basal levels and TCDD-induced changes in mRNA abundance. The α value was set at 0.05 for all statistical analyses.

Results

Experiment 1

Similar to previous analysis of hepatic responses to TCDD (Narasimhan et al., 1994), *Cyp1A1* mRNA levels in the liver were consistently low in all vehicle-treated WT, *Per1^{ldc}*, *Per2^{ldc}* and *Per1^{ldc}/Per2^{ldc}* mice but were significantly increased ($p < 0.05$) in all TCDD-treated groups (Fig. 1A). However, genotype-based differences in hepatic *Cyp1A1* expression were evident within both the vehicle and TCDD treatment groups. Among vehicle-treated animals, the basal levels of *Cyp1A1* mRNA in the liver were significantly ($p < 0.05$) higher in *Per1^{ldc}* mutant mice than in WT animals. Following TCDD treatment, hepatic *Cyp1A1* expression in *Per1^{ldc}* and *Per1^{ldc}/Per2^{ldc}* mutant mice was significantly ($p < 0.05$) and about 2 times higher than that found in WT animals. Hepatic expression of another *p450* gene, *Cyp1B1*, was similarly induced by TCDD treatment. In all WT and mutant mice, TCDD had significant effect in increasing *Cyp1B1* mRNA abundance in the liver ($p < 0.05$) relative to the basal levels observed in vehicle controls (Fig. 1B). No genotype-based differences were observed in either the basal or TCDD-induced *Cyp1B1* expression in the liver. For both *AhR* and *Arnt*, mRNA levels in the liver were comparable among all vehicle-treated WT and mutant mice (Figs. 1C–D). Moreover, there was no significant effect of either TCDD or genotype on *AhR* and *Arnt* mRNA expression in the liver.

Experiment 2

The effects of siRNA inhibition on *Per1* and *Per2* expression in Hepa1c1c7 cells were first validated by qt-PCR analysis of mRNA abundance. Infection with siRNA constructs targeting *Per1* or *Per2* had a significant effect ($p < 0.05$) in inhibiting expression of these clock genes in

Hepa1c1c7 cells such that *Per1* and *Per2* mRNA levels were respectively decreased by 64–85% and 54–63% relative to the levels found in siCON-treated cells (Fig. 2A). Western analysis revealed that these siRNA-mediated decreases in *Per1* and *Per2* mRNA expression in Hepa1c1c7 cultures were associated with a corresponding inhibition of their protein products. Infection with *Per1* or *Per2* siRNA constructs produced significant reductions ($p<0.05$) in PER1 and PER2 content in Hepa1c1c7 cells such that levels of these proteins were respectively decreased by 32–40% and 74–88% in comparison with those found in siCON-infected cultures (Fig. 2B). Importantly, β -actin protein levels in Hepa1c1c7 cultures infected with *Per1* and *Per2* siRNA constructs were similar to those observed in siCON-treated cells.

Basal levels of *Cyp1A1* expression were observed in all vehicle-treated Hepa1c1c7 cultures with no major differences among cells infected with siCON or siRNA constructs for a given gene (Fig. 3A). TCDD treatment had an inductive effect on *Cyp1A1* expression in both siCON- and siRNA-infected Hepa1c1c7 cells and consistently produced significant increases ($p<0.05$) in *Cyp1A1* mRNA levels relative to those observed in control cultures. However, differences in TCDD-induced *Cyp1A1* expression were apparent among cultures subjected to siRNA inhibition of *Per1* or *Per2*. Consistent with the preceding analysis of mouse liver *in vivo*, TCDD-mediated *Cyp1A1* induction in both groups of *Per1* siRNA-infected Hepa1c1c7 cells was significantly greater ($p<0.05$) than that in cultures infected with siCON constructs. In contrast to the effect of *Per1* inhibition, TCDD-induced *Cyp1A1* expression was significantly lower ($p<0.05$) in *Per2* siRNA-infected Hepa1c1c7 cultures than in siCON-infected cells. The TCDD-mediated induction of *Cyp1A1* expression within the *Per1* and *Per2* siRNA-infected groups was respectively increased by about 30% and decreased by 30–40% relative to controls.

Cyp1B1 expression was relatively low but variable among vehicle-treated Hepa1c1c7 cultures (Fig. 3B). Comparison of vehicle-treated groups revealed that *Cyp1B1* mRNA levels tended to be higher in *Per1* siRNA-infected Hepa1c1c7 cells than in siCON-infected cultures. Furthermore, *Cyp1B1* expression in *Per2* siRNA-infected cells was significantly lower ($p<0.05$) than the basal levels observed in siCON infected cultures. TCDD-mediated induction of *Cyp1B1* expression in Hepa1c1c7 cells followed the same general pattern as *Cyp1A1*. Relative to vehicle treatment, TCDD had a significant effect ($p<0.05$) in inducing *Cyp1B1* expression in both siCON- and siRNA-infected Hepa1c1c7 cultures. This TCDD-induced *Cyp1B1* expression in siRNA-infected Hepa1c1c7 cells was differentially altered by the inhibition of *Per1* or *Per2* expression. Following TCDD treatment, *Cyp1B1* mRNA levels were significantly higher ($p<0.05$) in Hepa1c1c7 cells infected with the *Per1a* siRNA construct but were significantly lower ($p<0.05$) in *Per2* siRNA-infected cultures than those observed in siCON-infected controls. TCDD-induced *Cyp1B1* expression was increased by about 100% in *Per1a* siRNA-infected cells and decreased by about 60% in both *Per2* siRNA-infected groups relative to controls.

The expression and TCDD-mediated regulation of *AhR* and *Arnt* in Hepa1c1c7 cells were also differentially affected by siRNA inhibition of *Per1* or *Per2*. Among vehicle-treated groups, *AhR* and *Arnt* expression were comparable in all siCON- and *Per1* siRNA-infected cultures but were altered by siRNA inhibition of *Per2* (Figs. 3C–D). Following vehicle treatment, *AhR* mRNA abundance in *Per2* siRNA-infected Hepa1c1c7 cells and *Arnt* expression in cultures infected with the *Per2a* siRNA construct were significantly lower ($p<0.05$) than those found in siCON-infected controls. Similar to the vehicle-treated groups, *AhR* and *Arnt* expression in TCDD-treated Hepa1c1c7 cells were not affected by siRNA inhibition of *Per1* but were significantly decreased ($p<0.05$) in *Per2* siRNA-infected cultures such that mRNA levels of these genes were respectively reduced by 53–58% and by 33–64% relative to those observed in siCON-infected cells.

Discussion

The impact of the circadian clock on drug and toxin metabolism has been repeatedly observed in studies demonstrating that the toxicity and efficacy of drugs varies depending on the time of administration (Levi and Schibler, 2007). This diurnal variation in the responses to drug treatment is consistent with evidence indicating that the functional activity of many drug metabolizing enzymes is rhythmically regulated by the circadian clock. For example, the activity of the hepatic P450 monooxygenase varies between day and night, and this diurnal fluctuation is abolished in rats with complete lesions of the master circadian clock in the suprachiasmatic nucleus (SCN) (Furukawa et al., 1999). In addition, known components of clock output pathways with PAR basic leucine zipper (PAR bZip) domains have been shown to control the expression of cytochrome *p450*s and other drug metabolizing enzymes (Gachon et al., 2006). Despite the wealth of descriptive observations on the rhythmic variation in the effects of drugs or toxins and the activity of metabolizing enzymes, the present study provides the first indication that specific molecular components of the circadian clockworks are involved in regulating toxin response pathways in the liver. Our results demonstrate that the targeted disruption or siRNA inhibition of *Per* gene expression alters AhR-mediated responses to TCDD in the mouse liver and Hepa1c1c7 hepatoma cells *in vitro*. The inductive effects of TCDD on hepatic expression of the cytochrome *p450* genes, *Cyp1A1* and *Cyp1B1*, were potentiated in *Per1^{ldc}* and *Per1^{ldc}/Per2^{ldc}*, but not in *Per2^{ldc}* mice. *Per1* involvement in the regulation of the AhR-mediated toxin metabolism was similarly evident in *in vitro* experiments demonstrating that siRNA inhibition of *Per1* in Hepa1c1c7 cells amplifies the TCDD-induced expression of these *p450* genes. It is interesting that siRNA inhibition of *Per2* had the opposite effect, producing decreases in the TCDD-mediated induction of *Cyp1A1* and *Cyp1B1* expression. The observed difference in the effects of *Per1* and *Per2* inhibition on responses of the AhR signaling pathway to TCDD is compatible with previous evidence for functional distinctions between these clock genes. In the circadian clockworks, the *Per1* and *Per2* genes are essential for normal timekeeping function but regulate different molecular processes (Zheng et al., 1999; Shearman et al., 2000; Bae et al., 2001). The targeted disruption of *Per2*, but not *Per1*, has been shown to abolish SCN rhythms in the expression of other clock genes (Bae et al., 2001). *Per1* and *Per2* are also marked by functional differences in the regulation of non-clock processes because sensitization to cocaine is abolished in *Per1* knockout mice but is enhanced in *Per2* mutant mice (Abarca et al., 2002). The collective implications of the present study are that in relation to its clock gene homolog, *Per1* plays a predominant and distinct role as a negative or inhibitory factor regulating TCDD-mediated activation of the AhR signaling pathway in the liver.

Although the present observations are indicative of a functional link between the *Per* genes and the AhR signaling pathway, the mechanism by which *Per1*, *Per2*, or other clock genes modulate responses of this pathway to TCDD is unknown. Because steroid hormones influence activation of the AhR signaling pathway *in vivo* (Gorski et al., 1988; Christou et al., 1995; Prough et al., 1996) and *Per1*-deficient mice are distinguished by alterations in steroid hormone levels and cycles (Dallmann et al., 2006), *Per*-mediated hormonal disturbances may be responsible for the potentiation of AhR-mediated responses to TCDD in *Per1^{ldc}* and *Per1^{ldc}/Per2^{ldc}* mice. However, our *in vitro* observations are incompatible with this possibility because siRNA inhibition of *Per1* produced comparable changes in TCDD-mediated induction of *Cyp1A1* and *Cyp1B1* in Hepa1c1c7 cultures despite the absence of glucocorticoids or other steroids that characterize the hormonal milieu *in vivo*.

Another potential explanation is that the *Per* genes may modulate hepatic responses to TCDD via interaction(s) with specific components of the AhR signaling pathway. In this regard, the *Per* genes may influence TCDD-mediated activation of the AhR signaling pathway in the liver by altering receptor levels. Current evidence for the effects of *Per2* siRNA inhibition in

decreasing *AhR* mRNA levels and TCDD-induced *p450* expression in Hepa1c1c7 cultures raises the possibility that *Per2* function in toxin response pathways may occur via its modulation of receptor levels. *Per1* may also have an important impact on AhR expression, perhaps by affecting the degradation process. Although its role in AhR degradation has not been corroborated, *Per1* function in regulating the stability of other PAS genes is supported by the finding that targeted disruption of *Per1* decreases the light-induced degradation of PER2 in the mouse SCN (Masubuchi et al., 2005). Similar to the functional significance of their interactions with other PAS proteins that form circadian clock mechanism (Bae et al., 2001), PER1 and PER2 may interact with AhR and ARNT and modulate toxin responses by affecting their dimerization or the binding of AhR:ARNT heterodimers with XREs. Interactions of the *Per* proteins with AhR and ARNT have not been documented in the liver, but *Drosophila* PER has been shown to form dimers with mammalian AhR and ARNT via the PAS domain and to impede the DNA binding activity of AhR:ARNT complexes (Lindebro et al., 1995). Other post-transcriptional events such as the stability and decay of target gene mRNAs may be important in the *Per*-mediated modulation of the AhR signaling pathway and its activation by TCDD. Although *Per1*, *Per2*, or other core components of the clock mechanism have not been directly linked to the regulation of mRNA stability, the clock-controlled gene, *Nocturnin*, encodes a poly(A)-specific ribonuclease that regulates mRNA decay and/or translational silencing (Baggs and Green, 2003; Oishi et al., 2003; Wang et al., 2001).

In summary, our findings suggest that regulation of the clock genes, *Per1* and *Per2*, may have an important impact on xenobiotic responses in the liver. Based on *in vivo* and *in vitro* observations, *Per1* appears to function as an inhibitory factor in AhR-mediated regulation of hepatic responses to TCDD. *Per2* may have the opposite effect, albeit only *in vitro*, and act as a positive regulator of TCDD activation of AhR signaling. These functional interactions between the *Per* genes and the AhR signaling pathway may also have implications for the biological consequences of xenobiotic exposure. PAHs are capable of inducing tumorigenesis and AhR is centrally involved in mediating their carcinogenic effects (Shimizu et al., 2000). In conjunction with evidence for the altered regulation of cell cycle genes and increased tumor development in *Per2*-deficient mice suggesting that this clock gene may function as a tumor suppressor (Fu et al., 2002; Lee, 2006), the present observations raise the possibility that the *Per* genes via their modulatory influence on AhR-regulated responses to toxins may represent important factors in the cancer risk associated with xenobiotic exposure.

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Fig. 1. Effects of targeted mutations of *Per1* (*Per1^{ldc}*), *Per2* (*Per2^{ldc}*), and *Per1/Per2* (*Per1^{ldc}/Per2^{ldc}*) on the expression and TCDD-induced responses of *p450* genes in the mouse liver. The relative abundance of *Cyp1A1* (A), *Cyp1B1* (B), *AhR* (C), and *Arnt* (D) mRNA was analyzed in the liver of WT, *Per1^{ldc}*, *Per2^{ldc}*, and *Per1^{ldc}/Per2^{ldc}* mice following treatment with vehicle (oil) or TCDD. Data are expressed as the mean ($\pm$ SEM) for each experimental group. The plotted values for the relative mRNA abundance correspond to the ratios of species-specific *Cyp1A1*, *Cyp1B1*, *AhR*, or *Arnt/CypA* mRNA signal that were adjusted in relation to the average for TCDD-treated WT mice, which was arbitrarily set at 100. Symbols denote genotype comparisons within each treatment group in which *Cyp1A1* mRNA levels in the liver following treatment with vehicle (+) or TCDD (*) were significantly greater ($p < 0.05$) in mutant mice than in WT animals.

Fig. 2.

Effects of siRNA inhibition of *Per1* (top panels) and *Per2* (bottom panels) on the expression and TCDD-induced responses of *p450* genes in Hepa1c1c7 cells. (A) Real-time PCR determinations of *Per1* and *Per2* mRNA abundance in Hepa1c1c7 cells infected with siCON and siRNA constructs. The plotted values for the relative mRNA abundance (mean±SEM) correspond to the ratios of species-specific *Per1* or *Per2*/β-actin mRNA signal that were adjusted in relation to the average for the siCON-infected cells, which was arbitrarily set at 100. (B) Representative Western blot results and densitometric analysis of PER1 or PER2 protein levels in siCON- and siRNA-treated Hepa1c1c7 cells. The optical density of PER1 and PER2 immunoreactive signal in Hepa1c1c7 cultures ($n=3$) infected with siCON or siRNA

constructs was determined and compared to that for β -actin. The plotted values represent the relative optical density (mean $\pm$ SEM) and correspond to the ratios of PER1 or PER2/ β -actin immunoreactive signal that were adjusted in relation to the average for the siCON-infected cells, which was arbitrarily set at 100. Asterisks denote values for relative mRNA abundance or protein levels in siRNA-infected cells that were significantly decreased ($p<0.05$) in comparison with those observed in siCON-infected cells.

Fig. 3.

Effects of siRNA inhibition of *Per1* or *Per2* on the expression and TCDD-induced responses of *p450* genes and primary components of the AhR signaling pathway in Hepa1c7 cells. *Cyp1A1* (A), *Cyp1B1* (B), *AhR* (C) and *Arnt* (D) mRNA abundance were analyzed in siCON- or siRNA-infected cells following treatment with vehicle (DMSO) or TCDD. The plotted values represent the relative mRNA abundance (mean±SEM) and correspond to the ratios of *Cyp1A1*, *Cyp1B1*, *AhR* or *Arnt*/β-actin mRNA signal that were adjusted in relation to the average for TCDD-treated siCON cells, which was arbitrarily set at 100. Symbols denote comparisons of DMSO control (+) and TCDD-treated (*) cultures in which *Cyp1A1*,

Cyp1B1, *AhR* or *Arnt* mRNA abundance in siRNA-infected cells was significantly different ($p<0.05$) from that found in siCON-infected cells.

Table 1

Oligonucleotide sequences for siRNAs

Gene	siRNA sequences	
<i>Per1</i>	<i>siPer1a</i>	Sense: 5'-GCAUAUCACAUCCGAAUACTT-3'
		Anti-sense: 5'-GUAUUCGGAUGUGAUAUGCTC-3'
	<i>siPer1b</i>	Sense: 5'-GCUCUUCAUUGAAUCUCGGTT-3'
		Anti-sense: 5'-CCGAGAUUCAAGAAGAGCTG-3'
<i>Per2</i>	<i>siPer1a</i>	Sense: 5'-CGGGUGUCCUAAGACAUUCTT-3'
		Anti-sense: 5'-GAAUGUCUUAGGACACCCGTG-3'
	<i>siPer1b</i>	Sense: 5'-GGAAGAUUCUUUCAUCAUTT-3'
		Anti-sense: 5'-AUGAUGAAAGAUUCUUCCTG-3'

Table 2

Quantitative RT-PCR Primers

Gene	Primer sequences
<i>Cyp1A1</i>	Forward: 5'-CCTCTTTGGAGCTGGGTTT-3' Reverse: 5'-AGGCTCCACGAGATAGCAGT-3'
<i>Cyp1B1</i>	Forward: 5'-TCTTTACCAGATACCCGGATG-3' Reverse: 5'-CACAACCTGGTCCAACCTCAG-3'
<i>AhR</i>	Forward: 5'-CAAATCAGAGACTGGCAGGA-3' Reverse: 5'-AGAAGACCAAGGCATCTGCT-3'
<i>Arnt</i>	Forward: 5'-GCCAGCCTGAGGTCTTTCAA-3' Reverse: 5'-AATCTTCATTGTGTAGGTGTTGCT-3'
<i>Cyp A</i>	Forward: 5'-TGTGCCAGGGTGGTGACTT-3' Reverse: 5'-TCAAATTTCTCTCCGTAGATGGACTT-3'
<i>β-actin</i>	Forward: 5'-CTTCCTTCTTGGGTATGGAATC-3' Reverse: 5'-ACGGATGTCAACGTCACACT-3'
<i>Per1</i>	Forward: 5'-AAACCTCTGGCTGTTCTACCA-3' Reverse: 5'-AATGTTGCAGCTCTCCAAATACC-3'
<i>Per2</i>	Forward: 5'-ATGCTCGCCATCCACAAGA-3' Reverse: 5'-GCGGAATCGAATGGGAGAAT-3'