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Title Page

Circadian Regulation of Benzo[a]Pyrene Metabolism and DNA Adduct Formation in Breast Cells and the Mouse Mammary Gland

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Running Title

Circadian Regulation of BaP Metabolism

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A list of abbreviations:

AhR: aryl hydrocarbon receptor

BaP: Benzo-a-Pyrene

PAHs: Polycyclic aromatic hydrocarbons

ZT: Zeitgeber

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Abstract

The circadian clock plays a role in many biological processes, yet very little is known about its role in metabolism of drugs and carcinogens. The purpose of this study was to define the impact of circadian rhythms on benzo-a-pyrene (BaP) metabolism in the mouse mammary gland and develop a circadian *in vitro* model for investigating changes in BaP metabolism resulting from cross-talk between the molecular clock and aryl hydrocarbon receptor. Female 129sv mice (12 weeks old) received a single gavage dose of 50 mg/kg BaP at either noon or midnight, and mammary tissues were isolated 4 or 24 hours later. BaP-induced *Cyp1a1* and *Cyp1b1* mRNA levels were higher 4 hours after dosing at noon than at midnight and this corresponded with parallel changes in *Per* gene expression. In our *in vitro* model, we dosed MCF10A mammary cells at different times after serum shock to study how time of day shifts drug metabolism in cells. Analysis of *CYP1A1* and *CYP1B1* gene expression showed the maximum enzyme-induced metabolism response 12 and 20 hours after shock, as determined by EROD activity, metabolism of BaP and formation of DNA-BaP adducts. The pattern of *PER*, *BMAL* and AhR-induced *CYP* gene expression and BaP metabolism was similar to BaP-induced *Cyp1A1* and *Cyp1B1* and molecular clock gene expression in mouse mammary gland. These studies indicate time of day exposure influences BaP metabolism in mouse mammary gland and describes an *in vitro* model that can be used to investigate the circadian influence on the metabolism of carcinogens.

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Introduction

The circadian clock plays a key role in coordinating many biological processes from behaviors to cellular metabolism and mitosis (Okamura et al., 2002; Reppert and Weaver, 2001). In vertebrates, these physiological responses are controlled by the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. Peripheral tissues including the liver, heart, kidney and mammary gland contain functional endogenous clocks (Metz et al., 2006; Yamazaki et al., 2000; Zylka et al., 1998), which regulate numerous physiological processes including cell proliferation and apoptosis. The molecular basis of the circadian clock centers on a "core loop" of several members of the Per-Arnt-Sim (PAS) family of proteins. The so-called "positive elements", CLOCK and BMAL1 (MOP3/ARNTL1) heterodimerize via their PAS domains and activate transcription of target genes through "E-box" enhancer sequences. CLOCK/BMAL1 dimers also stimulate transcription of the "negative elements" of the circadian clockworks, including the *PERIOD* genes, *PER1* and *PER2*, which contain PAS domains, and the PER-independent cryptochromes (CRY1 and CRY2). PER/CRY heterodimers exert a negative feedback function and repress *CLOCK/BMAL1* dependent transactivation. As PER and CRY proteins are degraded, this repression is released and the process begins again with a periodicity of about 24 hours.

Studies are now suggesting that frequent jet lag or shifts of daily rhythms as a result of rotating shift work can lead to many deleterious health outcomes. Approximately 15 million women in the U.S. work alternative shifts (i.e. night shifts), with a higher number of African Americans (21%) working relative to Caucasians (14%). Disruption of circadian rhythms can result in significant physiological consequences including sleep disorders, heart disease (Tobaldini et al., 2016), gastrointestinal disease (Knutsson, 2003) diabetes (Turek et al., 2005),

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immunosuppression (Moldofsky et al., 1989) reproductive dysfunction (Knutsson, 2003) (Baker and Driver, 2007) and increased cancer risk (Navara and Nelson, 2007). Once seen as controversial, the link between circadian disruption and cancer has gained increasing support from animal and human epidemiological studies. This was supported by the Nurses' Health Study, which showed that the risk of developing breast cancer is higher in women alternative shifts (Dickerman and Liu, 2012; Knutsson, 2003; Knutsson et al., 2012; Leonardi et al., 2012; Megdal et al., 2005; Poole et al., 2011; Reed, 2011; Schernhammer et al., 2001; Stevens, 2009; Wang et al., 2011), with the most compelling evidence of increased breast cancer risk in women working night shifts for 20 years or more (Davis et al., 2001; Hansen, 2001; Kolstad, 2008; Schernhammer et al., 2001). This led the International Agency for Research on Cancer (IARC), a unit of the World Health Organization, to declare shift work a probable carcinogen (Stevens, 2009; Straif et al., 2007).

Polycyclic aromatic hydrocarbons (PAHs), including benzo-a-pyrene (BaP), found in cigarette smoke and as a by-product of combustion and grilled food, are well characterized as carcinogens. Analysis of adjacent normal tissue from breast cancer patients has shown an increase in BaP-DNA adducts as compared to controls, suggesting an association between PAH exposure and breast cancer risk (Li et al., 1996). PAHs, such as BaP, mediate their effects primarily through interaction with the aryl hydrocarbon receptor (Camus et al., 1984) to induce CYP1A1 and CYP1B1 which in turn metabolically activate BaP to it's carcinogenic metabolites leading to cancer initiation and progression (Chaloupka et al., 1995; Noren and Meironyte, 2000; Safe et al., 1998; Safe and Wormke, 2003).

As we move towards a "24 hour society", understanding the effects of circadian rhythms and the consequences of circadian disruption on the outcome of chemical exposures should be an

important consideration in risk assessment of environmental and occupational contaminants. *Toxicology in the 21st Century* has urged researchers to develop "better toxicity assessment methods to quickly and efficiently test whether certain chemical compounds have the potential to disrupt processes in the human body that may lead to negative health effects" (Krewski et al., 2010). Therefore, it is imperative to study PAH metabolism and timing of exposure to relevant environmental contaminants such as BaP to identify windows of susceptibility that can lead to DNA damage, and perhaps the development of disease. Despite the fact that circadian rhythms are associated with critical biological processes, very little is known about its temporal effects on metabolism of carcinogens, such as BaP. The purpose of this study was to define the impact of circadian rhythms on BaP metabolism and develop an *in vitro* screening model to determine circadian-dependent changes in BaP metabolism and DNA-adduct formation resulting from cross-talk between the molecular clock and the aryl hydrocarbon receptor (Camus et al.) pathway.

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Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM-F12), Dulbecco's phosphate buffered saline (PBS), benzo[a]pyrene (BaP), ethoxyresorufin, 3,3′-methylene-bis(4-hydroxycoumarin) (dicumarol) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 3-Hydroxybenzo[a]pyrene (3OH), benzo(a)pyrene-*trans*-7, 8-dihydrodiol(+/−) (t7,8), benzo[a]pyrene-r-7,*t*-8-dihydrodiol-*t*-9,10-epoxide(±), (Tobaldini et al.) (BPDE), and pyrene (Pyr) were purchased from Midwest Research Institute (Kansas City, MO) which operates the Chemical Carcinogen Reference Standard Repository. Analytical data provided with each standard was reported as >99% pure by HPLC and UV/visible spectra. These UV/visible spectra were also confirmed prior to use in cells. Tissue culture flasks, 2-well Lab-Tek chambered cover glass slides were purchased from Thermo Fisher Scientific (Waltham, MA) and multi well plates were purchased from Corning Inc. (Corning, NY). BaP, 3OH, t7,8 were each prepared as 1 mM stocks in DMSO. Ethoxyresorufin was prepared as a 1 mM stock in methanol and diluted to 5 μM for EROD activity measurement.

Experimental Design for In Vivo Studies

Female 129sv mice were obtained from Charles River (Wilmington, MA). All animals were housed 3 per cage and maintained under a standard 12-hour photoperiod (LD 12:12; lights-on at 0600hr or Zeitgeber Time [ZT] 0). Animals were provided with access to food and water *ad libitum*. The University Laboratory Animal Care Committee at Texas A&M University approved the procedures used in this study. Mice were gavaged with BaP (50mg/kg in sesame

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seed oil or control) at ZT18 (midnight) or ZT6 (noon). Mammary glands were then harvested 4 hours or 24 hours after dosing of BaP or vehicle (Vh) control. These data are represented as ZT6 4 for animals dosed at noon and harvested 4 hours later, ZT6 24 for animals dosed at noon and harvested 24 hours later, ZT18 4 for animals dosed at midnight and harvested 4 hours later, and finally ZT18 24 for animals dosed at midnight and harvested 24 hours later. Mammary gland tissues were then isolated for RNA and real-time PCR analysis was performed.

Cell Culture

MCF10A human breast epithelial cells and MCF7 breast tumor cells were purchased from the American Tissue Type Culture Collection (Rockville, MD). MCF10A cells were grown in DMEM/F12 media supplemented with 5% Donor Horse Serum (DHS), epidermal growth factor (100 mg/ml), hydrocortisone (1mg/ml), cholera toxin (1mg/ml), insulin (10mg/ml), and penicillin streptomycin. MCF7 cells were grown in 10% Fetal Bovine Serum (FBS) DMEM. Cells were incubated at a constant temperature of 37^{o}C with a humidified atmosphere of 5% CO_2 and 95% air. Lentiviral transduction of MCF10A cells using PER2 shRNA was performed as previously described (Hill et al, Laffin et al MCB 2008).

Clock and Drug Metabolism Genes Under Serum Shocked and Normal Culture Conditions

MCF10A and MCF7 cells were grown to confluence in 6-well plates. At confluence, the cells were rinsed and the media on the cells was dropped to 0.5% DHS for MCF10A cells and 0.5% FBS for MCF7 cells overnight. The next morning cells were then rinsed and shocked with 50% DHS or FBS for two hours, cells were rinsed and changed back to 0.5% DHS or FBS and dosed with 1 μM BaP or DMSO control at the indicated time. In the no shock cells, cells were

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grown in normal 5% or 10% media until cells were 70% confluent and dosed with 1 μM BaP or DMSO control.

RNA Extraction, cDNA Synthesis, and Real-Time PCR

Total RNA isolation was performed using the High Pure RNA Isolation Kit (Roche Diagnostics). Reverse transcription was performed using 1μg of RNA template with the iScript cDNA Synthesis Kit (Bio-Rad). PCR primers that were used for the *in vivo* and *in vitro* studies are listed in Table 1 as well as we described (Metz et al., 2006). Quantitative PCR was performed using 50ng cDNA using goTaq (Promega) in a CFX384 (BioRad) as we described (Metz et al., 2006).

Ethoxyresorufin-O-deethylase (EROD) Activity

EROD activity is a biomarker of exposure to planar halogenated and polycyclic aromatic hydrocarbons (PHHs and PAHs, respectively) and provides evidence of aryl hydrocarbon receptor-mediated induction of cytochrome P450-dependent monooxygenases (Donato et al., 1993). To identify the induction of EROD activity, cells were plated on 96 well-plates at 10,000/well for 24 hours and rhythms were induced as indicated above and treated for the indicated times. Following two washes with PBS, plates were then loaded with a mixture of 5 μM ethoxyresorufin and 10 μM dicumarol for 30min. EROD activity was measured using a BioTek Synergy 4 plate reader (Biotek Instruments Inc., Winooski, VT, USA) with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The Thermo Scientific Pierce Coomassie Protein Assay Kit was then used to measure the total protein concentration per well using the Biotek Synergy 4 plate reader set to an absorbance of 590 nm and EROD fluorescence

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intensities measured were normalized accordingly. Eight samples per treatment were collected and at least 3 experiments were performed on different days.

Circadian Rhythm Effects on BaP Metabolites

To determine BaP metabolism *in situ* within living cells, we utilized a mathematical approach using multiphoton microscopy combined with advanced linear spectral unmixing analysis (Barhoumi et al., 2009; Barhoumi et al., 2014; Barhoumi et al., 2011; Wu et al., 2011). To evaluate the effect of circadian rhythms on BaP metabolism, cells were plated in 2-well Lab-Tek chambered cover glass slides and treated according to the experimental design. Slides were transferred to the stage of a Zeiss 780 META NLO laser-scanning microscope (Carl Zeiss Microimaging, Thornwood, NY). Spectral analysis of an area of 225 x 225 μm (typically containing 25 to 40 cells) was performed while irradiating cells with a Chameleon tunable Ti:Sapphire laser (Coherent Inc., Santa Clara, CA) at an excitation wavelength of 740 nm (which is roughly equivalent to 370 nm in single photon excitation with a continuous wavelength laser system). Using the lambda stack algorithm software available with the Zeiss 780 META NLO instrument, a fluorescence emission spectrum ranging from 399-600 nm with a 9 nm bandwidth was recorded for each scanned area. Fifteen to thirty areas were scanned per treatment. All images were collected with a Plan-Apo 40x/1.4 NA oil immersion objective. Images were then saved and later analyzed using the linear unmixing method described previously (Zimmermann et al., 2014). BaP metabolites present in each treatment were then identified using the linear unmixing process based upon the use of a spectral database of BaP and metabolite standards generated under the same experimental conditions as previously described (Barhoumi et al., 2011).

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Fluorescence Lifetime Imaging (FLIM)

Cells were cultured in 2-well Lab-Tek chambered cover glass slides and treated according to our experimental design. An area was selected and scanned with an inverted LSM 780 microscope and a 1.4NA 40x oil objective. Two-photon excitation was provided by a Chameleon (Coherent) Ti:sapphire laser tuned to 760 nm. Emission events were registered by an external hybrid detector (HAMAMATSU R10467U-40) powered by PCI based internal board. The PMT signal is collected by Spartan6 based FastFLIM box (ISS). FLIM images (256 x 256 pixels) were acquired with a pixel dwell time of 6.3µs. Lifetime of bound and free NAD(P)H (Blacker et al., 2014) was determined with the frequency domain technique using the VistaVision software (ISS, Champaign, Illinois). 6-10 images were collected per time point and lifetime was measured using the region average available with ISS VistaVision Suite version 4.1.

Statistical Analysis

Data was analyzed using JMP Pro version 11. Where appropriate, data is represented by the mean ± SEM. Data were normalized to Claudin-7 for *in vivo* studies and normalized to beta-Actin for *in vitro* studies and then normalized to a fold change of "1" for qPCR analysis. For analysis of the mammary gland *in vivo*, statistical analyses were performed using a two-way ANOVA to determine whether mRNA levels for a given gene were significantly different across different time points and treatments. A Tukey post hoc analysis was used if significant main effects were observed between BaP treated animals or vehicle control. To analyze EROD and lifetime data, a one-way ANOVA was used with a Tukey post hoc analysis to determine

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significance over time. In addition, BaP metabolites were analyzed using a two-way ANOVA with a Bonferroni post hoc analysis. The α value was set at 0.05 for all statistical analyses.

Results

Circadian Regulation of BaP-induced Ah-responsive Genes in the Mouse Mammary Gland

To investigate the impact of the circadian clock on drug metabolism genes in the mouse mammary gland, 12-week-old 129sv female mice received a single gavage dose of 50 mg/kg BaP at either 12:00 PM (ZT6) or 12:00 AM (ZT18), and mammary tissues were isolated 4 or 24 hours later (Figure 1). Confirmation of circadian rhythm gene expression found that *Per1* expression at ZT6 24, ZT18 4, and ZT18 24 was significantly different as compared to ZT6 4 (Figure 1A), whereas *Bmal* expression was out of phase with *Per1* peaking at ZT18 4 (Figure 1B), verifying our previous studies (Metz et al., 2006). BaP-induced *Cyp1a1* was significantly higher (172%) in mammary tissue 4 hours after treatment at ZT6 compared to treatment at ZT18 and similar differences were observed at 24 hours after treatment even though overall activity was decreased (Figure 1C). We found that *Cyp1b1* expression was significantly higher (169%) in animals dosed at ZT6 compared to ZT18 whereas these differences were not observed after 24 hours (Figure 1D). With the exception of the latter treatment group the induction (*Cyp1a1/Cyp1b1*) was parallel to the temporal changes observed for *Per1* gene expression (Figure 1A). Analysis of *Ahrr* gene expression showed a trend between ZT6 and ZT18, but no significant difference in time or Bap-dependent induction (Figure 1E).

Establishment of an MCF10A Circadian PAH Metabolism Model

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It has also been reported that circadian rhythms can also be induced in cell culture by serum shock, which has been extensively used as an *in vitro* approach to dissect the molecular pathways regulated by circadian rhythms (Balsalobre et al., 1998; Duffield et al., 2002; Gachon et al., 2004; Tamanini, 2007b). Analysis of the oscillatory expression of circadian regulated genes and reporter constructs in serum shocked primary and established cell lines, including normal breast and breast cancer cells, have similar circadian patterns of gene expression observed *in vivo* (Balsalobre et al., 1998; Goh et al., 2007; Huang et al., 2009; Jakubcakova et al., 2007; Nader et al., 2009; Nagoshi et al., 2004; Pan et al., 2010; Rossetti et al., 2012; Tamanini, 2007a; Wu et al., 2007; Xiang et al., 2012; Yin et al., 2010; Zvonic et al., 2007). To investigate an *in vitro* model for studying circadian effects on BaP metabolism (Figure 2), we shocked normal human breast MCF10A cells and treated with BaP and then isolated mRNA every 4 hours for 24 hours. Analysis of molecular clock genes in MCF10A cells showed that *PER1* and *PER2* expression peaked at 0 hour and 24 hours following serum shock (Figure 2A, B), whereas *BMAL* and *CLOCK* were inversely expressed with maximum induction at 12 hours and 20 hours, respectively (Figure 2C, D). Similar to previous studies (Rossetti et al., 2012), we found that MCF7 cells did not show a significant difference in circadian rhythm gene expression upon serum shock (data not shown), therefore, we focused the remainder of our BaP-induced circadian rhythm studies on the MCF10A cell line.

Utilizing the MCF10A cell circadian rhythm model, we compared BaP-induced AhR genes with no shock (Figure 3A), immediately following shock (0 hour; Figure 3B) and at the peak (12 hours; Figure 3C) and trough (20 hours; Figure 3D) of *PER* and *BMAL* gene expression, replicating *in vivo* differences in day and night. Analysis of un-shocked normal cycling MCF10A cells treated with either 1□M BaP or DMSO harvested every 4 hours for 24

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hours after dosing showed that *CYP1A1* and *CYP1B1* expression peaked 4 hours after dosing and *AHRR* mRNA was highest 12 hours after exposure to BaP. In MCF10A cells dosed immediately after shock, we observed a shift in peak *CYP1A1* and *CYP1B1* gene expression to 16 hours after dosing and a 2-and 4-fold increase in *CYP1A1* levels and *AHRR* mRNA compared to that observed in no shocked cells (Figure 3A). The highest level of *CYP1A1* and *CYP1B1* gene expression was observed in cells dosed 12 hours after shock (Figure 3C) correlating with *PER* expression *in vitro* and in the mouse mammary gland (Figure 1A). In contrast, *AHRR* levels were lower as compared to the 0 hour treatment. Surprisingly, treatment of cells with BaP at the highest level of *PER* expression, 20 hours following shock (Figure 3D), resulted in decreased *CYP1A1*, *CYP1B1*, and *AHRR* mRNA at levels comparable to the saw mRNA at levels comparable to those observed in the no shock cells (Figure 3A). Together, these data demonstrate that induction of circadian and BaP-induced genes in the MCF10A circadian rhythm model correspond with expression patterns in the mouse mammary gland.

To confirm a direct relationship between the molecular clock and BaP metabolism, we knocked down *PER2* in MCF10A cells using a shRNA-PER2 as previously described (Xiang et al., 2012). Comparison of *PER2* and *BMAL* expression in control and *shPER2* cell lines showed a decrease in *PER2* following shock and expected increase in *BMAL* gene expression (Figure 4A). Immediately following shock, we observed no difference in BaP-induced *CYP1A1* and *CYP1B1* gene expression in the control and *shPER2* cells, whereas *AHRR* induction was significantly decreased with loss of *PER2* (Figure 4B). Treatment of *sh*PER2 cells with BaP 12 hours following shock, at the highest level of *CYP1A1* and *CYP1B1* expression, showed a sharp increase in gene expression that was not as sustained as compared to the control cells (Figure 4C). A similar was response was observed 20 hours after BaP treatment with higher induction of

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CYP1A1, *CYP1B1* and *AHRR* expression as compared to controls (Figure 4D) and correlates with the higher level of *BMAL* and decreased *PER2*.

To determine if BaP affects the molecular clock in our MCF10A circadian rhythm model, we analyzed *PER1* (Figure 5A,D), *PER2* (Figure 5B,E) and *BMAL* (Figure 5C,F) expression in serum shocked MCF10A cells dosed with BaP immediately after shock and harvested every 4 hours for 24 hours. Other than one data point that had a significant difference in *PER2* expression at 20 hours (Figure 5E), we did not see a change in either the phase or magnitude in the positive or negative arms of the clock. These data confirm our results in the mouse mammary gland demonstrating that activation of the AhR by BaP does not affect molecular clock genes.

Circadian Regulation of EROD Activity and Multiphoton Analysis of BaP Metabolites in MCF10A Cells

We have shown that BaP dependent induction of AhR target genes is under circadian control; however, we have not determined if this response translates over to metabolic activity. Metabolic activation of BaP is primarily mediated by induced *CYP1A1* protein that can be determined by its associated phase 1 CYP1A1-dependent enzyme, ethoxyresorufin-o-deetheylase (EROD). For theses studies, we compared EROD activity in normal cycling MCF10A cells and shocked MCF10A cells dosed with 1 □M BaP every 4 hours for 24 hours. Cells were harvested 24 hours after dosing to account for the delay in CYP1A1 enzyme activity. In the no shock cells, we observed a 234% increase in EROD 24 hours after treatment with BaP. However, in the shocked cells we found that EROD activity was significantly magnified with an over 822% increase 16 hours after dosing (Figure 6A).

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To determine if the circadian-dependent difference in EROD activity affected BaP metabolism, we utilized an established mathematical method using multiphoton microscopy combined with advanced linear spectral unmixing analysis (Barhoumi et al., 2009; Barhoumi et al., 2011; Wu et al., 2011). Utilizing this approach, we measured differences in the BaP phenol (3-hydroxybenzo[a]pyrene [3-OH BaP] metabolite and the bulky DNA adducted (benzo[a]pyrene-r-7,t-8dihyrodiol-t-9, 10-epoxide(+/-) (BPDE) identified by its pyrene backbone in normal cycling MCF10A cells and serum shocked MCF10A cells dosed with BaP every 4 hours and harvested 24 hours later (Tobaldini et al.). Our results show that the 3-OH BaP and DNA bound pyrene metabolites peaked at 20 hours (Figure 6B,C), 4 hours after the maximum observed EROD activity.

Discussion

Disruption of circadian rhythms can increase susceptibility to multiple cancers; however, it is surprising that little is known about the consequences of night shift work and cancer incidence in populations exposed to high levels of carcinogens. Industries with the potential for high PAH exposures, such as smelting, coal mining and processing, coke production, iron and steel foundries, tar production, petrochemical workers and road pavers, often operate around the clock. Individuals working in these industries show an increased risk for cancer overall, without taking shift work into account (Boffetta et al., 1997). Increased light at night due to urban sprawl and recent evidence that hand held devices can disrupt circadian rhythms may also contribute to increase risk of cancer and other diseases (Smolensky et al., 2015). Many toxicological studies take into account the time of dosing, harvesting and/or analyses to exclude circadian variation. Understanding the effects of circadian rhythms and the consequences of circadian disruption on

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the outcome of chemical exposures should be an important consideration in risk assessment of environmental and occupational contaminants. In this study, we show that time of day exposure influences BaP metabolism in the mouse mammary gland and introduce an *in vitro* model that can be used to screen and establish guidelines for reducing human risk from PAH exposure by determining times of the day PAHs or other compounds are more likely to exert toxic effects.

Epidemiological studies have demonstrated that disrupted circadian rhythms are associated with increased risks for cancer (Davis and Mirick, 2006). PAHs exert their genotoxic effects by activating AHR-mediated transcription of CYP genes (Nebert and Dalton, 2006; Nebert et al., 2004). We have previously shown that the degree of *Cyp* inducibility in mammary glands is dependent upon time of day animals are exposed and hyper-induced in *Per* mutant mice (Qu et al., 2007). We found that 24 hours after treatment with the prototypical AHR ligand, 2,3,7,8-tetrachloro-dibenzo-para-dioxin (TCDD), *Cyp1a1* and *Cyp1b1* induction was higher in the mammary glands of animals treated at 12:00 AM than in those dosed at 12:00 PM (Qu et al., 2007). The *in vivo* results obtained in this study expanded upon these observations (including a time point 4 hours after treatment) to observe the metabolism of BaP over time and correlates to the circadian patterns of *Per1* and *Bmal* inducibility of *Cyp1a1* and *Cyp1b1*. We observed that BaP-induced *Cyp1a1*, and *Cyp1b1* were higher 4 hours after dosing at ZT6 (noon) than at ZT18 (midnight) as compared to TCDD, which showed highest induction when dosed at ZT18 (Qu et al., 2007). A similar response was seen in lungs of mice dosed with BaP, which found a significant increase in *Cyp1a1* when dosed at ZT 21 and harvested two hours later as compared to ZT9 (Tanimura et al., 2011). Interestingly, this differential response may be due to tissue-specific differences in *AhR* circadian expression as *AhR* mRNA levels peak at ZT 21 in the lung and at ZT 6 in the mouse mammary gland and ovary (Qu et al., 2007; Tanimura et al., 2011;

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Tischkau et al., 2011). Moreover, since TCDD is poorly metabolized and therefore has a long half-life (reported as 8-14 days in mice, (Miniero et al., 2001)), the difference in TCDD and BaP in the mammary gland response may be due to the shorter-lived BaP (reported as 0.6-1.23 days (Camus et al., 1984)). These studies also do not take into account potential extra-mammary gland metabolism, including the liver, which may contribute to BaP metabolism; however, oral administration of BaP has been shown to induce BaP adducts in rodent mammary glands (Walker et al., 1992). These results suggest that circadian regulation of the AhR is tissue and ligand specific.

Time of exposure or disruption of circadian rhythms through shift work or individual clock components increases susceptibility to BaP-induced genotoxicity through the interaction of multiple pathways; however, to date, *in vivo* models have been the only method to measure the potential impact of circadian rhythms on environmental exposure. Taking advantage of the ability to induce the molecular clock in cell culture, we treated MCF10A cells at different times after serum shock to study how time of day exposure shifts drug metabolism in the cell using BaP as a model carcinogen. Analysis of *CYP1A1* and *CYP1B1* gene expression found the maximum BaP dependent response 12 and 20 hours after shock, corresponding with EROD activity (Figure 6A), and the metabolism of BaP and presence of the 3OH, and DNA bound Pyrene (Figure 6B,C). Indeed, the pattern of *PER1*, *PER2*, *BMAL* and AhR-induced *CYP* gene expression and BaP metabolism was similar to BaP-induced *Cyp1a1* and *Cyp1B1* and molecular clock gene expression in the mouse mammary gland (Figure1). These results showed that *Bmal* expression was higher at ZT18 and inversely expressed with *Per1* and BaP-induced *Cyp1a1*/*Cyp1b1* expression. This is consistent with our previous studies and other reports showing hyper-induced *Cyp1a1* in *Per* mutant mice, correlating with maximum Bmal

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transactivation and peak *Per* expression (Koike et al., 2012; Metz et al., 2006). There are few reports investigating circadian rhythm expression in humans, which is further complicated by the time of day difference in activity and food intake between rodents and humans. We have shown in virgin mouse mammary glands that *Per2* levels are under circadian control peaking at ZT12, which is similar to peak *PER2* expression in human mucosa cells (Cajochen et al 2006). Indeed, disruption of normal rhythms with light at night was shown to induce *PER2* in buccal cells, disrupting the molecular clock. Our observation that BaP-induced gene expression is influenced by the molecular clock supports the hypothesis that shift work or light at night could influence carcinogen metabolism. Thus our results are consistent with other studies showing that AhR and Bmal may interact to regulate gene expression (Tischkau et al., 2011).

There is conflicting evidence in the literature that activation of the AhR by TCDD and other AhR ligands can influence the regulatory expression of the core clock genes. In the mouse it was shown that TCDD suppressed *Per1* expression in hematopoietic and liver stem and progenitor cells and (Garrett and Gasiewicz, 2006; Xu et al., 2010). Similarly, activation of the AhR also suppressed *Per1* in a hypothalamic cell line (Solak et al., 2013). However, analysis of a *Per2-Luc* reporter mouse found that treatment with TCDD did not alter the amplitude or phase of *Per2* expression in multiple tissues and *AhR* knockout mice have an intact circadian response (Mukai et al., 2008; Pendergast and Yamazaki, 2012). Our results show that treatment with BaP did not effect molecular clock gene expression the mouse mammary gland (Figure 1) or cycling MCF10A cells (Figure 5).

Taken together, these results demonstrate the importance of conducting research in an *in vivo* and *in vitro* model to study circadian rhythms. The experiments performed in this study address the hypothesis that the circadian clock influences BaP-mediated metabolism and DNA

damage and repair and that time of day, or disruption of the circadian clock, impacts these responses of BaP metabolism. Environmental factors do not generally act alone, but interact in complex ways, therefore circadian rhythms and exposure to BaP and other PAHs must be studied at multiple levels ranging from isolated cells to animals models. As information becomes available regarding circadian rhythm effects on gene regulation, it is apparent that the molecular clock controls many aspects of normal cell function. With the molecular clock potentially regulating so many of these events, it is expected that these results will contribute to our understanding of PAH exposure and cancer. We determined the effects of PAHs as they interact with circadian "windows of susceptibility" and PAH gene expression and metabolism, and present a novel *in vitro* study design modeling the situation present in many vulnerable working populations such as shift workers in industrial settings or airline crews exposed to cosmic radiation and frequent jet lag.

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Author Contributions

Participated in research design: Schmitt, Barhoumi, Metz, and Porter.

Conducted experiments: Schmitt, Barhoumi, Metz, and Porter.

Contributed new reagents or analytic tools: Barhoumi and Porter.

Performed data analysis: Schmitt, Barhoumi, Metz, and Porter.

Wrote or contributed to the writing of the manuscript: Schmitt, Barhoumi, and Porter.

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Footnotes

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Legends for Figures

Figure I: *In Vivo* model of female mice dosed with BaP at either noon (ZT6) or midnight (ZT18). Mammary glands were harvested either 4 hours or 24 hours later. mRNA was analyzed by qPCR and relative level of each gene is plotted on the graphs using a two-way ANOVA. Values are mean +/- SE. Significance versus the respective control (Vh): *$p < 0.05$. Significance versus peak expression time point: †$p < 0.05$.

Figure II: *In vitro* expression and oscillation of clock genes in MCF10A control cells following serum shock or in standard culture conditions. Total mRNA was extracted every 4 hours up to 24 hours and analyzed by qPCR. The relative level of each gene expression is plotted on the graph.

Figure III: *In vitro* expression and oscillation of drug metabolism genes in MCF10A control cells that were not shocked (A), shocked and treated at 0h (B), 12h (C), and 20h (D) with BaP. Total mRNA was extracted every 4 hours thereafter for 24 hours and analyzed by qPCR. The relative level of each gene is plotted as fold induction.

Figure IV: *In vitro* expression and oscillation of PER2 and BMAL genes in MCF10A control cells vs. MCF10A *sh*Per2 cells (A). *In vitro* expression and oscillation of drug metabolism genes in MCF10A control cells vs. MCF10A *shPer2* cells that were shocked and treated at 0h (B), 12h (C), and 20h (D) with BaP. Total mRNA was extracted every 4 hours thereafter for 24 hours and analyzed by qPCR. The relative level of each gene is plotted as fold induction.

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Figure V: *In vitro* expression and oscillation of drug metabolism genes in MCF710A Cells. Total mRNA was extracted every 4 hours thereafter for 24 hours and analyzed by qPCR. The relative level of each gene is plotted on the graphs (A,B,C). We also compared BaP treated MCF10A cells with control MCF10A cells for PER1 (D), PER2 (E) and BMAL (F). Significance versus the respective control (Vh): *$p < 0.05$.

Figure VI: Biomarkers of MCF10A cells exposed to BaP after 24 hours. There is a peak in EROD activity of BaP exposure at 16 hours (A) as confirmed by 3OH-BaP (B) and Pyrene (C) metabolite spectra analysis. Significance versus control * † ††$p < 0.05$; Significance versus BaP * †$p<0.05$ for panel (A). Significance versus control *$p<0.05$ for (B) and (C).

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Tables

Table I. Human primers used in real-time reverse transcriptase-polymerase chain reaction analysis as shown below. Mouse primers have been identified elsewhere (Qu et al., 2007)

Gene	**Primers Sequence**
CYP1A1	Forward: 5'–CCTCTTTGGAGCTGGGTTT–3' Reverse: 5'–AGGCTCCACGAGATAGCAGT–3'
CYP1B1	Forward: 5'–TCTTTACCAGATACCCGGATG–3' Reverse: 5'–CACAACCTGGTCCAACTCAG–3'
AHRR	Forward: 5'–ACCGCGGATGCAAAAGTAAAAG–3' Reverse: 5'–GCTCCTTCCTGCTGAGTAATTGG–3'
CLOCK	Forward: 5'–AAGTTAGGCTGAAAGACGACG–3' Reverse: 5'–GAACTCCGAGAAGAGGCAGAAG–3'
CLAUDIN7	Forward: 5'–CCATGGCCAACTCGGGCCTGCAAC–3' Reverse: 5'–TCACACGTATTCCTTGGAGG–3'
PER1	Forward: 5'–TGGCTATCCACAAGAAGATTC–3' Reverse: 5'–GGTCAAAGGGCTGGCCCG–3'
PER2	Forward: 5'–GGCCATCCACAAAAAGATCCTGC–3' Reverse: 5'–GAAACCGAATGGGAGAATAGTCG–3'
BMAL	Forward: 5'–GGCTCATAGATGCAAAAACTGG–3' Reverse: 5'–CTCCAGAACATAATCGAGATGG–3'

Figure 1

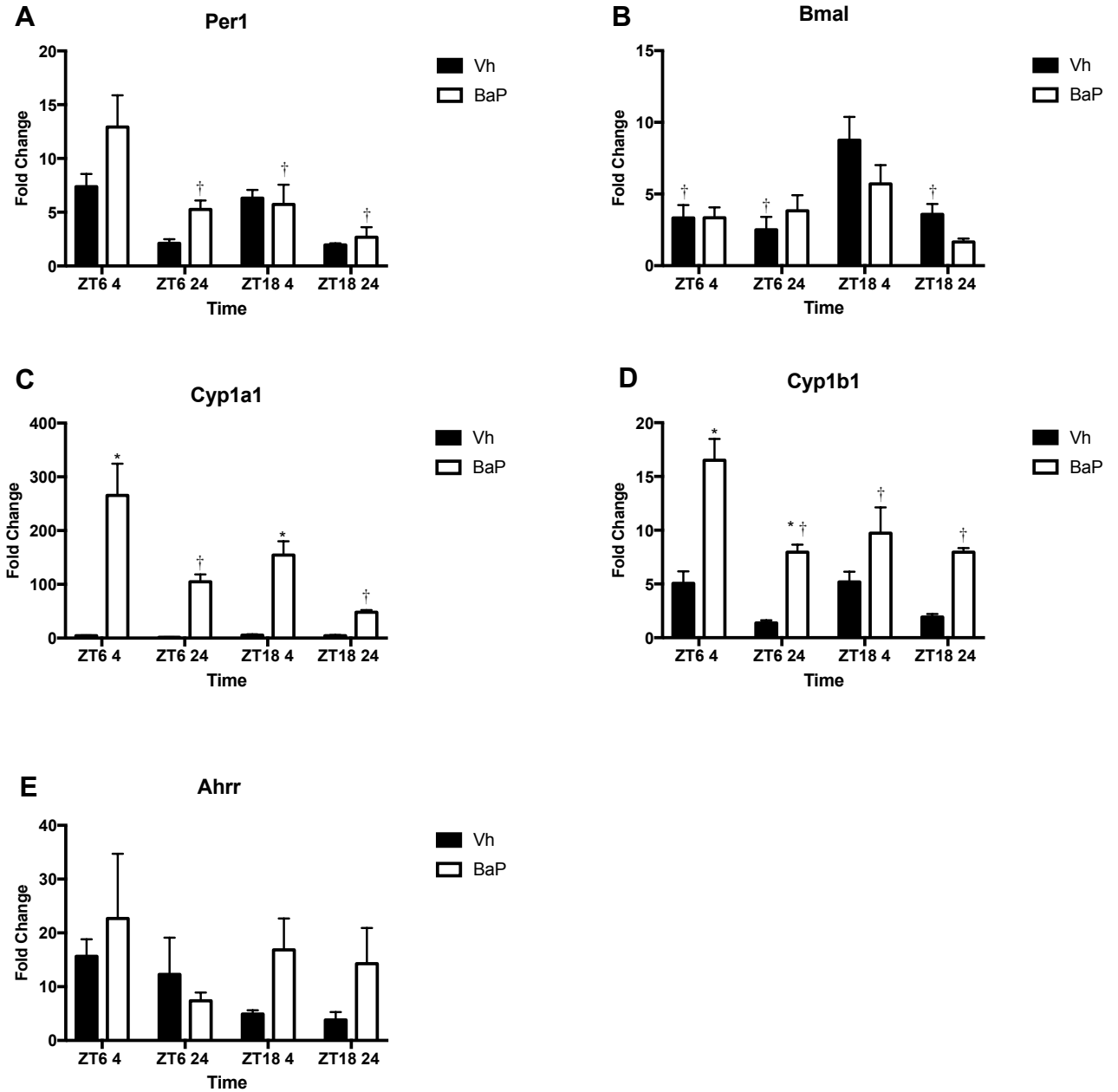

A
Per1
Fold Change
20
15
10
5
0
ZT6 4
ZT6 24
ZT18 4
ZT18 24
Time
Vh
BaP
B
Bmal
Fold Change
15
10
5
0
ZT6 4
ZT6 24
ZT18 4
ZT18 24
Time
Vh
BaP
C
Cyp1a1
Fold Change
400
300
200
100
0
ZT6 4
ZT6 24
ZT18 4
ZT18 24
Time
Vh
BaP
D
Cyp1b1
Fold Change
20
15
10
5
0
ZT6 4
ZT6 24
ZT18 4
ZT18 24
Time
Vh
BaP
E
Ahrr
Fold Change
40
30
20
10
0
ZT6 4
ZT6 24
ZT18 4
ZT18 24
Time
Vh
BaP

Figure 2

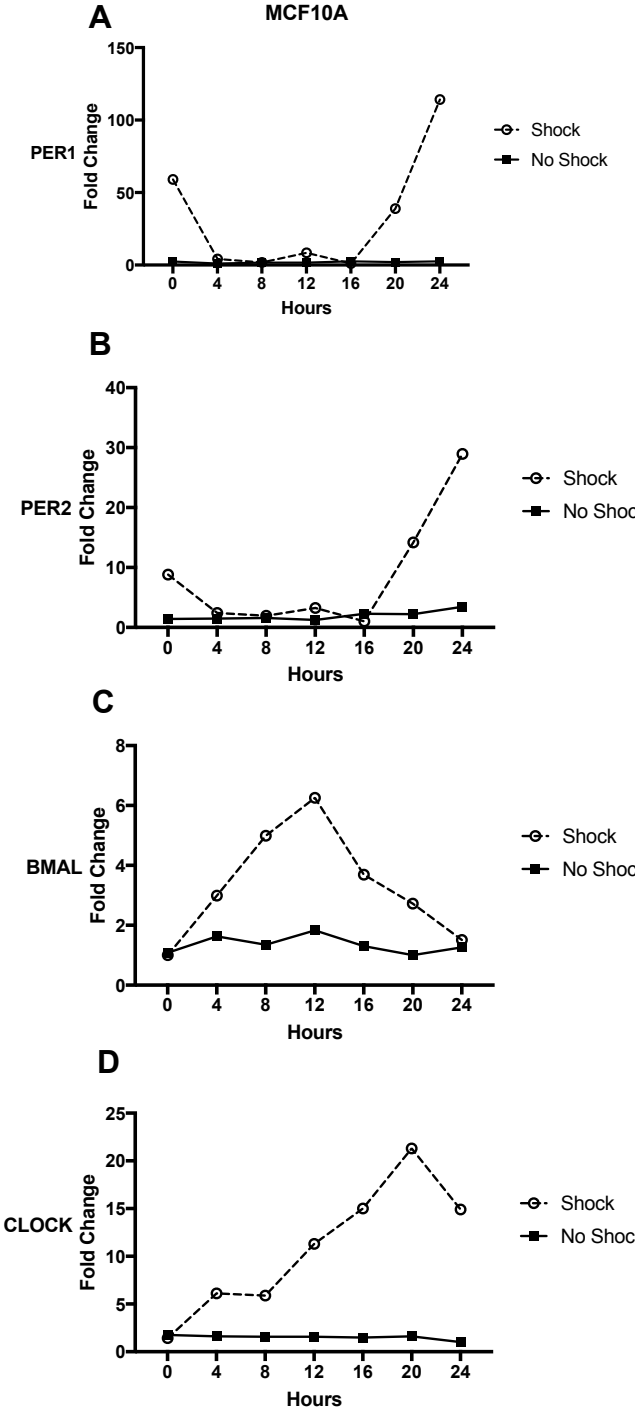

A
MCF10A
PER1
Fold Change
150
100
50
0
0
4
8
12
16
20
24
Hours
Shock
No Shock
B
PER2
Fold Change
40
30
20
10
0
0
4
8
12
16
20
24
Hours
Shock
No Shock
C
BMAL
Fold Change
8
6
4
2
0
0
4
8
12
16
20
24
Hours
Shock
No Shock
D
CLOCK
Fold Change
25
20
15
10
5
0
0
4
8
12
16
20
24
Hours
Shock
No Shock

Figure 3

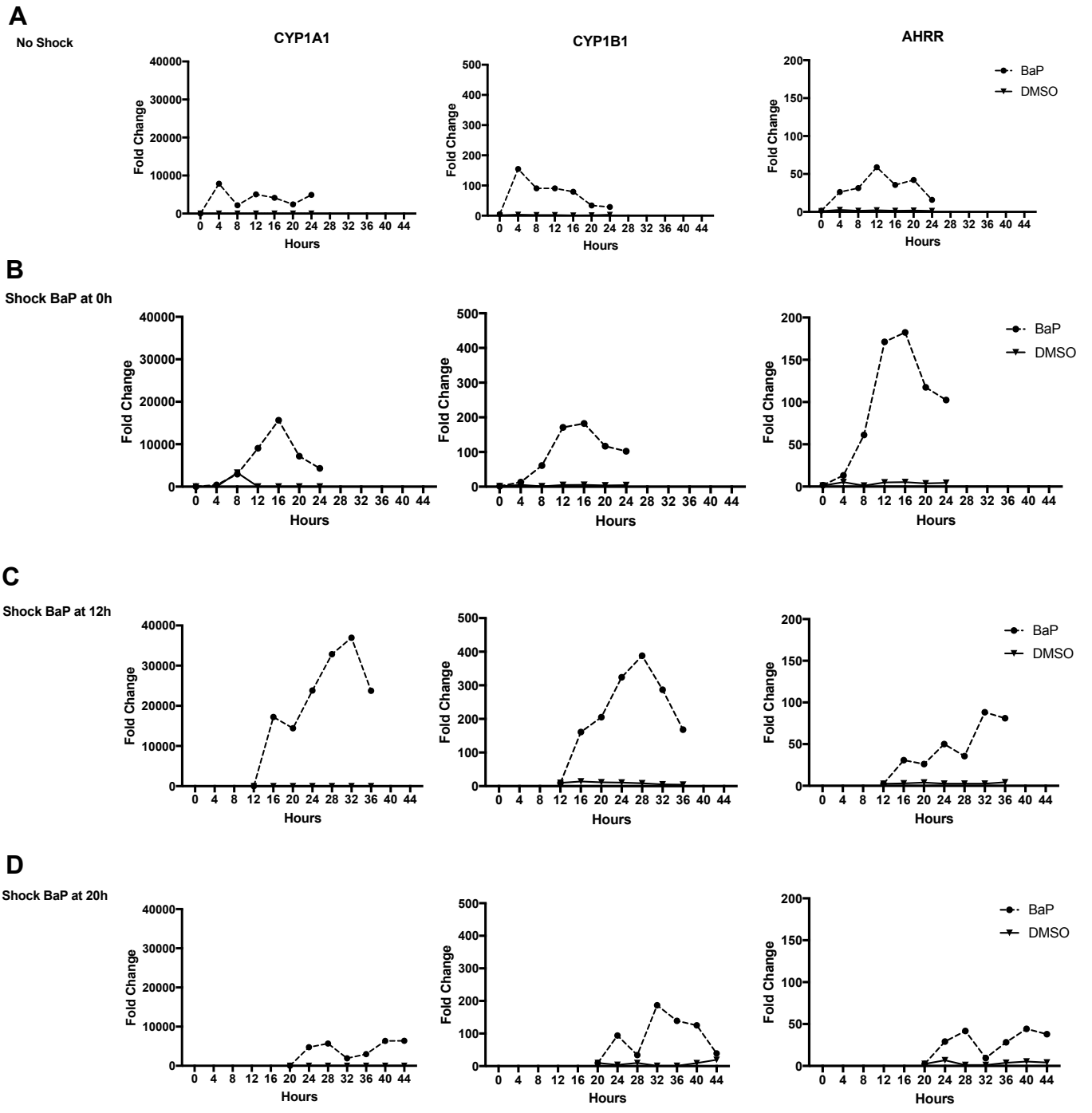

A
No Shock
CYP1A1
CYP1B1
AHRR
Fold Change
Hours
BaP
DMSO
B
Shock BaP at 0h
C
Shock BaP at 12h
D
Shock BaP at 20h

Figure 4

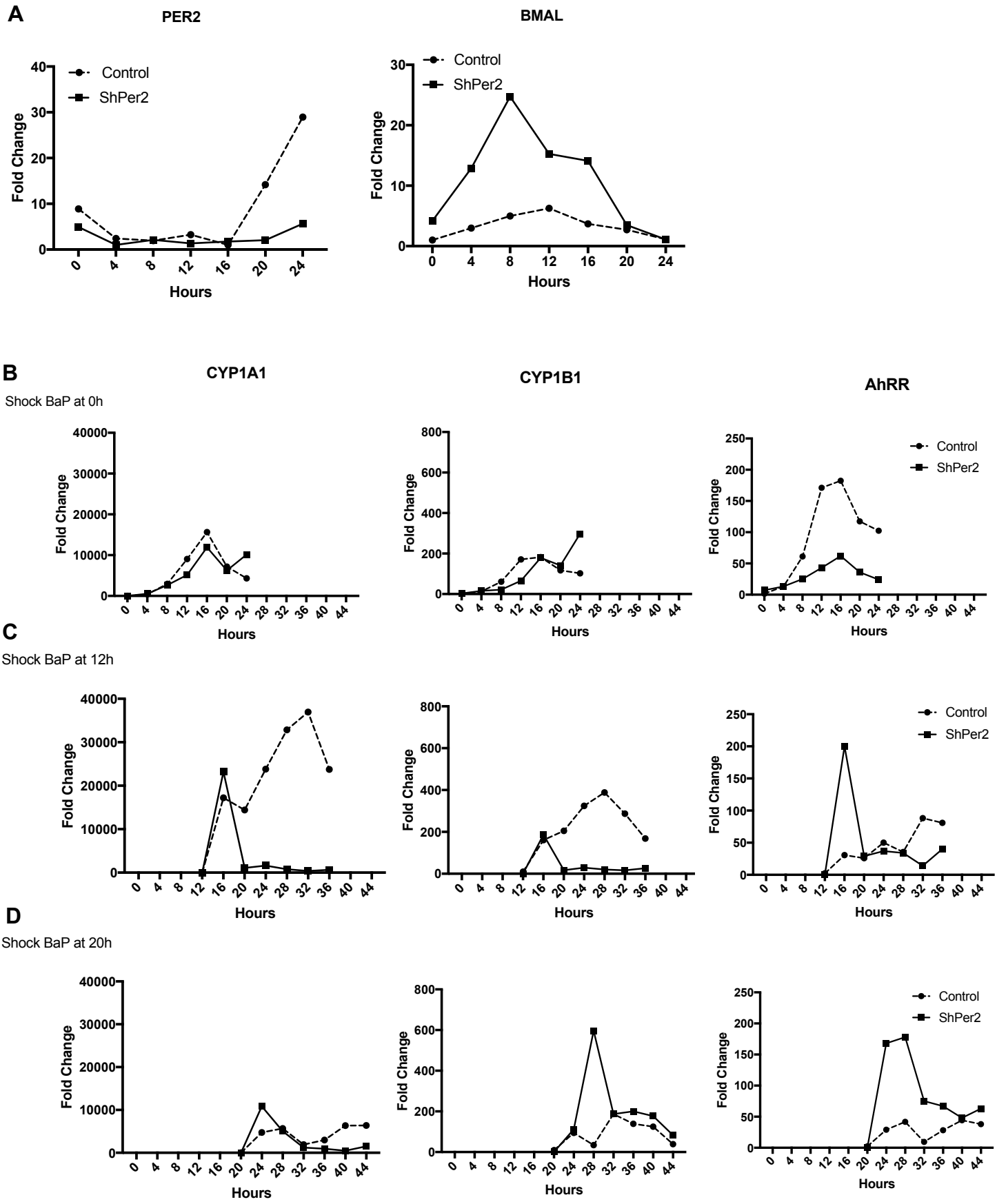

A
PER2
BMAL
Control
ShPer2
Fold Change
Hours
B
CYP1A1
CYP1B1
AhRR
Shock BaP at 0h
C
Shock BaP at 12h
D
Shock BaP at 20h

Figure 5

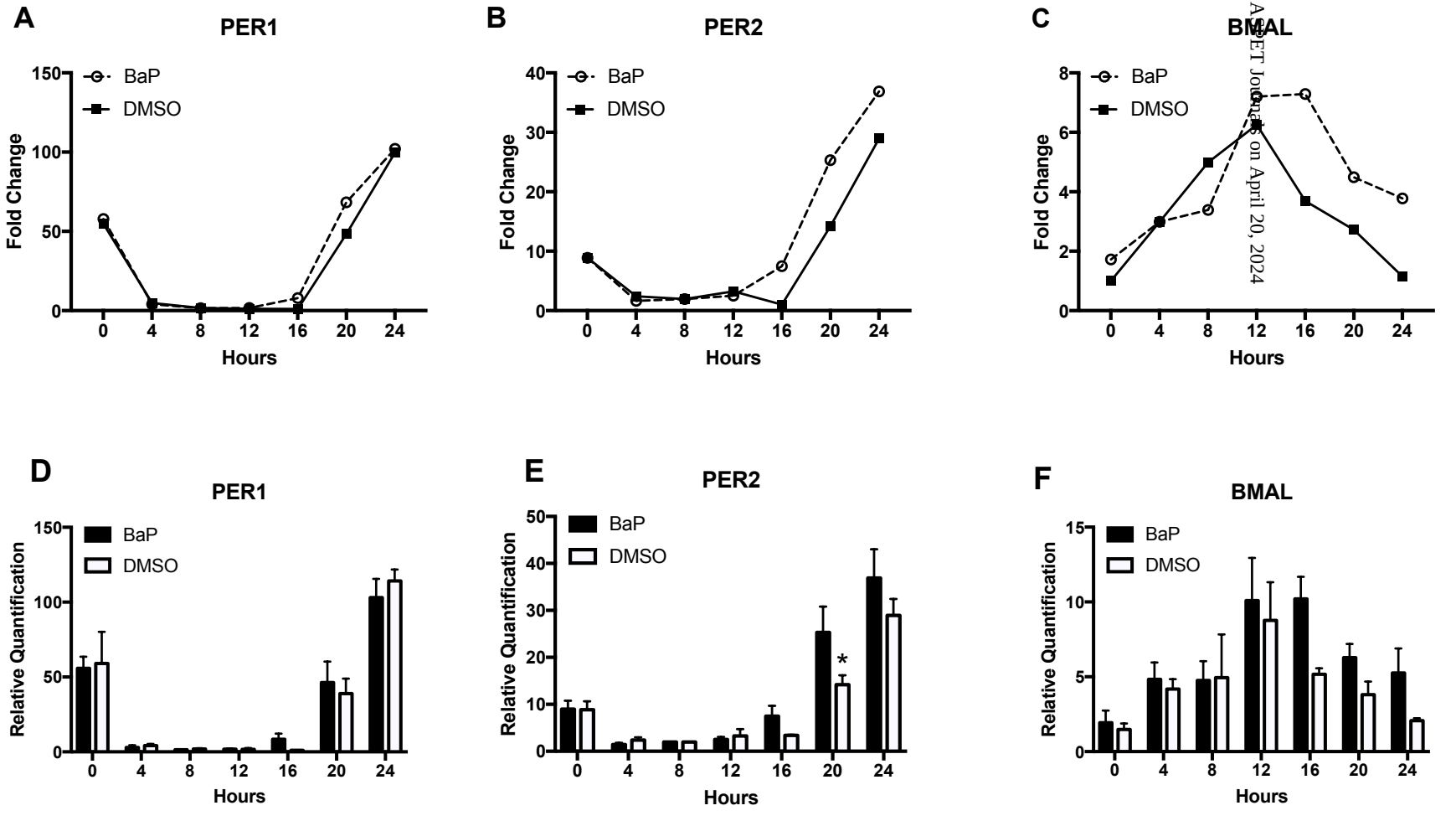
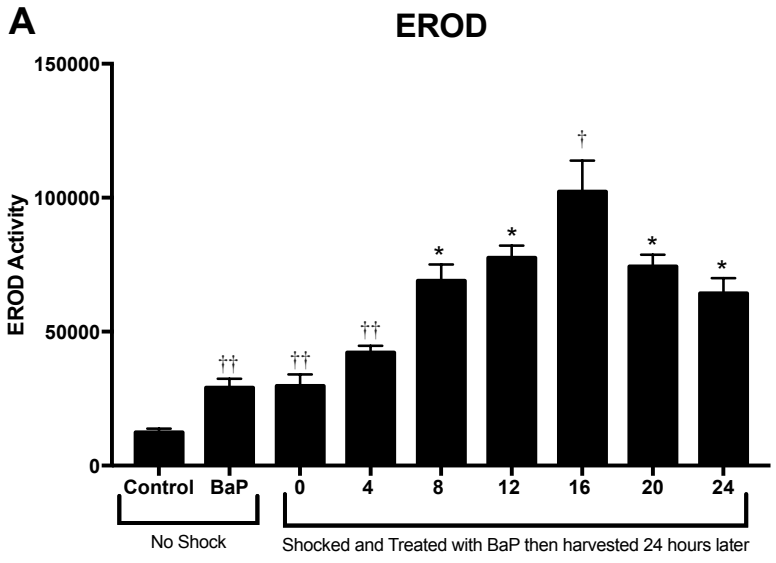
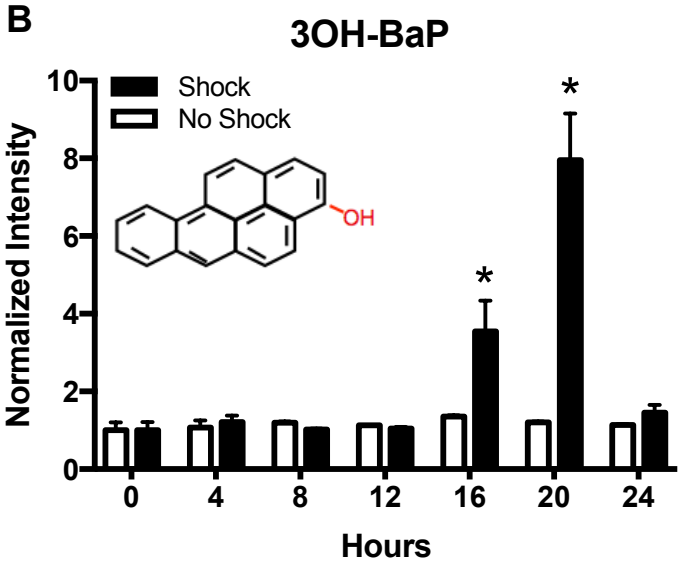
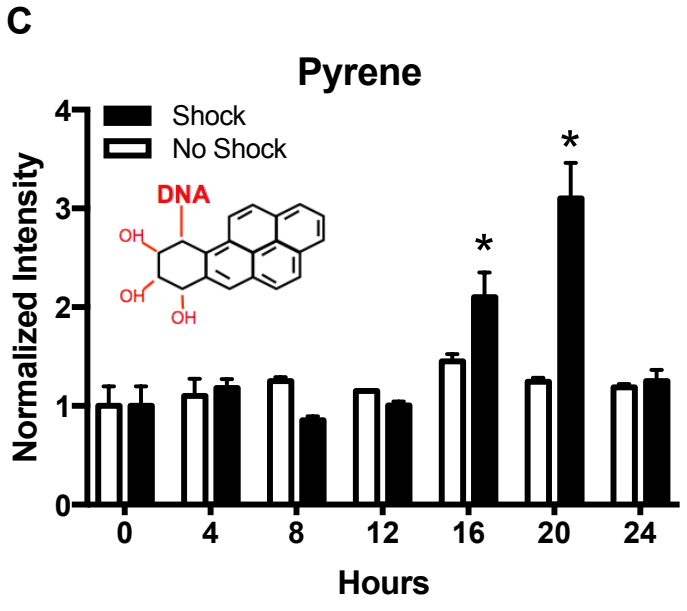


Figure 6


A
EROD
150000
100000
50000
0
EROD Activity
Control
BaP
0
4
8
12
16
20
24
No Shock
Shocked and Treated with BaP then harvested 24 hours later


B
3OH-BaP
Shock
No Shock
OH
Normalized Intensity
10
8
6
4
2
0
0
4
8
12
16
20
24
Hours


C
Pyrene
Shock
No Shock
DNA
OH
OH
OH
Normalized Intensity
4
3
2
1
0
0
4
8
12
16
20
24
Hours