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TITLE PAGE

DISRUPTION OF CLOCK GENE EXPRESSION ALTERS RESPONSES OF THE AHR SIGNALING PATHWAY IN THE MOUSE MAMMARY GLAND

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RUNNING TITLE PAGE

Running title: **Clock genes and regulation of the AhR signaling pathway**

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Abbreviations: AhR, Aryl hydrocarbon receptor; Cyp450, cytochrome p450; DMEM/F-12, Dulbecco's Modified Eagle Medium:Nutrient Mix F-12; DMSO, dimethyl sulfoxide; DREs, dioxin response elements; EGF, epithelial growth factor; PBS, phosphate buffered saline; PAHs, polycyclic aromatic hydrocarbons; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; XREs, xenobiotic response elements.

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ABSTRACT

The biological effects of many environmental toxins are mediated by genes containing Per-Arnt-Sim (PAS) domains, the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (ARNT). Because these transcription factors interact with other PAS genes that form the circadian clockworks in mammals, we determined whether targeted disruption of the clock genes, *Per1* and/or *Per2*, alters toxin-induced expression of known biological markers in the AhR signaling pathway. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a prototypical Ahr agonist, had an inductive effect on mammary gland expression of cytochrome *p450*, subfamily I, polypeptide 1 (*Cyp1A1*) mRNA regardless of genotype. However, TCDD-mediated *Cyp1A1* induction in the mammary glands of *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice was significantly (17.9- and 5.9-fold) greater than that in WT animals. In addition, TCDD-induced *Cyp1B1* expression in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mammary glands was significantly increased relative to that in WT mice. Similar to *in vivo* observations, experiments using primary cultures of mammary gland tissue demonstrated that TCDD-induced *Cyp1A1* and *Cyp1B1* expression in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mutant cells was significantly greater than that in WT cultures. *AhR* mRNA levels were distinctively elevated in cells derived from all mutant genotypes but were commonly decreased in WT and mutant cultures following TCDD treatment. In WT mice, an interesting corollary is that the inductive effects of TCDD on mammary gland expression of *Cyp1A1* and *Cyp1B1* vary over time and are significantly greater during the night. These findings suggest that clock genes, especially *Per1*, may be involved in TCDD activation of AhR signaling pathways.

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INTRODUCTION

Members of the Per-Arnt-Sim (PAS) family of transcriptional regulators are involved in development and in sensing and adapting to environmental conditions. PAS proteins control diverse biological processes such as morphogenesis, circadian rhythms, and responses to hypoxia and toxins (Crews and Fan, 1999). The PAS domain is a multi-functional protein motif governing ligand and DNA binding as well as interactions between PAS and non-PAS proteins. Most PAS proteins function as heterodimers consisting of a sensor protein associated with a general binding partner. For example, the aryl hydrocarbon receptor (*AhR*) partners with the AhR nuclear translocator (*Arnt*) to mediate the transcriptional activation of xenobiotic metabolizing enzymes while circadian locomotor output cycles kaput (*Clock*) associates with brain, muscle ARNT-like protein 1 (*Bmal1*) to form core elements of the circadian clock mechanism in mammals. Not surprisingly, PAS protein-regulated pathways are interconnected 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).

The importance of the PAS genes, *AhR* and *Arnt*, responding to environmental toxins such as polycyclic aromatic hydrocarbons (PAHs) is well-documented. Following entry into the cell, PAHs bind the AhR, which is complexed with 90 kD heat shock proteins (*Hsp90*) and the aryl hydrocarbon receptor interacting protein (*Aip*). Upon ligand binding, this complex dissociates, and PAH-bound AhR translocates to the nucleus and partners with ARNT. AhR-ARNT heterodimers bind to xenobiotic response elements (XREs) in target gene promoters affecting their expression. Principal targets of AhR signaling are cytochrome *p450* enzymes of the A and B subfamily including *Cyp1A1*, *Cyp1A2* and *Cyp1B1*. Cytochrome *p450*s catalyze oxidation of PAHs to reactive metabolites suitable for conjugation by phase II detoxifying

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enzymes including glutathione *S*-transferases and UDP-glucuronosyltransferases. The resulting conjugates are generally less reactive, more hydrophilic molecules that are easier to excrete. If not rendered less reactive or excreted, oxidative PAH metabolites can form DNA adducts leading to mutations and increased cancer risk.

The PAS genes, *Clock*, *Bmal1*, Period 1 (*Per1*) and *Per2*, are important components of the circadian clock mechanism in mammals. These PAS genes form interacting positive- and negative-feedback loops in which the transcription of core components is rhythmically regulated by their protein products. PER1 and PER2 form heterodimeric complexes with the protein products of the Cryptochrome (*Cry*) genes and following a delay, these complexes are translocated to the nucleus (Kume et al., 1999; Yagita et al., 2000). CRY proteins then inhibit the transcription of *Clock* and *Bmal*1 (Griffin et al., 1999). In turn, CLOCK and BMAL1 close the feedback loop by forming heterodimers that positively regulate the rhythmic transcription of the *Per* and *Cry* genes via the activation of E-box elements (Gekakis et al., 1998; Hogenesch et al., 1998; Jin et al., 1999). CLOCK:BMAL1 complexes also mediate the activation of clock-controlled genes that serve as outputs from the clock and function to regulate downstream rhythmic processes throughout the body.

Recent evidence suggests that molecular components of the circadian clock serve important functions in other PAS gene-regulated processes including development, tumorigenesis and drug metabolism. For example, *Per1* and *Per2* have been implicated in mammary gland development and differentiation based on changes in their expression during different stages of development and of the cell cycle. *Per1* and *Per2* involvement in the regulation of neoplastic growth is supported by the observations that *Per2*-deficient mice are more susceptible to the development of spontaneous and γ-radiation-induced tumors (Fu et al.,

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2002) and that PER1 and PER2 expression is down regulated in human breast tumors relative to normal surrounding tissue (Chen et al., 2005).

Because PAHs are potent carcinogens, and PAS proteins can interact with one another, we examined whether core elements of the clock mechanism also play some role in PAH responses mediated by the PAS gene, *AhR*. Previous studies indicate that *Drosophila* PER forms dimers with AhR and ARNT via the PAS domain and this process interferes with the DNA binding activity of AhR/ARNT heterodimers (Lindebro et al., 1995). Clock gene function in AhR signaling is also suggested by studies demonstrating that BMAL1 interacts with AhR (Hogenesch et al., 1997). Consequently, experiments were conducted to determine whether targeted disruption of the clock genes, *Per1* and/or *Per2*, affects the activation of cytochrome *p450*s and other components of the AhR signaling pathway in the mammary gland by the protypical AhR ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Our results demonstrate that disruption of the circadian clock produces hyperinduction of host responsiveness to environmental toxicants.

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MATERIALS AND 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) derived from breeding pairs generously provided by Dr. David Weaver (University of Massachusetts Medical School, Worcester, MA). Establishment and characterization of these transgenic mice has 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 0600 hr) 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 mammary gland *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 10µg/kg body weight. Previous studies showed a single dose of 5µg/kg TCDD or higher for 24 hours significantly induces hepatic *Cyp1A1* expression in mice (Narasimhan et al., 1994). In the current study, animals received an intraperitoneal injection of vehicle (corn oil) or TCDD about 6 hours after lights-on in the LD12:12 cycles (1200 hr; Zeitgeber Time [ZT] 6). Twenty-four hours after treatment, animals were sacrificed by cervical dislocation at ZT6) and mammary gland tissues were collected in RNA Stabilization Reagent (RNA *later*, Qiagen, Valencia, CA) for later extraction of total RNA. For each tissue sample, approximately 30 mg of mammary tissue was homogenized and

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processed for extraction of total cellular RNA using the RNeasy Lipid Tissue 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.

Experiment 2: Effects of targeted disruption of *Per1*, *Per2* and *Per1/Per2* on TCDD-induced responses of the AhR signaling pathway in primary cultures of the mouse mammary gland

Mammary gland cells were collected from 12- to 14-week old female mice, and primary cultures of these cells were established using methods similar to those described previously (Pullan and Streuli, 1996; Seagroves et al., 1998). For each experiment, mammary gland cultures were obtained from WT mice and compared with those from the mutant mice (*Per1*^{ldc}, *Per2*^{ldc} or *Per1*^{ldc}/*Per2*^{ldc}) at the same age (@N=3). Briefly, cells were extracted from mouse mammary glands and cultured on serum/fetuin-coated 6-well-plates in DMEM/F12 medium [Invitrogen, Carlsbad, California] containing 5µg/ml insulin [Sigma-Aldrich, St. Louis, MO], 1µg/ml hydrocortisone [Sigma-Aldrich], 5ng/ml epithelial growth factor (EGF) [QED Biosciences, San Diego, CA], 50µg/ml gentamicin [Invitrogen], 100U/ml penicillin/streptomycin [Invitrogen] and 5% FBS at 37°C in a humidified incubator with 5% CO_2. Confluent cultures were treated with vehicle (N=3; dimethyl sulfoxide [DMSO], Sigma-Aldrich) or 20nM TCDD (N=3) for 24 hours. After treatment, cultures were collected by trypsinization and total RNA was extracted using RNeasy mini kit [Qiagen]. The dose and duration of TCDD treatment in these experiments were based on previous observations indicating that robust increases in *Cyp1A1* and *Cyp1B1* mRNA and protein levels occur within human mammary epithelial cells *in vitro* following exposure to TCDD for 24 hours (Chen et al.,, 2004).

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Experiment 3: Time-dependent effects of TCDD treatment on the AhR signaling pathway in the mouse mammary gland *in vivo*

To determine whether TCDD-induced effects on the AhR signaling pathway *in vivo* vary as a function of treatment time, WT mice were injected intraperitoneally with vehicle or 10ug/kg body weight TCDD at the midpoint of either the light phase (1200hr; ZT 6; N=22) or dark phase (0000hr; ZT 18; N=12) and mammary gland tissues were collected 24 hours after treatment as described in Experiment 1.

Quantitative RT-PCR analysis

Quantification of relative 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 (1ug) 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 (CypA) or β-actin was amplified from the same samples. Primer sequences for PCR amplification of target and control genes are listed in Table 1.

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

In Experiments 1 and 2, statistical analyses were first performed on the raw data using two-way analyses of variance (ANOVAs) with treatment (vehicle vs. TCDD) and genotype (WT,

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Per1^{ldc}, *Per2*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc}) 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 control and TCDD groups of the same genotype. The fold differences in gene expression between these treatment groups were then analyzed using one-way ANOVA and, if required, Fisher's least significant difference (LSD) post hoc analyses to determine whether genotype had a significant effect on TCCD-induced changes in mRNA levels for a given gene. In Experiment 3, the raw data were first analyzed using two-way ANOVAs with treatment (vehicle vs. TCDD) and time (ZT 6 vs. ZT 18) as two independent variables. If significant main effects were identified, planned comparisons using independent *t*-tests were applied to compare gene expression between control and TCDD groups at the same treatment time. For the *p450* genes, the fold differences in TCDD-induced gene expression were also analyzed using independent *t*-tests to determine the significance of treatment time. The α value was set at 0.05 for all statistical analyses.

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RESULTS

Effects of targeted disruption of *Per1, Per2* and *Per1/Per2* on TCDD-activated AhR signaling pathway in the mouse mammary gland *in vivo*. Expression and TCDD-mediated induction of key genes in the AhR signaling pathway was compared between WT, *Per1*^{ldc}, *Per2*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mutant mice (Bae et. al., 2001). Consistent with previous findings (Narasimhan et al., 1994), basal levels of *Cyp1A1* mRNA expression were observed in the mammary glands of all vehicle-treated WT and mutant mice (Fig. 1A). Relative to vehicle controls, TCDD had a robust effect in inducing *Cyp1A1* expression within the mammary gland. In both WT and mutant mice, mammary gland levels of *Cyp1A1* mRNA were significantly greater (p<0.05) in TCDD-treated animals than vehicle controls. Genotype-related differences were evident in the absolute values of TCDD-induced *Cyp1A1* expression in the mammary gland (Fig. 1A). In the mammary glands of *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mutant mice, the TCDD-induced *Cyp1A1* expression was significantly (p<0.05) and about 3 times higher than that found in WT animals. Analysis of the fold difference in gene expression between the TCDD- and vehicle-treated groups for each genotype revealed further distinctions in the activation of the AhR signaling pathway among mutant mice with targeted disruptions of the *Per1* and *Per2* genes (Fig. 1B). The fold differences in the TCDD-induced *Cyp1A1* expression within the mammary gland were significantly greater in *Per1*^{ldc} (p<0.05) and *Per1*^{ldc}/*Per2*^{ldc} (p<0.05) mutant mice than in WT animals. In fact, the inductive effects of TCDD on *Cyp1A1* expression within the mammary glands of *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mutant mice were respectively increased by 17.9- and 5.9-fold relative to that found in WT mice.

TCDD-mediated effects on mammary gland expression of another *p450* gene in the AhR signaling pathway, *Cyp1B1*, followed a similar trend. *Cyp1B1* mRNA levels in the mammary

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gland were consistently low and similar among all vehicle-treated WT and mutant mice (Fig. 1A). In comparison with vehicle controls, TCDD treatment produced significant increases ($p<0.05$) in mammary gland levels of *Cyp1B1* mRNA in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice but not in WT and *Per2*^{ldc} animals. Despite the lack of significant variation among genotype-based comparisons, the fold differences in TCDD-induced *Cyp1B1* expression within the mammary glands of *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mutant mice was respectively increased by 2.5- and 2.2-fold relative to that found in WT animals (Fig. 1B).

Two major regulators of TCDD-induced responses, *AhR* and *Arnt* were also analyzed in our study. Similar levels of *AhR* expression were observed in mammary glands of all vehicle-treated WT and mutant mice (Fig. 2). No significant differences in mammary gland levels of *AhR* mRNA were evident among vehicle control and TCCD-treated mice, regardless of their genotype. Similar to *AhR*, *Arnt* mRNA expression in the mammary gland was comparable in all mice with no major treatment- or genotype-based differences (Fig. 2).

Effects of targeted disruption of *Per1*, *Per2* and *Per1/Per2* on TCDD-activated AhR signaling pathway in primary cultures of the mouse mammary gland. Because the AhR signaling pathway is influenced by steroid hormones such as glucocorticoids and 17β-estradiol *in vivo* (Gorski et al., 1988; Christou et al.,, 1995; Prough et al., 1996) and because serum levels and the rhythmic regulation of steroid hormones are altered in *Per1*-deficient mice (Dallmann et al., 2006), parallel *in vitro* analysis was conducted to indirectly address the role of *Per*-mediated hormonal changes in the potentiation of TCDD-induced AhR signaling in mutant mice. Primary cultures of the mouse mammary gland were used to determine whether the observed amplification of TCDD-induced *p450* expression in *Per* mutant mice persists *in vitro* in the absence of hormonal influences. Basal levels of *Cyp1A1* expression were observed among all

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vehicle-treated mammary gland cultures derived from WT and mutant animals (Fig. 3A). Relative to vehicle controls, treatment with 20nM TCDD for 24 hours induced significant increases ($p<0.05$) in *Cyp1A1* expression in all mammary gland cultures. Genotype-based distinctions were evident in the fold differences in *Cyp1A1* expression between TCDD- and vehicle-treated cultures. Consistent with the results of our *in vivo* study, the fold differences in the TCDD-induced *Cyp1A1* expression were significantly greater in mammary gland cultures derived from *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} ($p<0.05$) mutant mice than in those from WT animals (Fig. 3B). The TCDD-mediated induction of *Cyp1A1* mRNA levels was 5.7- and 4.2-fold higher in *Per1*^{ldc} and *Per1*^{ldc} /*Per2*^{ldc} mammary cells than in WT cultures. In mammary gland cultures from *Per2*^{ldc} mice, the fold differences in the induction of *Cyp1A1* expression by TCDD were reduced relative to WT cells.

Similar to *Cyp1A1*, *Cyp1B1* expression was consistently low in vehicle-treated mammary cells (Fig. 3A). TCDD had a significant effect in inducing *Cyp1B1* expression in all WT and mutant cultures ($p<0.05$). Interactions between treatment and genotype were comparable to those observed *in vivo*. TCDD treatment produced increases in *Cyp1B1* expression in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mammary cells that were significantly greater ($p<0.05$) than those found in WT cultures (Fig. 3A). Further analysis revealed that the fold differences in TCDD-induced *Cyp1B1* expression were significantly greater ($p<0.05$) in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mammary gland cultures than in WT cells (Fig. 3B). Fold differences in TCDD-mediated *Cyp1B1* induction in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} cells were respectively 2.3- and 3.9-times higher than that in WT cultures.

The central regulators of TCDD-induced signaling, *AhR* and *Arnt*, were differentially expressed and affected by this toxin in primary cultures of the mouse mammary gland. Among vehicle-

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treated mammary cells, it is interesting that *AhR* mRNA expression in all mutant cultures were significantly greater ($p<0.05$) than WT levels. The highest levels of *AhR* expression in vehicle-treated cells were observed in cultures derived from *Per1*^{ldc} mice. TCDD had a significant effect in reducing *AhR* mRNA levels in both WT and mutant mammary cells ($p<0.05$) (Fig. 4). In response to TCDD exposure, *AhR* expression was reduced to comparable levels among WT and mutant cells with exception of cultures derived from *Per1*^{ldc} mice. Following treatment, *AhR* mRNA levels in *Per1*^{ldc} mammary gland cultures were significantly (about 2 times) higher ($p<0.05$) than those found in WT cells exposed to TCDD. In contrast to *AhR*, there was no significant effect of either treatment or genotype on *Arnt* expression in mammary gland cultures (Fig. 4). Similar levels of *Arnt* mRNA were expressed by both WT and mutant cells following treatment with vehicle or TCDD.

Time-dependent effects of TCDD treatment on the AhR signaling pathway in the mouse mammary gland *in vivo*. Because the results of our *in vivo* and *in vitro* experiments indicate that changes in *Per* gene expression affect TCDD-induced responses of the AhR signaling pathway, we next determined whether the inductive effects of this toxin on *p450* gene expression varies endogenously in accord with the diurnal *Per* rhythms that are known to occur in mouse mammary gland (Metz et al., 2006). In oil-injected WT mice, *Cyp1A1* mRNA levels in the mammary gland were low irrespective of treatment time (Fig. 5A). TCDD administered during the day and at night both triggered significant increases in mammary gland levels of *Cyp1A1* mRNA ($p<0.05$). Interestingly, treatment time had a significant effect ($p<0.05$) on the fold differences in the TCDD-mediated *Cyp1A1* induction in the mammary gland such that the increase in the expression of this *p450* gene triggered by toxin injection at ZT 18 was 8.6-fold higher than that following treatment at ZT 6 (Fig. 5B). Unlike the pattern for *Cyp1A1, Cyp1B1*

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expression in the mammary glands of vehicle-treated animals was marked by significant variation over time ($p<0.05$) with mRNA levels at ZT 6 that were substantially greater than those at ZT 18. Consequently, TCDD had no significant effects on mammary gland levels of *Cyp1B1* mRNA when treatment was administered at ZT 6 but induced significant increases ($p<0.05$) in expression of this *p450* gene following exposure at ZT 18 (Fig. 5A). Moreover, the fold differences of TCDD-induced *Cyp1B1* expression within the mammary gland at ZT 18 were significantly ($p<0.05$) and about 2 times higher than those observed at ZT 6 (Fig. 5B).

AhR expression in the mammary gland was also marked by time-dependent variation in vehicle-treated WT mice. AhR mRNA levels in the mammary glands of vehicle controls were significantly greater during the day at ZT 6 than during the night at ZT 18 ($P<0.05$) (Fig. 6). TCDD administration did not alter *AhR* expression in the mammary gland and no time-dependent differences were evident in its effects on this gene. In vehicle-treated mice, *Arnt* expression in the mammary gland showed no sign of diurnal variation (Fig. 6). *Arnt* mRNA levels in the mammary gland were not affected by TCDD administration or the time of treatment.

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DISCUSSION

Previous studies have linked the PAS genes, *Per1*, *Per2*, *Bmal1* and *Clock*, not only with the generation of circadian rhythms but also with the regulation of various non-clock functions. Mice with deletions or mutations of these genes exhibit alterations in the circadian regulation of locomotor activity (Bae et al., 2001; Reppert and Weaver, 2002) in conjunction with a myriad of other physiological or behavioral disturbances including decreased body weight, shortened life span, increased tendon calcification (McDearmon et al., 2006), premature aging, tissue hyperplasia (Fu et al., 2002; Lee, 2006), increased alcohol consumption (Spanagel et al., 2005) and altered responses to other drugs of abuse (Kondratov et al., 2007). Consistent with these observations, the present study revealed that targeted disruption of the *Per* genes modifies mammary gland responses to the environmental toxin, TCDD. It is interesting that the inductive effects of TCDD on expression of the cytochrome *p450* genes, *Cyp1A1* and *Cyp1B1*, were potentiated in mammary glands and in primary cultures of mammary cells from *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc}, but not *Per2*^{ldc}, mice. Similar to primary analyses of these mutant mice indicating that the *Per1* and *Per2* genes influence different molecular processes but are indispensable for normal clock function (Zheng et al., 1999; Shearman et al., 2000; Bae et al., 2001), our findings suggest that *Per1* plays a distinct role in modulating TCDD activation of the AhR signaling pathway.

The mechanism by which the *Per* genes interact with components of the AhR signaling pathway and influence its activation by TCDD is currently unknown. However, a possible explanation is that the potentiation of TCDD-induced *Cyp1A1* and *Cyp1B1* expression in the mammary gland is associated with the altered function of the circadian clockworks in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice. Similar to the findings of Bae et al. (2001), these mutant mice exhibited arrhythmic

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patterns of wheel-running activity after 1-2 weeks of exposure to constant darkness (data not shown). Because up to 10% of the transcriptome is clock-controlled in peripheral tissues (Duffield, 2003) and some of these genes with oscillatory profiles are essential elements of critical biochemical processes mediating drug metabolism and responses to xenobiotic agents (Gachon et al., 2006; Menger et al., 2007), the disruptive effects of the *Per1* mutation on circadian clock function may extend to the rhythmic regulation of the AhR signaling pathway in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice. This hypothesis is indirectly supported by the present observations that AhR expression and TCDD-mediated induction of *p450* genes in the mammary gland are marked by diurnal variation. In the mammary glands of WT mice, AhR mRNA levels are lower and TCDD-induced *Cyp1A1* and *Cyp1B1* expression is greater during the night than during the day. Because the diurnal variation in the TCDD-mediated *p450* induction in the mammary gland is inversely related to the temporal pattern of *Per1* gene expression, in which tissue mRNA levels peak during the day and remain low throughout the night (Metz et al., 2006), the disruption of *Per1* expression and rhythmicity in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice may be responsible for the potentiated activity of the AhR signaling pathway in response to this toxin. To further explore this possibility, it will be necessary to determine whether the rhythmic variation in TCDD-induced *p450* gene expression in the mammary gland is also abolished in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice.

A related explanation for the present findings is that the disruption of *Per1* gene expression or clock function in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice may indirectly mediate the potentiation of TCDD-induced *p450* expression in the mammary gland, perhaps by altering the levels and/or circadian cycles of hormones that influence the AhR signaling pathway. The potential involvement of *Per*-mediated hormonal changes in the altered TCDD responses in *Per1*^{ldc} and

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Per1^{ldc}/*Per2*^{ldc} mice is compatible with the observations that steroid hormones modulate AhR signaling *in vivo* (Gorski et al., 1988; Christou et al., 1995; Prough et al., 1996) and that steroid hormone levels and cycles are altered in *Per1*-deficient mice (Dallmann et al., 2006). However, the results of our *in vitro* study do not appear to support this possibility because the potentiation of TCDD-induced *Cyp1A1* and *Cyp1B1* expression persists in mammary cultures from these mutant mice despite the absence of hormonal signals that occur *in vivo*.

Alternatively, the potentiation of TCDD-induced *p450* gene expression in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mice may not be associated with the disruption of the circadian clockworks but instead related to changes in *Per* gene interactions with specific components of the AhR signaling pathway. Our findings raise the possibility that *Per1* may directly inhibit TCDD activation of the AhR signaling pathway. This inhibition could occur via interactions between *Per1* and PAS gene components of the AhR signaling pathway at several different levels. Because the function of PER1 in regulating circadian rhythmicity is distinctly mediated through its interactions with other PAS proteins in the feedback loop (Bae et al., 2001), PER1 may similarly interact with the PAS proteins, AhR and ARNT, and perhaps inhibit their dimerization. *Per1* may also directly influence the AhR signaling pathway by inhibiting the binding of AhR:ARNT complexes to the DREs of target genes. This hypothesis is corroborated by the observation that *Drosophila* PER impedes the formation and DNA binding activity of AhR:ARNT complexes by dimerizing with AhR and ARNT via the PAS domain (Lindebro et al., 1995). Our *in vitro* results suggest that AhR expression is another prospective target for *Per1* in down-regulating TCCD-mediated activation of the AhR signaling pathway because AhR mRNA expression in mammary gland cells derived from *Per1*^{ldc}, *Per2*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} mutant mice was substantially higher than that found in WT cultures. Further analysis will be necessary to

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specifically determine whether the *Per* genes modulate TCDD-mediated induction of *p450* gene expression by inhibiting AhR expression, the formation of AhR:ARNT heterodimers or the binding of these complexes with DREs.

In summary, our data indicate that the targeted disruption of *Per1* potentiates the inductive effects of TCDD on *p450* gene expression in the mammary gland *in vivo* and *in vitro*. Because the induced expression of the *p450* genes, *Cyp1A1* and *Cyp1B1*, has been associated with increased cancer risk (Schrenk, 1998), this finding may have further implications for the involvement of the *Per* genes in carcinogenesis. Previous studies have shown that *Per2* suppresses tumor development by regulating responses to DNA damage (Fu et al., 2002). Moreover, human breast cancer tissue is distinguished by *Per1* promoter methylation and associated alterations in PER1 protein levels relative to that found in adjacent normal cells (Chen et al., 2005). Together with the present evidence for diurnal fluctuations in AhR expression and TCDD-induced *Cyp1A1* and *Cyp1B1* expression within the mammary gland, these observations suggest that the *Per* genes, perhaps via their function in the circadian clockworks, may play an important role in regulating responses to environmental toxins and in modulating their carcinogenic effects.

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ACKNOWLEDGEMENTS

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FOOTNOTES

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LEGENDS FOR FIGURES

FIGURE 1. Effects of targeted mutations of *Per1* (*Per1*^{ldc}), *Per2* (*Per2*^{ldc}), and *Per1*/ *Per2* (*Per1*^{ldc}/*Per2*^{ldc}) on TCDD-induced expression of *p450* genes in the mouse mammary gland. For *Cyp1A1* and *Cyp1B1*, the relative mRNA abundance (A) and fold differences (B) in their expression after TCDD treatment were analyzed in the mammary glands from WT, *Per1*^{ldc}, *Per2*^{ldc}, and *Per1*^{ldc}/*Per2*^{ldc} 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* or *Cyp1B1*/*CypA* mRNA signal that were adjusted in relation to the average for TCCD-treated WT mice, which was arbitrarily set at 100. The values for fold differences in TCDD-induced *Cyp1A1* expression are represented at x100. Asterisks denote comparisons for each genotype, in which *p450* gene expression in the mammary gland of TCDD-treated mice was significantly greater ($p<0.05$) than that observed in oil-treated controls. For each genotype, fold differences in *p450* gene 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. The fold differences in the TCDD-induced *Cyp1A1* and *Cyp1B1* expression within the mammary gland were significantly greater in *Per1*^{ldc} and *Per1*^{ldc}/*Per2*^{ldc} (†, $p < 0.05$) mutant mice than in WT animals.

FIGURE 2. Relative abundance of *AhR* and *Arnt* mRNA in the mammary glands of oil- and TCDD-treated *Per1*^{ldc}, *Per2*^{ldc}, and *Per1*^{ldc}/*Per2*^{ldc} mice. Data are expressed as the

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mean (±SEM) for each experimental group. The plotted values for the relative mRNA abundance correspond to the ratios of species-specific *AhR* or *Arnt*/*CypA* mRNA signal that were adjusted in relation to the average for TCCD-treated WT mice, which was arbitrarily set at 100. TCDD treatment or genotype had no significant effects on mammary gland levels of *AhR* and *Arnt* mRNA.

FIGURE 3. Effects of targeted mutations of *Per1*, *Per2*, and *Per1*/ *Per2* on TCDD-mediated induction of *p450* genes in mouse mammary cells *in vitro*. For *Cyp1A1* and *Cyp1B1*, the relative mRNA abundance (A) and fold differences (B) in their expression after TCDD treatment were analyzed in primary cultures of mammary tissue derived from WT, $Per1^{ldc}$, $Per2^{ldc}$, or $Per1^{ldc}/Per2^{ldc}$ 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* or *Cyp1B1*/β*-actin* mRNA signal that were adjusted in relation to the average for TCCD-treated cells from WT mice which was arbitrarily set at 100. Asterisks denote comparisons for each culture genotype, in which *p450* gene expression in TCDD-treated mammary cells was significantly greater ($p<0.05$) than that observed in DMSO-treated cultures. The fold differences in *p450* gene expression between the TCDD- and DMSO-treated cultures of each genotype were determined by normalizing all values to the average of DMSO-treated controls, which was arbitrarily set at 1. The fold differences in TCDD-induced *Cyp1A1* and *Cyp1B1* expression were significantly greater (†, $p<0.05$) in $Per1^{ldc}$ and $Per1^{ldc}/Per2^{ldc}$ mammary gland cultures than in WT cells.

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FIGURE 4. Relative abundance of *AhR* and *Arnt* mRNA in DMSO- and TCDD-treated mammary cultures derived from WT, *Per1*^{ldc}, *Per2*^{ldc}, or *Per1*^{ldc}/*Per2*^{ldc} 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 *AhR* or *Arnt*/*β-actin* mRNA signal that were adjusted in relation to the average for TCCD-treated cells from WT mice, which was arbitrarily set at 100. Asterisks denote comparisons for each culture genotype, in which *AhR* mRNA levels in TCDD-treated mammary cultures was significantly decreased ($p<0.05$) relative to that observed in DMSO-treated cells.

FIGURE 5. Effects of treatment time on TCDD-mediated induction of *p450* genes in the mouse mammary gland. For *Cyp1A1* and *Cyp1B1*, the relative mRNA abundance (A) and fold differences (B) in their expression after TCDD treatment during the daytime (ZT 6) and nighttime (ZT 18) were analyzed in the mammary glands of WT 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* or *Cyp1B1*/*CypA* mRNA signal that were adjusted in relation to the average for WT mice exposed to TCDD at ZT6, which was arbitrarily set at 100. The values for fold differences in TCDD-induced *Cyp1A1* expression are represented at x100. Asterisks denote treatment times, in which TCDD induced significant ($p<0.05$) increases in *Cyp1A1* and *Cyp1B1* expression within the mammary gland relative to that observed in oil-treated controls. For

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each treatment time, fold differences in *p450* gene 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. The fold differences in the TCDD-induced *Cyp1A1* and *Cyp1B1* expression within the mammary gland were significantly greater (†, $p < 0.05$) during the night at ZT 18 than during the day at ZT 6.

FIGURE 6. Time-dependent effects of TCDD treatment on *AhR* and *Arnt* mRNA expression in the mouse mammary gland. The relative abundance of *AhR* and *Arnt* mRNA in the mammary glands of WT mice was analyzed in response to TCDD treatment during the daytime (ZT 6) or nighttime (ZT 18). 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 *AhR* or *Arnt*/*CypA* mRNA signal that were adjusted in relation to the average for WT mice treated with TCDD at ZT6, which was arbitrarily set at 100. In oil-treated controls, mammary gland levels of *AhR* mRNA at ZT 18 were significantly lower (†, $p<0.05$) than that observed at ZT 6. TCDD and treatment time had no significant effect on *Arnt* mRNA expression in the mammary gland.

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TABLES

TABLE 1. Primers Used in Real-time RT-PCR Analyses

Gene	Primer sequences
Cyp1A1	Forward: 5'-CCTCTTTGGAGCTGGGTTT-3'
	Reverse: 5'-AGGCTCCACGAGATAGCAGT-3'
Cyp1B1	Forward: 5'-TCTTTACCAGATACCCGGATG-3'
	Reverse: 5'-CACAACCTGGTCCAACTCAG-3'
AhR	Forward: 5'-CAAATCAGAGACTGGCAGGA-3'
	Reverse: 5'-AGAAGACCAAGGCATCTGCT-3'
Arnt	Forward: 5'-GCCAGCCTGAGGTCTTTCAA-3'
	Reverse: 5'-AATTCTTCATTGTTGTAGGTGTTGCT-3'
Cyp A	Forward: 5'-TGTGCCAGGGTGGTGACTT-3'
	Reverse: 5'-TCAAATTTCTCTCCGTAGATGGACTT-3'
β-actin	Forward: 5'-CTTCCTTCTTGGGTATGGAATC-3'
	Reverse: 5'-ACGGATGTCAACGTCACACT-3'

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Figure 1

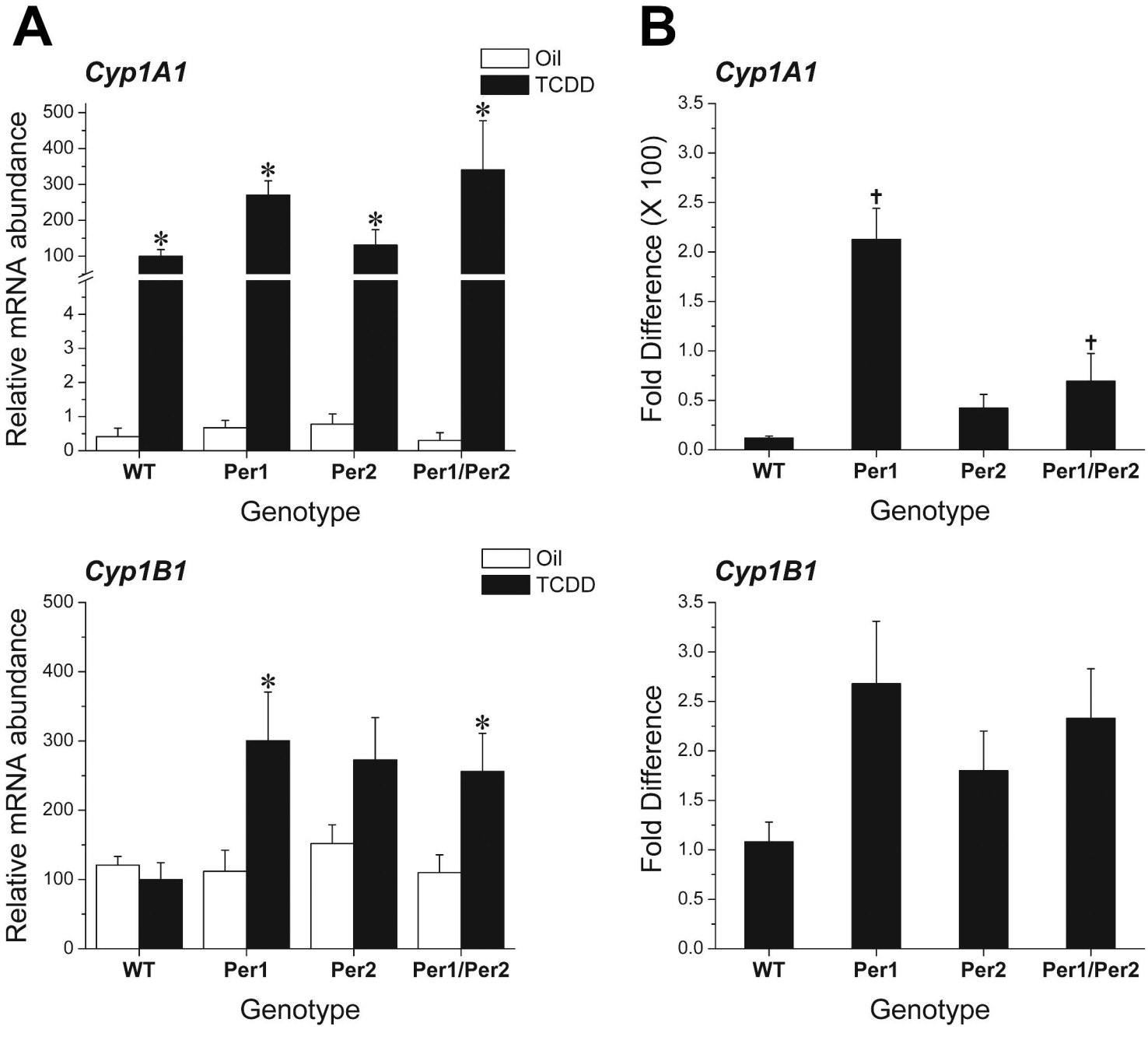

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Figure 2

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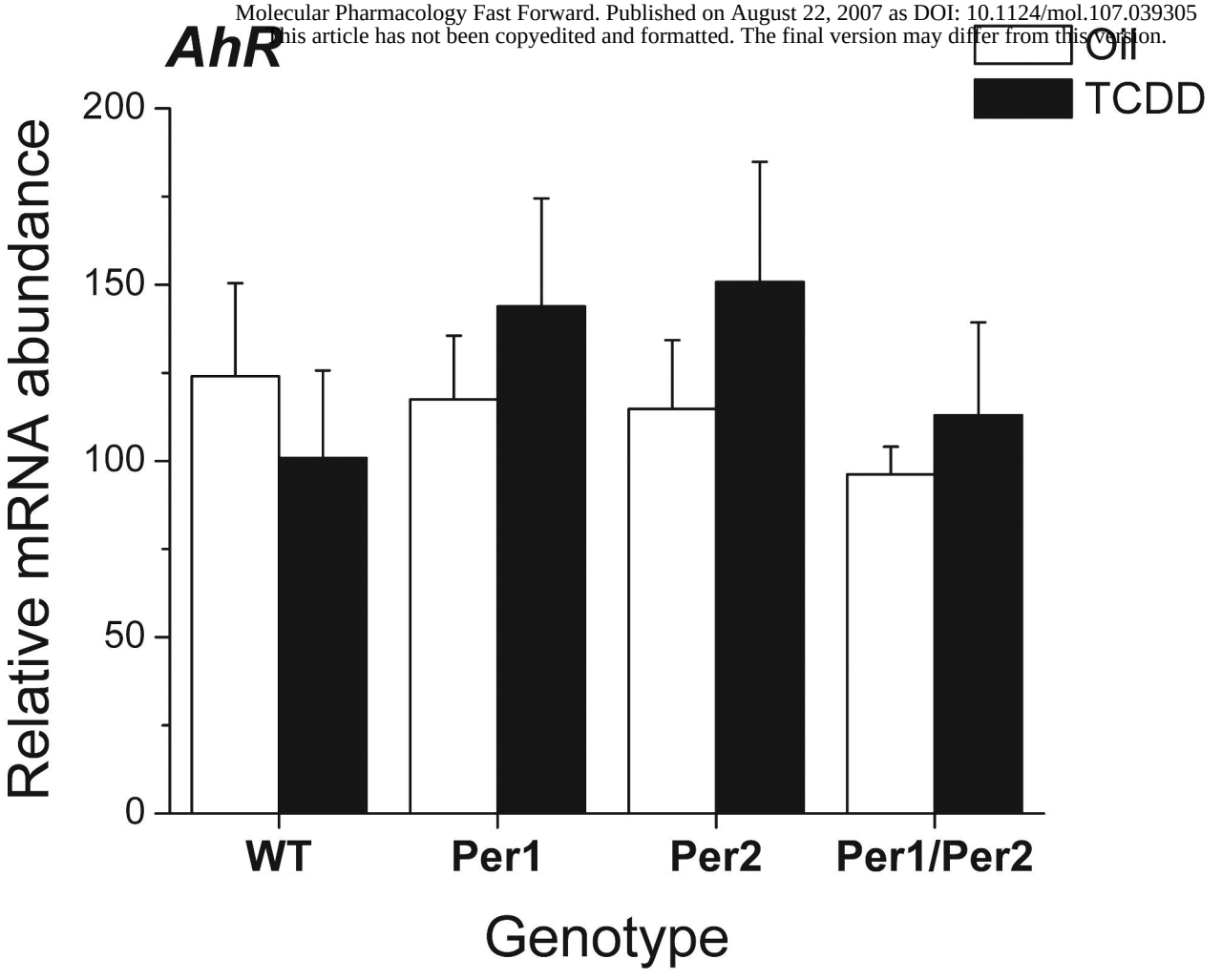

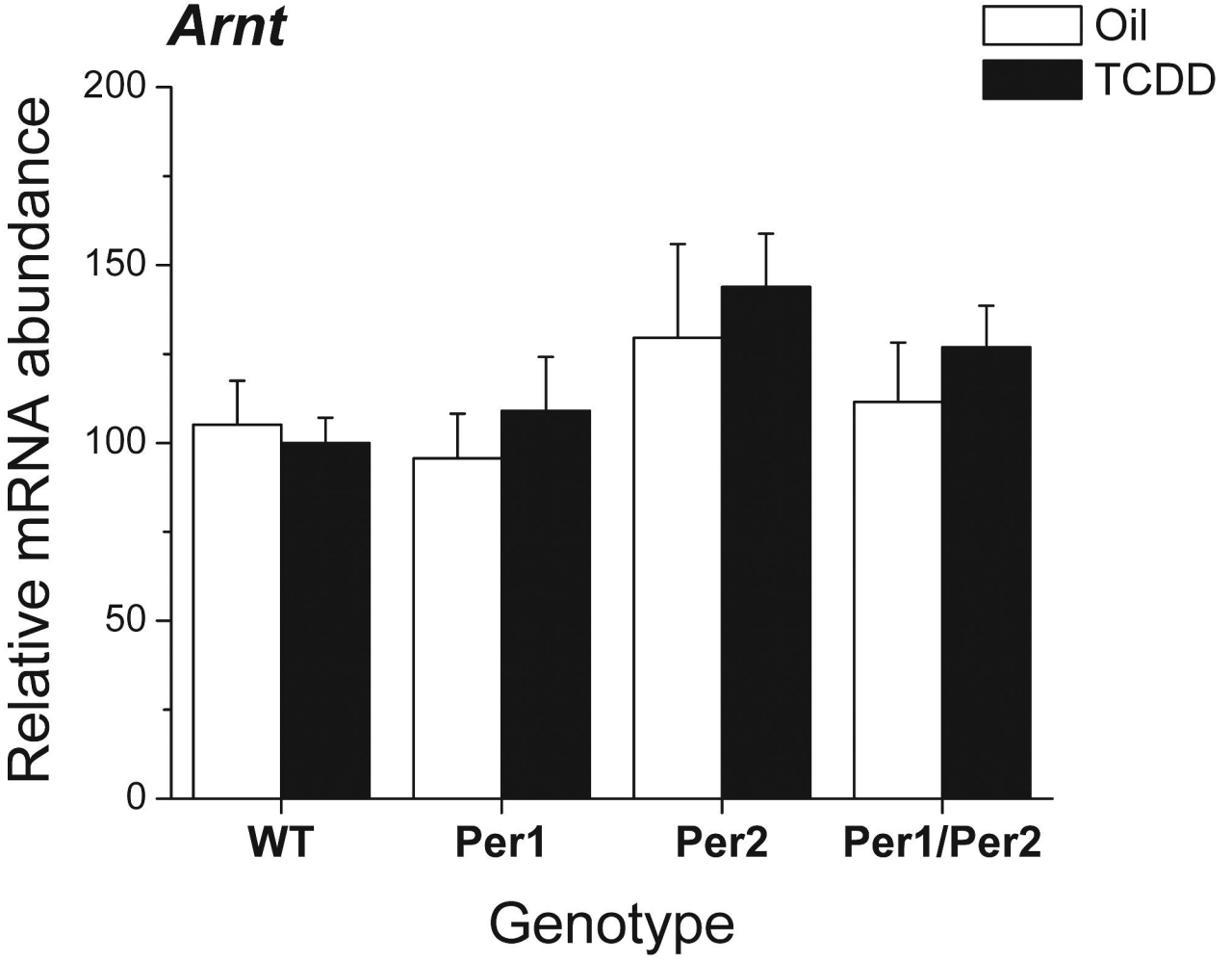

Figure 3

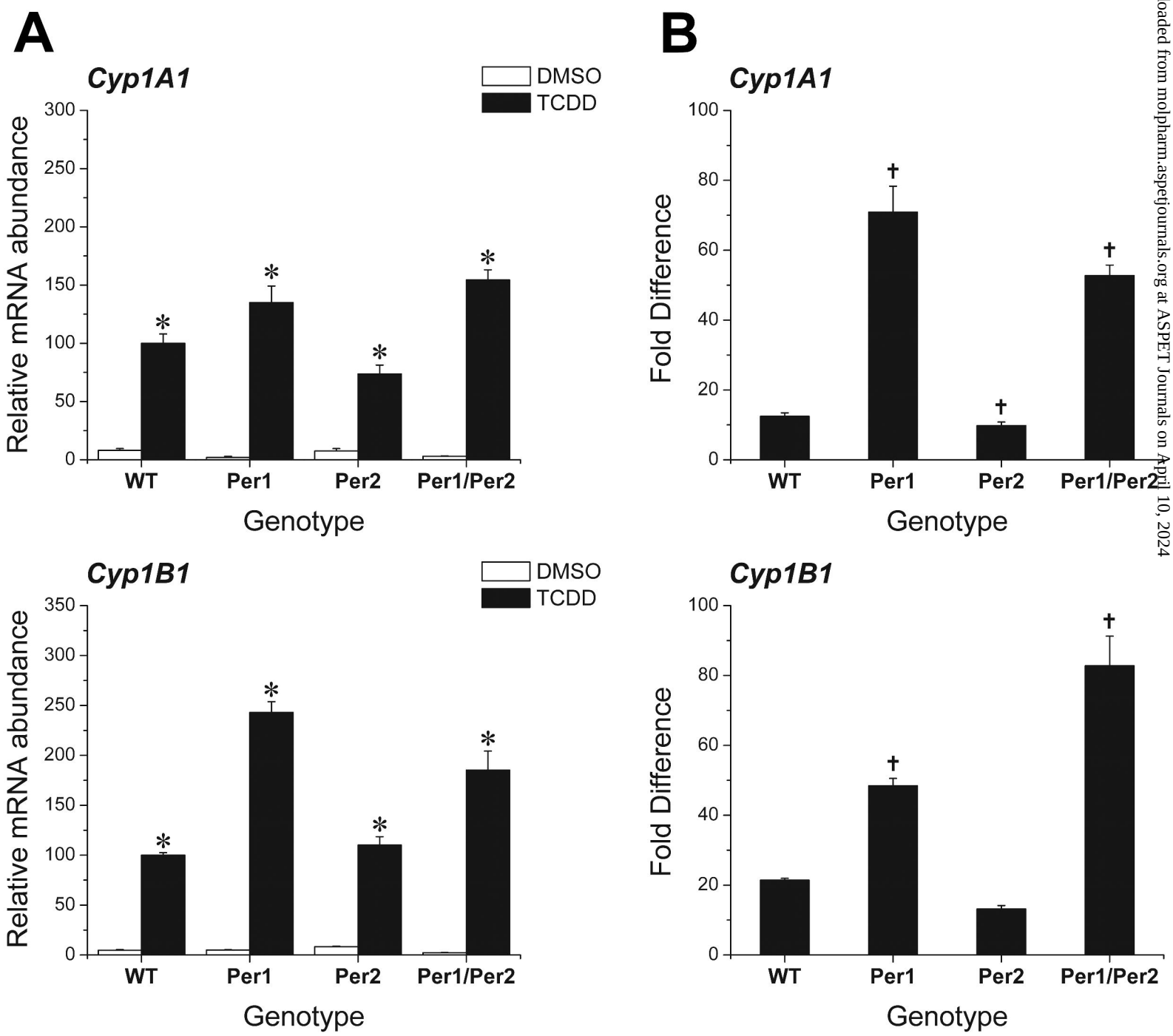

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Figure 4

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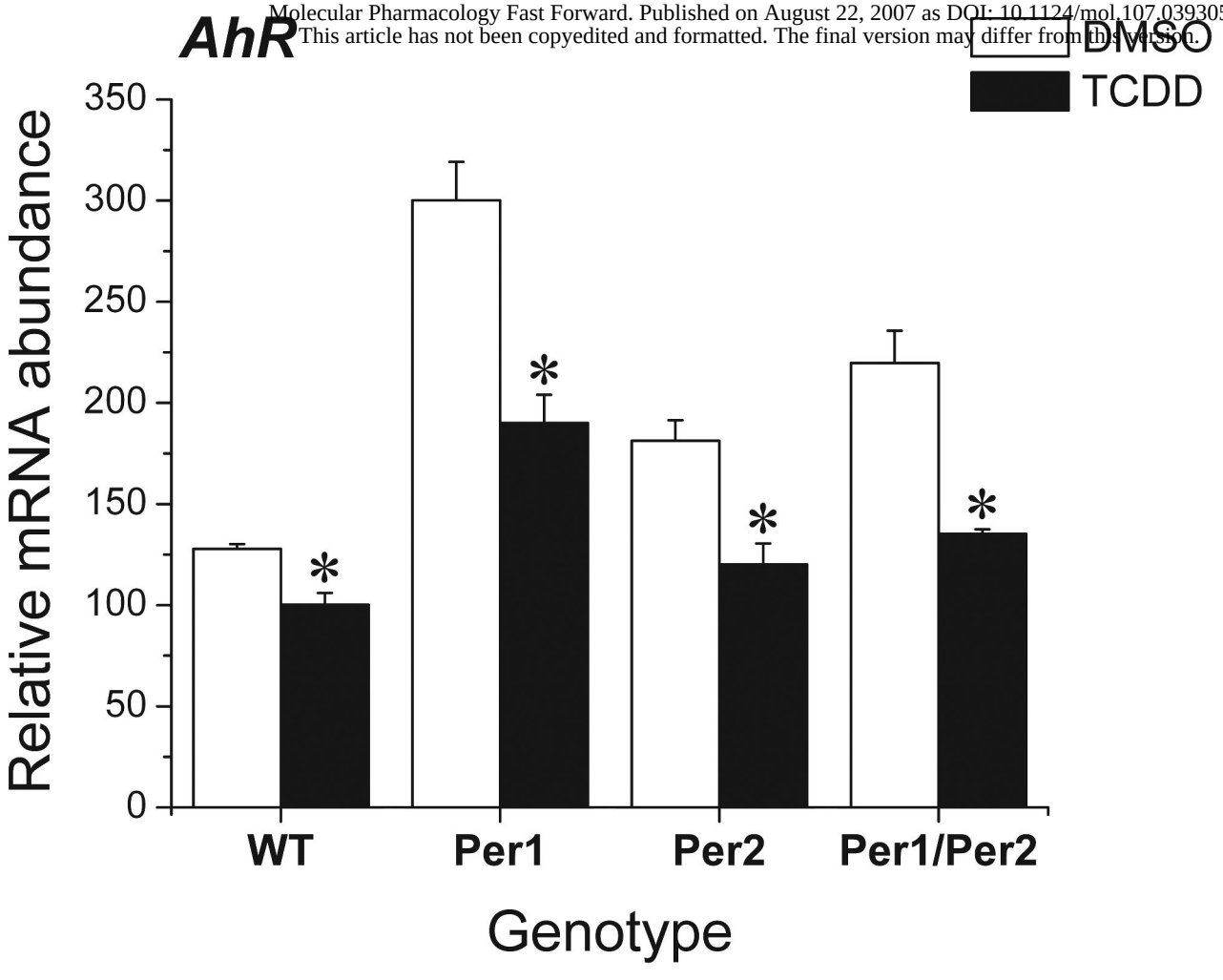

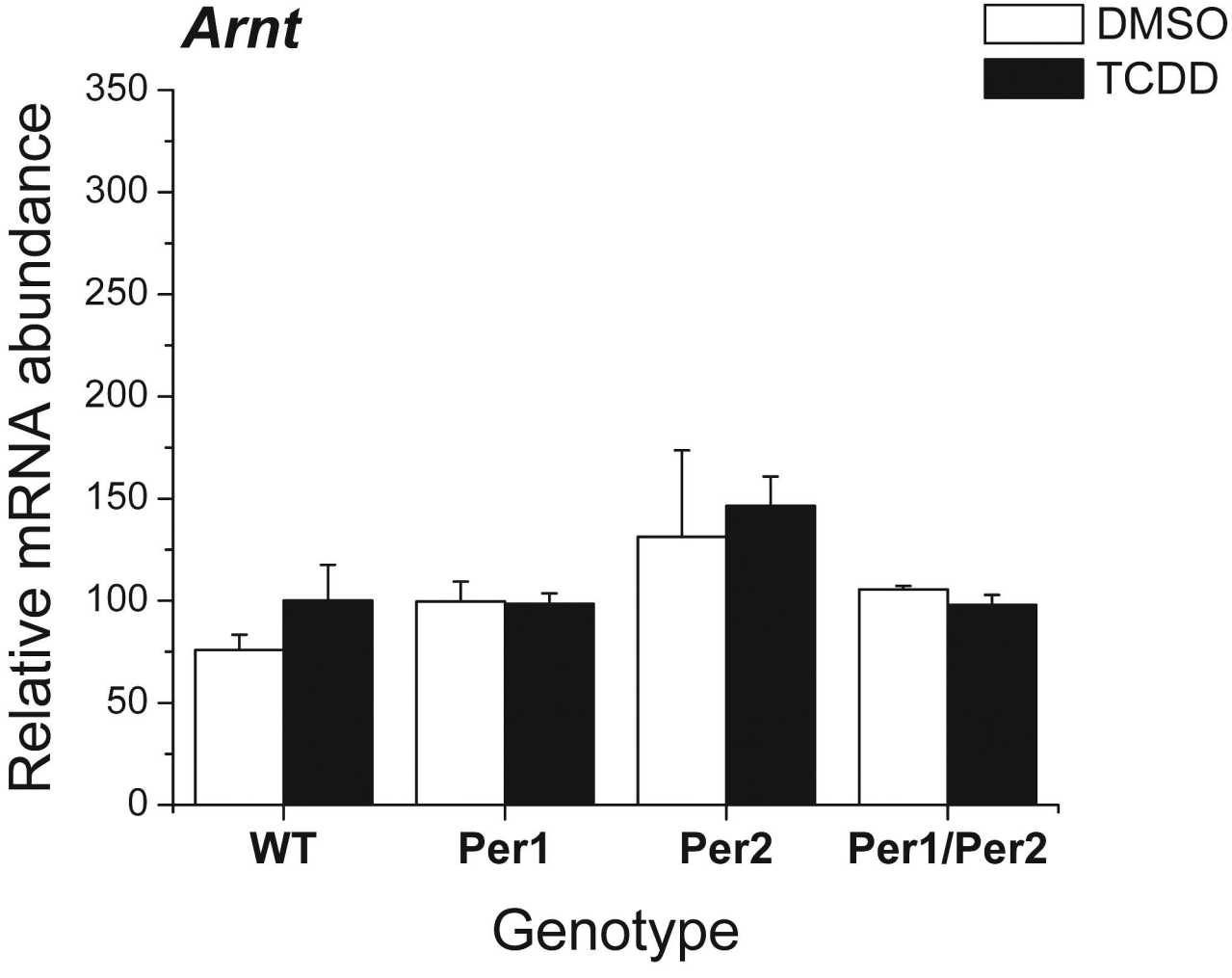

Figure 5

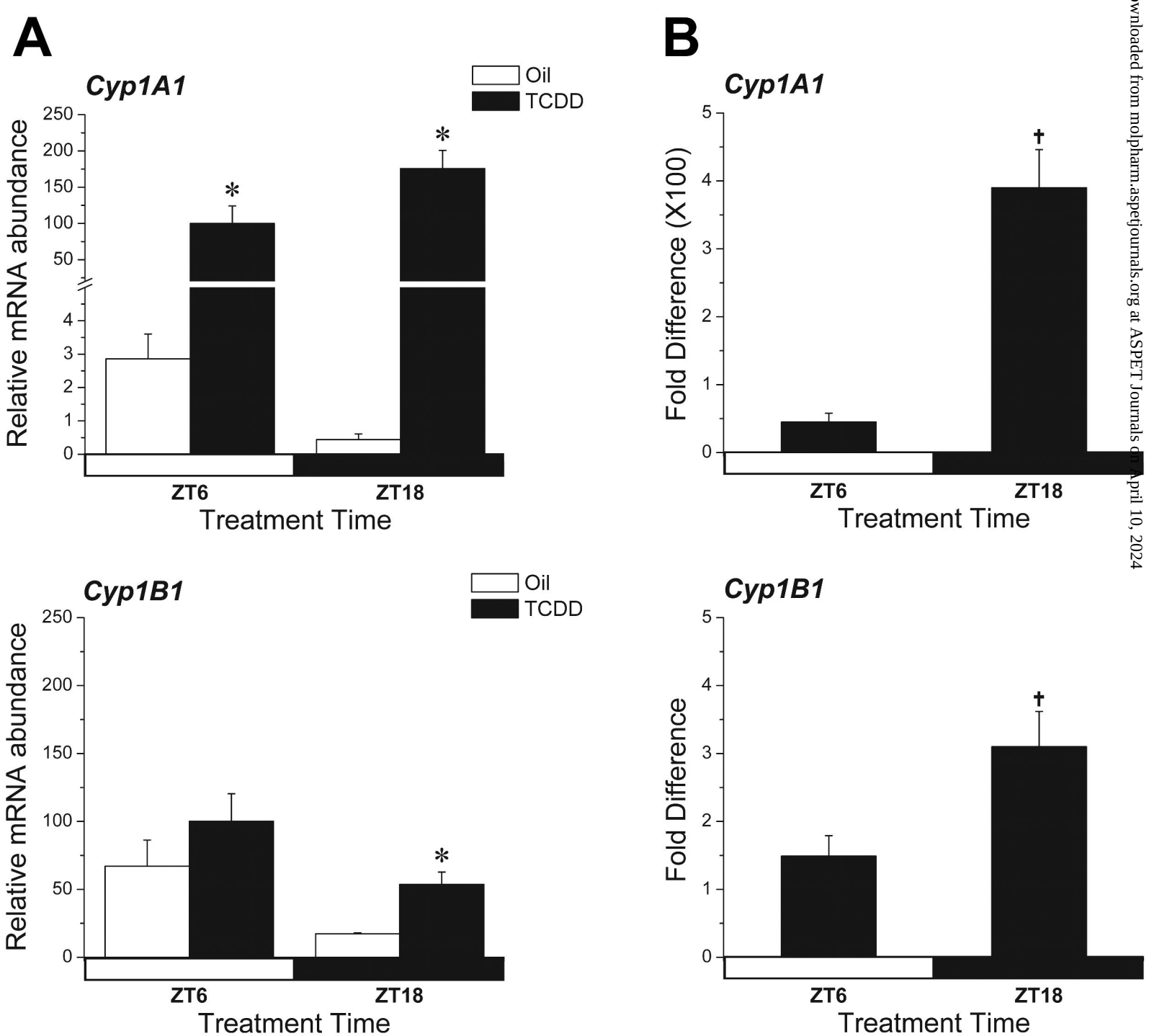

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Figure 6

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