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THE CLOCK GENES PERIOD 1 AND PERIOD 2 MEDIATE DIURNAL RHYTHMS IN DIOXIN-INDUCED *CYP1A1* EXPRESSION IN THE MOUSE MAMMARY GLAND AND LIVER

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Abstract

Transcription factors expressing Per-Arnt-Sim (PAS) domains are key components of the mammalian circadian clockworks found in most cells and tissues. Because these transcription factors interact with other PAS genes mediating xenobiotic metabolism and because toxin responses are often marked by daily variation, we determined whether the toxin-mediated activation of the signaling pathway involving several PAS genes, the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (ARNT), fluctuates rhythmically and whether this diurnal oscillation is affected by targeted disruption of key PAS genes in the circadian clockworks, Period 1 (*Per1*) and *Per2*. Treatment with the prototypical Ahr ligand, 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), had inductive effects on a key target of AhR signaling, *Cyp1A1*, in both the mammary gland and liver of all animals. In wild type mice, the amplitude of this TCDD-induced *Cyp1A1* expression in the mammary gland and liver was significantly greater (23–43 fold) during the night than during the daytime. However, the diurnal variation in the TCDD induction of mammary gland and liver *Cyp1A1* expression was abolished in $Per1^{ldc}$, $Per2^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mutant mice, suggesting that *Per1*, *Per2* and their timekeeping function in the circadian clockworks mediate the diurnal modulation of AhR-regulated responses to TCDD in the mammary gland and liver.

Keywords

circadian clock; PAS genes; xenobiotics; aryl hydrocarbon receptor; AhR nuclear translocator; cytochrome *p450* enzymes

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INTRODUCTION

In multi-cellular organisms, a variety of biochemical, physiological and behavioral processes undergo rhythmic fluctuations that closely parallel the time course of the daily solar cycle. These 24-hour or circadian rhythms are normally entrained or synchronized to external time cues but persist even under constant environmental conditions. The generation and photoentrainment of mammalian circadian rhythms is mediated by a hierarchical timekeeping system consisting of a master pacemaker located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus and local oscillators in most peripheral tissues and cells throughout the body. Genes with multi-functional Per-Arnt-Sim (PAS) domains comprise the essential "gears" of the molecular clockworks common to both the circadian pacemaker in the SCN and peripheral oscillators (Shearman et al., 1997; Zylka et al., 1998; Yamazaki et al., 2000). The PAS genes, circadian locomotor output cycles kaput (*Clock*), brain, muscle ARNT-like protein 1 (*Bmal1*), Period 1 (*Per1*) and *Per2*, form interlocked transcription-translation feedback loops in which their protein products rhythmically regulate expression of these genes, with exception of *Clock*. Evidence for their autoregulatory interactions and function in the clock mechanism is based on the observations that disruption or knockout of these PAS genes alters the regulation of other core components in the molecular feedback loops and abolishes circadian behavior (Reppert and Weaver, 2002). For example, mutant mice with targeted disruption of *Per2* are distinguished by altered rhythms of *Bmal1*expression and arrhythmic patterns of locomotor activity (Bae et al., 2001).

The circadian timekeeping system has a notable impact on biochemical and physiological processes associated with the metabolism of ingested xenobiotics such as drugs and environmental contaminants. The aryl hydrocarbon receptor (*AhR*) is a PAS gene component of the signaling pathway responsible for the metabolism of drugs and environmental toxins such as polycyclic aromatic hydrocarbons (PAHs). Upon entry into the cell, PAHs bind the AhR and this process triggers nuclear translocation of AhR and its association with the protein product of another PAS gene, AhR nuclear translocator (*Arnt*). In the nucleus, AhR-ARNT heterodimers induce transcriptional activation of xenobiotic metabolizing enzymes including cytochrome *p450* enzymes of the 1A and 1B subfamily (CYP) by binding to xenobiotic response elements (XREs) in their promoter regions. Induction of cytochrome *p450*s produces oxidation of PAHs to reactive metabolites suitable for conjugation by phase II detoxifying enzymes. Rhythmic fluctuations have been observed in critical components of the AhR signaling pathway. In the liver, AhR protein expression oscillates on a diurnal basis with peak levels occurring during the day (Richardson et al., 1998). The activity of the hepatic *p450* monooxygenase also fluctuates rhythmically such that that levels are high during the night (Furukawa *et al*., 1999). Temporal profiling of the liver transcriptome has revealed that circadian rhythmicity is a distinctive property of other detoxification enzymes (Akhtar et al., 2002; Panda et al., 2002).

Rhythmic fluctuations in responses to xenobiotics are an apparent corollary of these oscillations in critical components of the AhR signaling pathway. For example, the toxicity and efficacy of many anesthetic and anticancer drugs vary rhythmically based on the time of administration (Levi and Schibler, 2007). Because the widespread distribution of the circadian clockworks throughout the body provides for the local temporal organization of tissue- and cell-specific processes, we examined an important target in the AhR signaling pathway, the cytochrome *p450* gene *Cyp1A1*, to determine whether its induction by the protypical AhR ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), is marked by diurnal variation and whether core elements of the circadian clockworks mediate rhythmic responses to this PAH. Previous studies suggest that the *Per* genes may interact with primary components of the AhR signaling pathway (Lindebro et al., 1995) and have an inhibitory influence on AhR-mediated responses to TCDD (Qu et al., 2007, 2009). Consequently, the present experiments used mice with targeted

disruption of *Per1* and/or *Per2* to determine whether these genes and their timekeeping function in the circadian clockworks are necessary for the diurnal modulation of AhR-mediated responses to TCDD in the mammary gland and liver.

MATERIALS AND METHODS

Animals

Experimental subjects were female wild type (WT) 129/sv mice (N=24) and *Per1*^{ldc} (N=25), *Per2*^{ldc} (N=26) and *Per1*^{ldc}/*Per2*^{ldc} (N=24) mutant mice 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 12h light: 12h dark cycle (LD 12:12; lights-on at 0600hr) 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.

Experimental Paradigm—Responses of the AhR signaling pathway were examined in mammary gland and liver tissue from 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 10μg/kg body weight. TCDD treatment was based on our previous studies showing that *Cyp1A1* expression in the mouse mammary gland and liver is significantly induced 24 hours after a single injection of TCDD at this dose (Qu et al., 2007, 2009). For each genotype in the current study, animals were randomly divided into treatment groups (N=5–9) that received an intraperitoneal injection of vehicle (corn oil) or TCDD at either 6 hours after lights-on (1200hr; Zeitgeber Time [ZT] 6) or 6 hours after lights-off in the LD 12:12 photoperiod (2400hr; ZT18). These treatment times were selected on the basis of: 1) our previous studies (Qu et al., 2007, 2009) and many other investigations in which the effects of TCDD were analyzed at ZT 6 or during the middle of the day; and 2) observations indicating that peak activity of critical components mediating AhR regulation of cytochrome *p450* genes occurs near the middle of the night (Furukawa *et al*., 1999). Twenty-four hours after treatment, animals were sacrificed by cervical dislocation at 1200hr (ZT6) or 2400hr (ZT18), and mammary gland and liver tissues were collected in RNA Stabilization Reagent (RNAlater, QIAGEN, Valencia, CA) for subsequent extraction of total RNA. For each sample, approximately 100mg of mammary gland tissue and 30mg of liver were 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 100μl RNase-free water, and then stored at −80°C.

Quantitative Real-Time PCR Analysis—Relative quantification of *Cyp1A1* mRNA abundance 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 (1μg) was reverse transcribed using Superscript II (Invitrogen) and random hexamers. For each sample, the cDNA equivalent to 1.25ng total RNA per 12.5ul 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 (*Ppia*) was amplified from the same samples. Primer sequences were designed for PCR amplification of target and control genes using PrimerExpress software (ABI). The sequences of *Cyp1A1* primers are 5'-CCTCTTTGGAGCTGGGTTT-3′ (forward) and 5'-AGGCTCCACGAGATAGCAGT-3′ (reverse); the sequences of *Ppia* primers are 5'-TGTGCCAGGGTGGTGACTT-3′ (forward) and 5'-TCAAATTTCTCTCCGTAGATGGACTT-3′ (reverse).

The comparative C_T method was used to calculate the relative mRNA abundance for a given target gene. Using this method, the amount of *Cyp1A1* mRNA in each sample was normalized first to corresponding *Ppia* mRNA levels, and then relative to a calibrator consisting of pooled cDNA from multiple samples that was analyzed on each reaction plate.

Statistical Analysis—For each genotype, the raw data were first examined using two-way analyses of variance (ANOVAs) with treatment (vehicle vs. TCDD) and treatment time (ZT6 vs. ZT18) as two independent variables. Fisher's least significant difference (LSD) post hoc analyses were used if significant main effects were obtained. For analysis of fold differences in the TCDD-mediated induction of *Cyp1A1* mRNA expression within each genotype, independent *t*-tests were performed to determine whether the effects of TCDD treatment at ZT6 were significantly different from those at ZT 18. The α value was set at 0.05 for all statistical analyses.

RESULTS

Cyp1A1 mRNA expression remained at basal levels in the mammary glands of all vehicle-treated WT and mutant mice irrespective of treatment time (Fig. 1A). In vehicle-treated WT and mutant mice, *Cyp1A1* expression in the mammary gland at ZT 6 was not significantly different from that observed at ZT 18. Two-way ANOVAs revealed a main effect of TCDD treatment on mammary gland levels of *Cyp1A1* mRNA in both WT and mutant mice [WT: $F(1,13)=131.65$, $Per1^{ldc}$: $F(1,16)=27.53$, $Per2^{ldc}$: $F(1,22)=9.15$, $Per1^{ldc}/Per2^{ldc}$: $F(1,20)=23.36$; $p<0.05$]. Based on post-hoc analyses, *Cyp1A1* expression in the mammary gland was significantly increased ($P<0.05$) in all animals treated with TCDD at ZT 6 and ZT 18 relative to those found in time-matched vehicle controls (Fig. 1A). Genotype-based differences were evident in the effects of treatment time on the amplitude of the TCDD-mediated *Cyp1A1* induction in the mammary gland. WT animals were distinguished by time-dependent differences in the effects of TCDD on mammary gland expression of this *p450* gene such that the fold difference in the *Cyp1A1* induction triggered by toxin injection at ZT 18 was significantly ($t_7 = 2.36$; $P = 0.000014$) and about 23 times higher than that following toxin treatment at ZT 6 (Fig. 1B). In contrast, $Per1^{ldc}$, $Per2^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mutant mice exhibited negligible changes in the amplitude of TCDD-mediated *Cyp1A1* induction in the mammary gland at ZT 18 relative to that observed in response to treatment at ZT 6. The fold differences in the TCDD-mediated *Cyp1A1* induction in the mammary gland at ZT 18 were decreased in $Per1^{ldc}$ animals and were slightly increased in $Per2^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mice (Fig. 1B) relative to those observed at ZT 6. Within each mutant genotype, there was no significant effect of treatment time on the fold differences in the TCDD-triggered *Cyp1A1* induction in the mammary gland.

Similar to the pattern observed in the mammary gland, hepatic *Cyp1A1* expression in WT, $Per1^{ldc}$, $Per2^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mice was consistently low in vehicle controls with no evidence of significant variation at ZT 6 and ZT 18. However, TCDD treatment had a significant effect in inducing *Cyp1A1* expression in the liver of WT and mutant mice [WT: $F(1,20)=92.99$, $Per1^{ldc}$: $F(1,21)=100.20$, $Per2^{ldc}$: $F(1,18)=19.04$, $Per1^{ldc}/Per2^{ldc}$: $F(1,18)=40.91$; $p<0.05$] relative to that observed time-matched controls (Fig. 2A). WT and mutant mice were again marked by differences in the effects of treatment time on the amplitude of TCDD-induced *Cyp1A1* expression in the liver. In WT mice, the fold difference in the TCDD-mediated induction of *Cyp1A1* mRNA levels in liver tissue at ZT 18 was significantly higher ($t_{11} = 2.20$; $P = 0.000003$) than that observed in response to toxin treatment at ZT 6 (Fig. 2B). The amplitude of the TCDD induction of hepatic *Cyp1A1* expression in WT mice was about 42.9 times greater during the night than during the daytime. However, mutant mice with targeted disruption of the *Per1* and/or *Per2* genes showed no evidence of time-dependent variation in the toxin-mediated induction of hepatic *Cyp1A1* expression. Within each mutant

genotype, the fold differences in the TCDD-mediated induction of hepatic *Cyp1A1* expression at ZT 18 were almost equivalent to those observed in response to toxin treatment at ZT 6 (Fig. 2B). In comparison with daytime effects, TCDD treatment during the nighttime produced only small changes in the amplitude of the *Cyp1A1* induction in liver tissue from *Per1*^{ldc}, *Per2*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice (9.5%, −3.8% and 2.7%, respectively). Furthermore, comparisons within each mutant genotype revealed that treatment time had no significant effect on the fold differences in the TCDD-mediated *Cyp1A1* induction in the liver (Fig. 2B).

DISCUSSION

The present study demonstrates that the inductive effects of PAH exposure on cytochrome *p450* expression vary rhythmically in the mammary gland and liver and that the integrity of molecular feedback loops comprising the circadian clockworks is essential for this rhythmic modulation of AhR-regulated responses to environmental toxins. Diurnal fluctuations in the TCDD-mediated induction of *Cyp1A1* expression were disrupted in the mammary gland and liver of *Per1*^{ldc}, *Per2*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice. This finding is compatible with previous studies demonstrating that these mutant mice are distinguished by altered function of the underlying molecular clockworks and an associated loss of circadian rhythmicity in other biological processes such as wheel-running activity (Bae et al., 2001) and steroid hormone levels (Dallmann et al., 2006). Because 4% to 9% of the transcriptome is clock-controlled in peripheral tissues such as the liver (Panda et al., 2002; Storch et al., 2002; Duffield, 2003), it is possible that targeted disruption of the *Per* genes also abolishes rhythmic expression of some genes in biochemical pathways mediating drug metabolism and responses to xenobiotics. Thus, the observed loss of diurnal rhythmicity in the inductive effects of TCDD on AhR-mediated signaling in *Per1*^{ldc}, *Per2*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice may stem from the disruptive effects of the *Per1* and *Per2* mutations on the circadian clock in peripheral tissues and its oscillatory regulation of key components within this signaling pathway.

In conjunction with its role in linking the circadian clockworks to the pathway mediating xenobiotic responses, *Per1* has been implicated as a modulator of TCDD-mediated induction of AhR signaling. In both the liver and mammary gland, targeted disruption of this clock gene potentiates the inductive effects of TCDD treatment on cytochrome *p450* genes (Qu et al., 2007, 2009), raising the possibility that *Per1* may have an inhibitory influence on the activation of AhR-regulated responses to this toxin. Several observations from the present study are compatible with the putative role of *Per1* as a negative or inhibitory factor in the TCDD-induced activation of AhR signaling. First, TCDD-mediated *p450* induction in the mammary gland and liver oscillates in an inverse relationship to the circadian pattern of *Per1* gene expression; in WT mice, inductive effects of TCDD on mammary gland expression of *Cyp1A1* are low during the day and high at night corresponding respectively to times when the rhythmic peak and trough in tissue levels of *Per1* mRNA are known to occur (Hara et al., 2001; Metz et al., 2006). The inhibitory influence of *Per1* on the AhR signaling pathway is further suggested by the observation that targeted disruption of this clock gene abolished diurnal fluctuations in the responses to toxin treatment by differentially affecting TCDD induction of *Cyp1A1* expression during the daytime. Relative to that observed in WT mice, TCDD-mediated *Cyp1A1* induction in the mammary gland and liver of *Per1*^{ldc} mutants was increased at ZT 6, presumably due to the disruption of *Per1* expression and the rhythmic peak that normally occurs at this time, but was largely unchanged at ZT 18 (Fig. 1A and 2A). The mechanism by which *Per1* exerts an inhibitory influence on the AhR signaling pathway is unknown. Because steroid hormones modulate AhR signaling *in vivo* (Gorski et al., 1988; Christou et al., 1995; Prough et al., 1996) and steroid hormone levels and cycles are altered in *Per1*-deficient mice (Dallmann et al., 2006), *Per*-mediated hormonal disturbances may be responsible for the potentiated and non-rhythmic induction of AhR-regulated responses to TCDD in mutant mice. However, the results of previous *in vitro* studies are incompatible with

this possibility because comparable changes in TCDD-mediated induction of *Cyp1A1* persist in mammary cultures from these mutant mice despite the absence of glucocorticoids or other steroids that characterize the hormonal milieu *in vivo* (Qu et al., 2007). Alternative explanations are that *Per1* function as an inhibitory factor in the TCDD-induced activation of the AhR signaling pathway may involve *Per1*-mediated changes in: 1) AhR or ARNT expression, 2) formation, DNA binding activity and stability of AhR:ARNT complexes, 3) post-transcriptional regulation of target genes, or 4) negative feedback regulation by the AhR Repressor (AhRR) which inhibits recruitment of AhR:ARNT heterodimers onto the XRE (Ciolino and Yeh, 1999; Lee and Safe, 2001; Pollenz, 2002; Fujii-Kuriyama and Mimura 2005).

Coupled with our previous findings (Qu et al., 2007, 2009), the present evidence for *Per1* function in the rhythmic modulation of TCDD-induced AhR signaling suggests that this and perhaps other PAS genes in the circadian clockworks may play a role in determining the biological outcome of xenobiotic exposure. PAHs, when oxidized to form DNA adducts, pose increased cancer risk and AhR is necessary for their carcinogenic effects (Shimizu *et al*., 2000). Similar to the observed role of *Per2* in regulating tumorigenesis (Fu *et al*., 2002; Lee, 2006), *Per1* has been implicated as a suppressor of tumor development. Clinical studies demonstrate that human breast, lung and hepatocellular tumors are distinguished by decreased *Per1* expression relative to that found in matching noncancerous tissues from the same patients (Gery et al., 2006; Winter et al., 2007; Lin et al., 2008). Moreover, overexpression of *Per1* was found to alter mRNA or protein levels of key genes in the cell cycle pathway, inhibit cellular proliferation and increase the rate of apoptosis in several human cancer cell lines (Gery et al., 2006). Collectively, these findings and the observed disruption of diurnal fluctuations in TCDD induction of *Cyp1A1* expression in $Per1^{ldc}$ mutants suggest that *Per1* may provide a key functional link between the circadian clockworks and pathway mediating the biological effects of xenobiotics, and that its modulatory influence on AhR-regulated responses to toxins may represent an important factor in the cancer risk associated with xenobiotic exposure.

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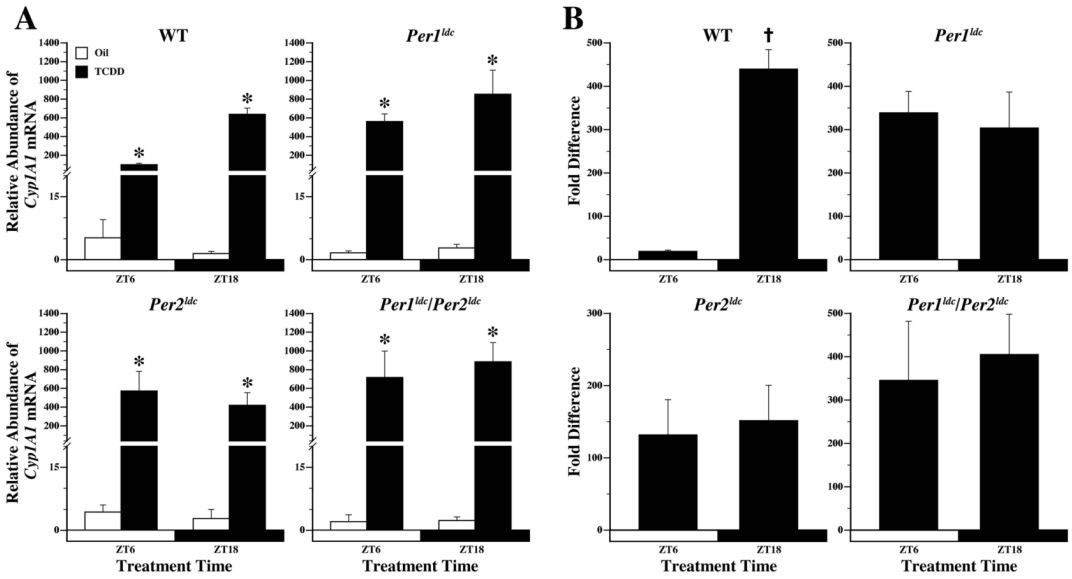

FIGURE 1.

Effects of targeted mutations of *Per1* (***Per1^{ldc}***), *Per2* (***Per2^{ldc}***), and *Per1*/*Per2* (*Per1^{ldc}*/*Per2^{ldc}*) on time-dependent changes in the TCDD-mediated induction of *Cyp1A1* expression in the mouse mammary gland. The relative abundance of *Cyp1A1* mRNA following vehicle or TCDD treatment (A) and fold differences in *Cyp1A1* expression after TCDD treatment (B) during the middle of the day (ZT 6) and night (ZT 18) were analyzed in the mammary glands of WT and mutant mice. Data are expressed as the mean (±SEM) for each experimental group. The plotted values for the relative mRNA abundance correspond to the ratios of species-specific *Cyp1A1*/*Ppia* mRNA signal that were adjusted in relation to the average for WT mice exposed to TCDD at ZT6, which was arbitrarily set at 100. For each treatment time, fold differences in *Cyp1A1* expression between the TCDD- and oil-treated groups were determined by normalizing all values to the average of oil-treated controls, which was arbitrarily set at 1. Asterisks denote time-matched comparisons within each genotype in which TCDD induced significant ($P<0.05$) increases in *Cyp1A1* expression within the mammary gland relative to that observed in oil-treated controls. WT, but not mutant, mice exhibited fold differences in the TCDD-mediated *Cyp1A1* induction within the mammary gland that were significantly greater († $P<0.05$) during the night at ZT 18 than during the day at ZT 6.

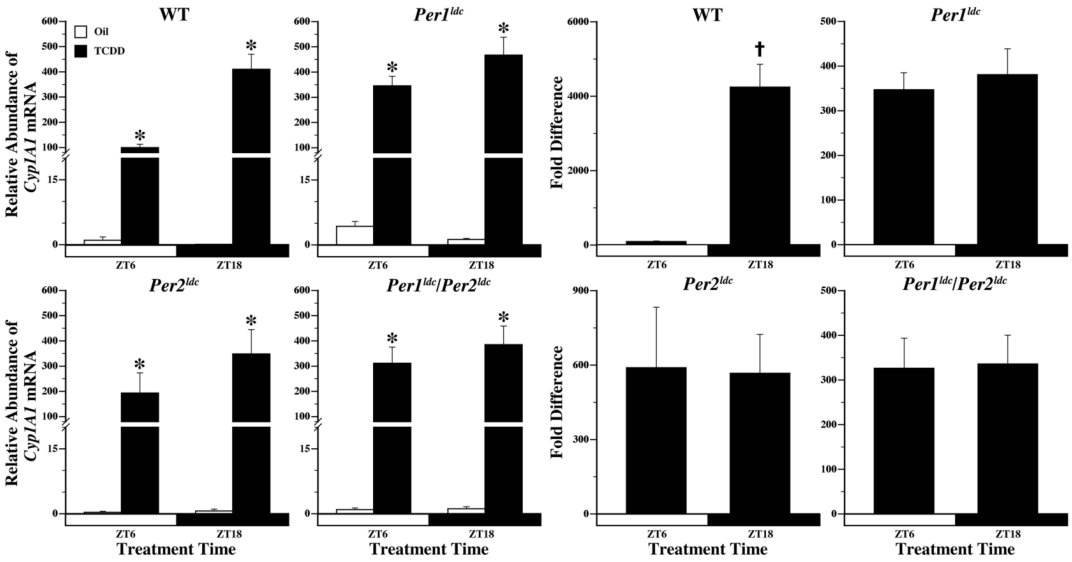

FIGURE 2.

Effects of targeted mutations of *Per1* (*Per1*^{ldc}), *Per2* (*Per2*^{ldc}), and *Per1*/*Per2* (*Per1*^{ldc}/*Per2*^{ldc}) on time-dependent changes in the TCDD-mediated induction of *Cyp1A1* expression in the mouse liver. The relative abundance of *Cyp1A1* mRNA following vehicle or TCDD treatment (A) and fold differences in *Cyp1A1* expression after TCDD treatment (B) at ZT 6 and at ZT 18 were analyzed in liver tissue from WT and mutant mice. The plotted values represent the mean (±SEM) for each experimental group. The relative abundance of *Cyp1A1* mRNA and fold differences in the TCDD-induced expression of this *p450* gene in the liver were established as described in Figure 1. Asterisks denote time-matched comparisons within each genotype in which hepatic *Cyp1A1* mRNA abundance in TCDD-treated mice was significantly greater ($P<0.05$) than that observed in oil-treated controls. The fold differences in the TCDD-mediated *Cyp1A1* induction within the liver of WT, but not mutant, mice were significantly greater († $P<0.05$) during the night at ZT 18 than during the day at ZT 6.