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Daily Cycle of bHLH-PAS Proteins, Ah Receptor and Arnt, in Multiple Tissues of Female Sprague–Dawley Rats[1]

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The aryl hydrocarbon receptor (AhR) shares a common PAS domain with a number of genes that exhibit a pronounced circadian rhythm. Therefore, this study examined the daily cycle of AhR and AhR nuclear translocator (Arnt) protein expression in multiple tissues of female Sprague–Dawley rats. Rats were euthanized at 4, 7, and 11 AM and 4, 7, and 11 PM after which whole tissue homogenates were made from multiple tissues. Western blot analysis showed that the daily cycle of relative AhR protein expression exhibits a similar oscillation pattern in the liver, lungs, and thymus. The daily cycle of relative Arnt protein expression exhibits a similar oscillation pattern in the liver and lungs. The apparent daily cycle of AhR and Arnt protein expression in multiple tissues was not observed within the spleen. This preliminary report is the first study to suggest that the PAS proteins, AhR and Arnt, exhibit a daily oscillation pattern within multiple target tissues which may give insight into the tissue-specific toxic and biochemical responses mediated through this dimerization pair, as well as the physiological function of these proteins.

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The basic-helix-loop-helix (bHLH)-PAS proteins are named for a dimerization domain of approximately 250–300 amino acids, originally identified in the Drosophila periodicity (per) gene product, the aryl hydrocarbon receptor (AhR) and human AhR nuclear translocator (Arnt) proteins and the Drosophila single-minded (sim) gene product [1–3]. These PAS proteins are a class of transcription factors which modulate development and adaptation to environmental stimuli including metabolism of xenobiotics, hormone-receptor function, and intracellular homeostatic responses elicited by hypoxic conditions [4]. A linkage between the PAS domain and circadian rhythms has also been established [4]. For example, mutation in the PAS domain of the Drosophila per gene causes a prolonged circadian oscillation [5, 6]. Recently mammalian homologs of the Drosophila per gene have been cloned which possess a PAS domain and exhibit a circadian rhythm [7–13]. For example, a mutation in the mouse clock gene lengthens the circadian period and abolishes the persistence of rhythmicity [7].

Extensive studies on the molecular biology of CYP1A1 induction revealed that the liganded AhR is a unique member of the bHLH-PAS protein family [14, 15]. Analysis of AhR cDNA showed that this protein contains a bHLH motif contiguous with the PAS domains present in a variety of transcriptional regulators, including the Arnt and Arnt2 proteins, hypoxia-inducible factor 1α (HIF-1α) and HIF-2 α, orphan bHLH-PAS protein MOP3 and the *Drosophilia* factors, per and sim [1–3, 16–24]. The liganded:AhR complex translocates into the nucleus of the target cell by forming a heterodimer with the Arnt protein, binds dioxin responsive elements (DREs) upstream of target genes and acts as a ligand-activated transcription factor (LTF) [15, 25].

The AhR has been identified in both mammalian and nonmammalian species and in a large number of tissues [26–31]. In addition, the expression of Arnt in

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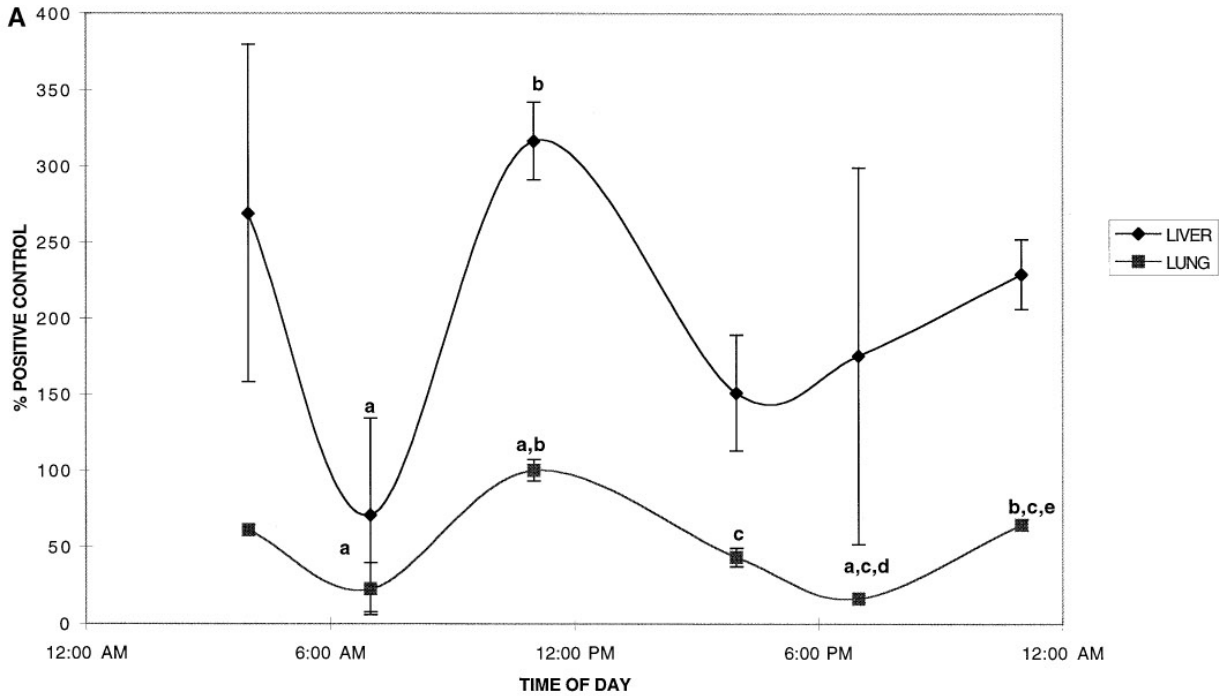
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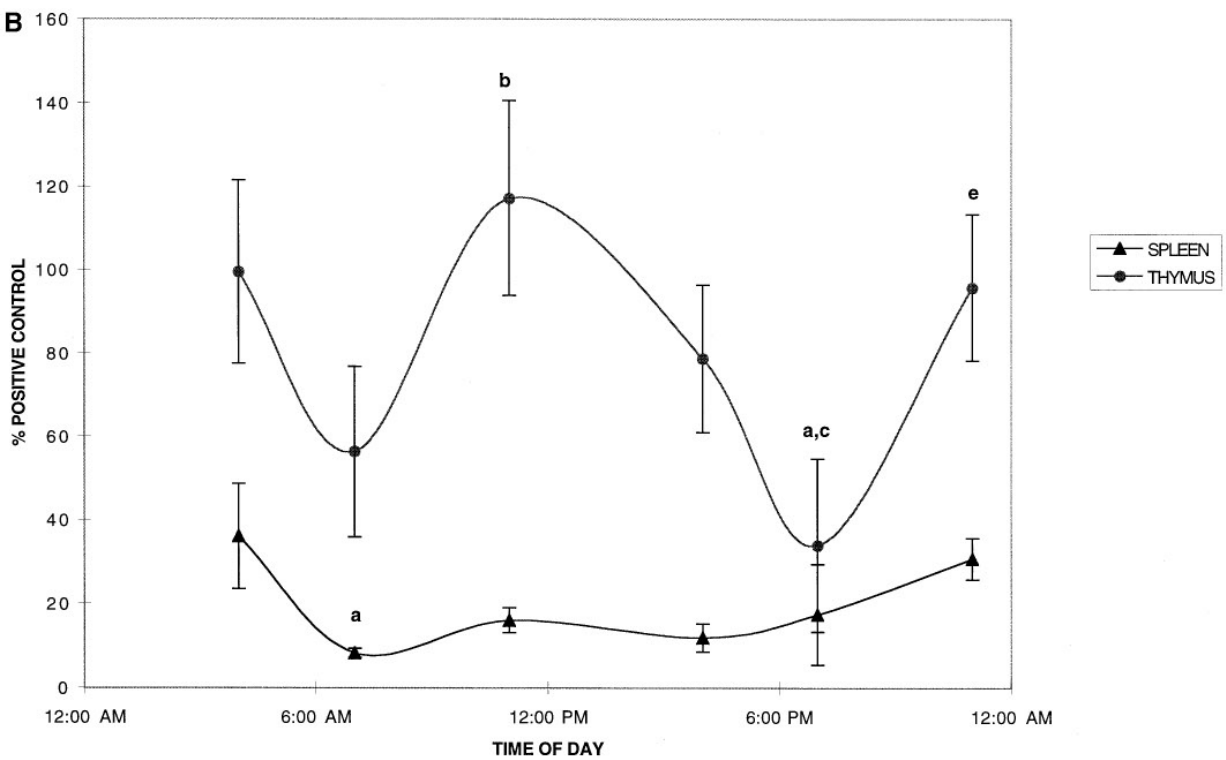
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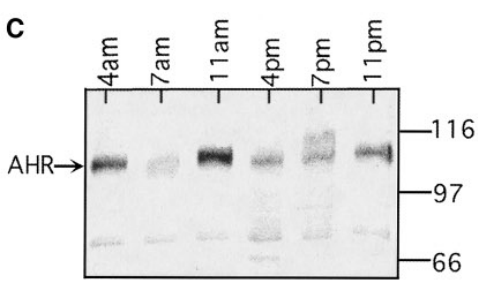
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multiple species and tissues has been reported [28–32]. Recent studies from this laboratory suggest daily time-dependent changes in AhR and Arnt proteins in multiple tissues from control animals [28]. In addition, three mammalian Drosophila per homologs exhibit a pronounced circadian oscillation in multiple tissues, including liver, skeletal muscle, testis, brain and retina [33]. Since, the AhR shares a common PAS domain with a number of genes that exhibit a pronounced circadian rhythm, the focus of this study was to examine the daily cycle of AhR and Arnt protein expression in multiple tissues.

MATERIALS AND METHODS

Animals. Twelve-week old female Sprague–Dawley rats (225–250 g), were purchased from Charles River Laboratories (Raleigh, NC). Rats were maintained at the National Health and Environmental Effects Research Laboratory (NHEERL) of the U.S. Environmental Protection Agency (Research Triangle Park, NC) and followed a diurnal cycle of 12 h of light/dark (6 AM/6 PM) at an ambient temperature (22 ± 1°C) and relative humidity of 55± 5%. Animals were placed in polycarbonate cages, each holding two animals, with hardwood bedding (Beta Chips, North Eastern Products, Warrensburg, NY) and acclimated for 1 week prior to usage. Rats had free access to Purina 5001 Rodent Chow (Ralston Purina, St. Louis, MO) and water. Rats were euthanized at 4, 7, and 11 AM, 4, 7, and 11 PM by CO_2 asphyxiation (n = 3 animals/time point). All animals within each time point were euthanized within a 15 minute period. The liver, lung, spleen, and thymus were removed, weighed and immediately placed on dry ice. All samples were stored at −80°C until use.

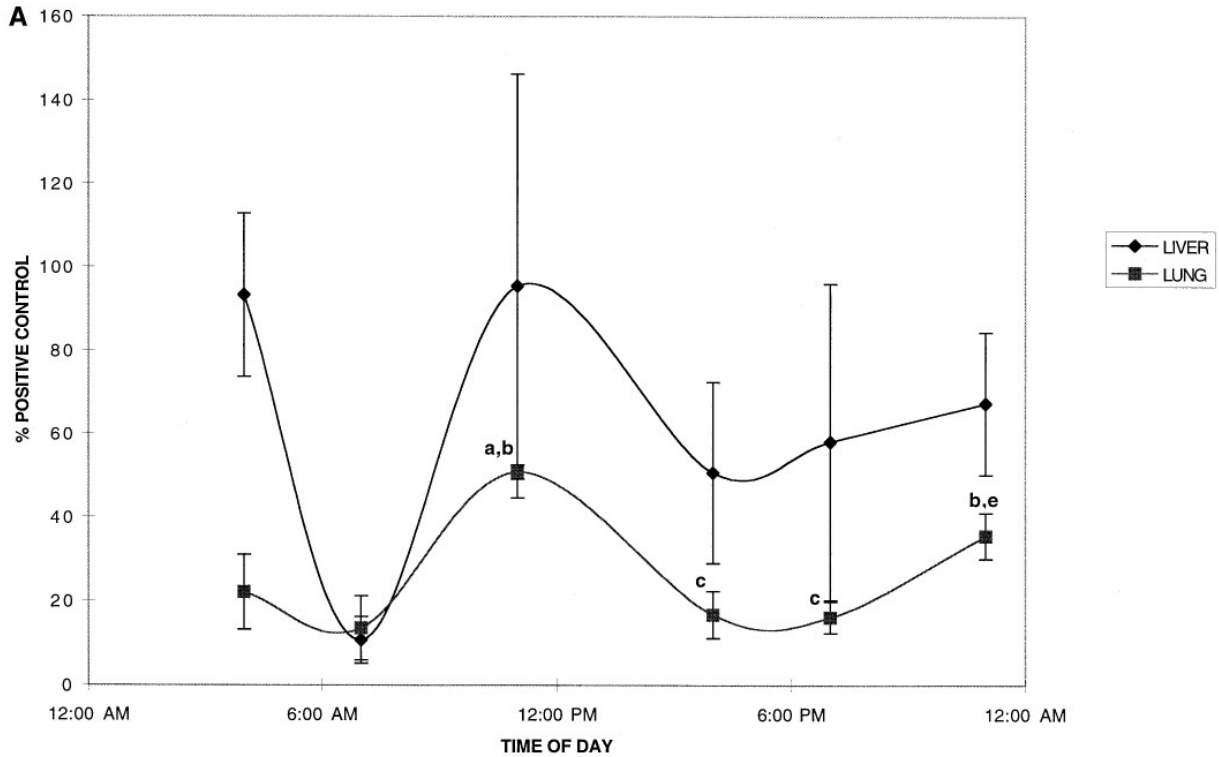
Preparation of tissue lysates. The procedure for preparation of tissue lysates is essentially as described in [28]. Frozen tissues were homogenized in an ice-cold 1× lysis buffer supplemented with aprotinin (100 U/ml) and leupeptin (50 μM) until thoroughly disrupted as previously described [28]. Samples were re-homogenized after the addition of phenylmethylsulfonyl fluoride (100 μM) to the tissue homogenate. A portion of the tissue homogenate (1 ml) was removed and sonicated after the addition of 10% Igepal CA-630 (Sigma Chemical Co., St. Louis, MO) as described [28]. The resulting tissue homogenates were centrifuged at 5000 rpm for 2 min at 4°C to remove any unbroken tissue. A small amount of the supernatant (10 μl) was removed to determine protein concentration as described [34] using BSA as the standard. The remaining amount of supernatant was combined with an equal volume of 2× sample loading buffer, heated at 95°C for 5 min and defined as total tissue lysate [28].

Gel electrophoresis and Western blotting. The procedure for determination of AhR and Arnt protein expression is essentially as described in [28]. Whole tissue homogenates (30–150 μg total protein loaded depending on the tissue) were resolved by SDS–PAGE using a 10% acrylamide resolving gel and a 4% acrylamide stacking gel as described [35]. Proteins were transferred onto 0.2 μm nitrocellulose membrane at 120 mA for 1 h and 30 min using a Hoefer SemiPhor Semi-Dry transfer unit (Pharmacia Biotech Inc., Piscataway, NJ) as described [28]. Membranes were blocked for 1 h in 5% blocking grade non-fat milk supplemented with DL-histidine (20 mM) and probed with a 1:500 dilution of a rabbit polyclonal antibody against AhR or Arnt [28] for 2 h at room temperature. Membranes were probed with a 1:5000 dilution of biotinylated donkey anti-rabbit IgG (Amersham, Arlington Heights, IL) for 1 hr at room temperature followed by incubation with a 1:4000 dilution of streptavidin-horseradish peroxidase conjugate (Amersham, Arlington Heights, IL) for 30 min. AhR and Arnt protein expression was visualized by using the enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL) according to manufacturer's recommendation. AhR and Arnt protein expression in liver, lung, spleen and thymus tissue homogenates were quantified against a thymus tissue homogenate sample, which exhibits a high concentration of both AhR and Arnt protein expression [28], using Quantity One software (PDI Inc., Huntington Station, NY). All data are represented as the means±standard deviation. The autoradiographs were translated into TIFF formatted files using an Arcus II scanner (AGFA Gevaert NV, Mortsel, Belgium) at 300 dots per inch (dpi) and printed using Adope photoshop version 4.0 (Adobe Systems Inc., Mountain View, CA) 4.0 at 300 dpi (see Figs. 1C and 2C). The statistical intergroup comparisons were determined using a one-way analysis of variance (ANOVA) followed by Scheffe's test. The levels of probability of statistical significance are $P < 0.05$.

RESULTS AND DISCUSSION

The bHLH-PAS proteins are a family of transcription regulatory proteins that function in biological rhythms, and a number of other cellular processes including development [4, 36]. This PAS domain is found in a variety of transcriptional regulators including the AhR and Arnt proteins [1, 2, 17]. Although, the physiological role of the AhR has not been established, a linkage between the PAS domain of other proteins, and circadian rhythms has been observed [4]. In addition, recent studies showing that ultraviolet-induced tryptophan photoproducts are Ah receptor agonists suggest a potential interaction between ligand:AhR action and light-regulated daily time-keeping systems within mammalian cells [37, 38]. Therefore, since the AhR shares a common PAS domain with a number of

FIG. 1. Daily cycle of AhR protein expression in multiple tissues (liver and lungs A); (thymus and spleen B) obtained from female Sprague–Dawley rat was determined by Western blot analysis as described in the Materials and Methods. Representative Western Blot of the daily cycle of AhR protein expression in female Sprague–Dawley rats (C). Rats were euthanized at 4, 7, and 11 AM and 4, 7, and 11 PM and tissue homogenates were prepared from liver, lungs, thymus and spleen as described under Materials and Methods. Whole tissue homogenates (30–150 μg total protein loaded depending on the tissue) were resolved by SDS–PAGE as described under Materials and Methods. AhR protein expression in liver, lung, spleen and thymus tissue homogenates were quantified against a thymus tissue homogenate sample, which exhibits a high concentration of both AhR and Arnt protein expression [28]. All protein data are represented as the means ± standard deviation (n = 3). Immunoblots were scanned and printed as described under Materials and Methods, using Quantity One software (PDI Inc., Huntington Station, NY). Daily cycle of AhR protein expression in liver and lungs (A) and thymus and spleen (B) were graphed in Microsoft Excel 5.0 using curve smoothing of a scatter plot. The statistical intergroup comparisons were determined using a one-way analysis of variance (ANOVA) followed by Scheffe's test with $P < 0.05$. [a]statistically different than the 4 AM time-point. [b]Statistically different than the 7 AM time-point. [c]Statistically different than the 11 AM time-point. [d]Statistically different than the 4 PM time-point. [e]Statistically different than the 7 PM time-point.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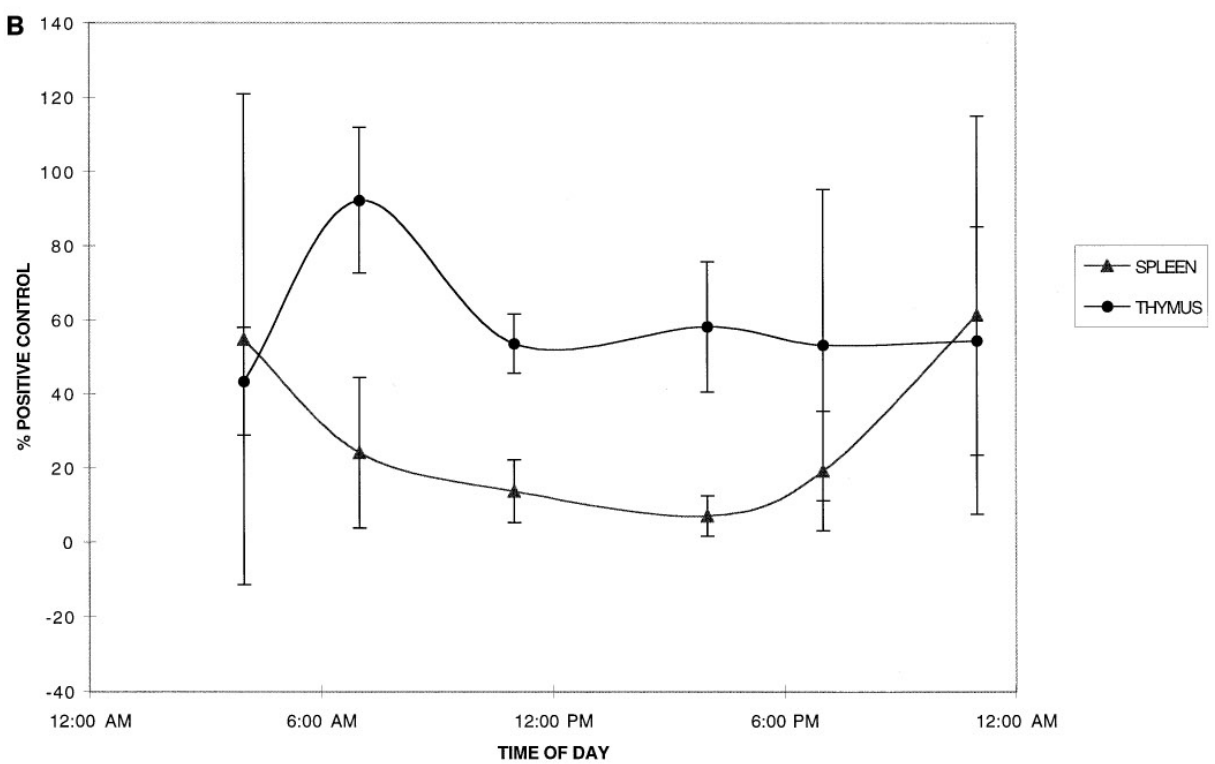
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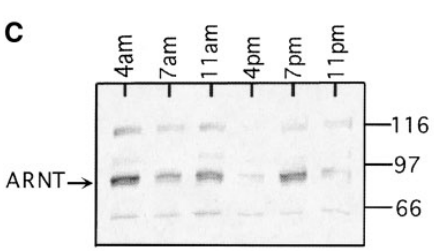
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genes that exhibit a pronounced circadian rhythm, the objective of this preliminary report was to examine the daily cycle of AhR and Arnt protein expression in multiple target tissues to gain insight into the toxic and biochemical responses elicited through the dimerization pair, as well as the physiological function of these proteins.

In the current study, the liver, lungs, thymus and spleen tissues were examined because recent studies from this laboratory suggest daily time-dependent changes in AhR and Arnt proteins in these tissues from control and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated animals which may play a role in the tissue-specific toxic and biochemical responses elicited by TCDD [28]. In addition, the concentration of AhR and Arnt proteins has been previously determined in these tissues [28]. The data suggests that the daily cycle of relative AhR protein exhibits a similar oscillation pattern in the liver, lungs and thymus (Figs. 1A and 1B). Figure 1C shows a representative western blot of the daily cycle of AhR protein in the liver of female Sprague–Dawley rats. Analysis of the relative AhR protein concentration in the liver, lungs and thymus (Figs. 1A and 1B) revealed a decrease in AhR protein concentration between the hours of 4 and 7 AM followed by an increase between the hours of 7 and 11 AM. Between the hours of 11 AM and 4 PM, a decrease in hepatic AhR protein concentration was observed (Fig. 1A). In contrast, to the liver (Fig. 1A), a prolonged decrease in AhR protein concentration was observed between the hours of 11 AM and 7 PM in the lungs (Fig. 1A) and thymus (Fig. 1B). However, an increase in AhR protein concentration from 7 to 11 PM was observed in all tissues (Figs. 1A and 1B). The apparent daily cycle of AhR protein concentration in the liver, thymus and lungs was not observed in the spleen (Fig. 1B)

The daily cycle of relative Arnt protein concentration exhibits a similar oscillation pattern in the liver and lungs (Fig. 2A). Figure 2C shows a typical western blot of the daily cycle of Arnt protein in the liver of female Sprague–Dawley rats. Analysis of the Arnt protein concentration in the liver and lungs (Fig. 2A) revealed a decrease in Arnt protein concentration between the hours of 4 and 7 AM followed by an increase between the hours of 7 and 11 AM. An increase in the relative Arnt protein concentration was observed in the thymus between the hours of 4 to 7 AM which was followed by a decrease between the hours of 7 and 11 AM that remained unchanged from 11 AM to 11 PM (Fig. 2B). Between the hours of 11 AM and 4 PM, a decrease in hepatic Arnt protein concentration was observed, which remained unchanged from 4 to 11 PM (Fig. 2A). In contrast, to the liver (Fig. 2A), a prolonged decrease in Arnt protein concentration was observed between the hours of 11 AM and 4 PM in the lungs (Fig. 2A) which was followed by an increase from 7 to 11 PM. The apparent daily cycle of Arnt protein concentration in multiple tissues was not observed in the spleen throughout the 24-h period (Fig. 2B).

This preliminary report illustrates that the PAS proteins, AhR and Arnt, exhibit apparent daily oscillations within multiple tissues of female Sprague–Dawley rats. However, these data also suggest some tissue-specific differences in AhR and Arnt protein oscillation patterns. For example, a slight oscillation in Arnt protein concentration within the thymus was only observed between the hours of 4 and 11 AM. In addition, within the spleen a lack of daily oscillation of the AhR and Arnt proteins was found. The reason for the tissue-specific differences in oscillation patterns of the AhR and Arnt proteins is unknown but may be related to the lack of coordinate cycling of these genes. For example, the change in daily oscillation of the Arnt protein is larger than that of the AhR (Figs. 1A, 1B, 2A, and 2B) and a 6- to 10-fold higher concentration of the AhR is found in the liver, thymus and spleen compared to the concentration of the Arnt protein within these tissues [28]. In addition, Pollenz *et al.* (1998) showed that TCDD-treatment results in a rapid depletion of the AhR protein in these tissues with little change in Arnt protein, suggesting a lack of coordinate regulation of these genes. A lack of coordinate regulation of AhR and Arnt gene expression was also observed in human embryonic palatal cells [32]. For example, immunohistochemical double-staining revealed that individual epithelial cells expressed both AhR and Arnt proteins. However, individual mesenchyme and nasal spine cartilege cells expressed either AhR or Arnt.

FIG. 2. Daily cycle of Arnt protein expression in multiple tissues (liver and lungs A); (thymus and spleen B) obtained from female Sprague–Dawley rat was determined by Western blot analysis as described in the Materials and Methods section. Representative Western Blot of the daily cycle of Arnt protein expression in female Sprague–Dawley rats (C). Rats were euthanized at 4, 7, and 11 AM and 4, 7, and 11 PM and tissue homogenates from liver, lungs, thymus and spleen were prepared as described under Materials and Methods. Whole tissue homogenates (30–150 μg total protein loaded depending on the tissue) were resolved by SDS–PAGE as described under Materials and Methods. Arnt protein expression in liver, lung, spleen and thymus tissue homogenates were quantified against a thymus tissue homogenate sample, which exhibits a high concentration of both AhR and Arnt protein expression [28]. All protein data are represented as the means $\pm$ standard deviation ($n = 3$). Immunoblots were scanned and printed as described under Materials and Methods, using Quantity One software (PDI Inc., Huntington Station, NY). Daily cycle of Arnt protein expression in liver and lungs (A) and thymus and spleen (B) were graphed in Microsoft Excel 5.0 using curve smoothing of a scatter plot. The statistical intergroup comparisons were determined using a one-way analysis of variance (ANOVA) followed by Scheffe's test with $P < 0.05$. [a]Statistically different than the 4 AM time-point. [b]Statistically different than the 7 AM time-point. [c]Statistically different than the 11 AM time-point. [d]Statistically different than the 4 PM time-point. [e]Statistically different than the 7 PM time-point.

Among the most characterized biochemical responses induced by TCDD through an AhR-dependent mechanism of gene regulation is the induction of cytochrome P450 gene expression in multiple tissues [14, 39] and previous studies have suggested that hepatic cytochrome P450 inducibility undergoes a circadian rhythm [40]. In a previous study, a time-dependent change in AhR protein concentration in multiple tissues from both corn oil- and TCDD-treated female Sprague–Dawley rats was observed during the light period [28]. In addition, functional studies using cultured mammalian cell lines showed that downregulation of the AhR was accompanied by a loss in TCDD-inducible reporter gene expression upon subsequent challenge with a subsequent dose of chemical [28]. However, in the current study, the apparent oscillation pattern of AhR and Arnt proteins in multiple target tissues was not accompanied by a significant loss in constitutive CYP1A1 or CYP1A2 gene expression (data not shown). These data agree with the lack of diurnal variation of constitutive cytochrome P450 expression observed in adult male golden Syrian hamsters [41].

Although, the full function and significance of the daily oscillation pattern of the AhR and Arnt proteins on gene regulation is unknown, this preliminary report suggesting a daily oscillation pattern of AhR and Arnt protein expression in multiple tissues may give insight into the normal physiological function of these proteins. In addition, the recent characterization of the AhR and Arnt knockout mice may lead to identification of the normal physiological action of these proteins and function in gene regulation. For example, the AhR knockout mouse exhibits altered cell growth and development [42, 43] and mice deficient in the PAS protein, Arnt, die by GD-10 [44] demonstrating a key role of these PAS proteins in development. Therefore, even a slight modulation of the concentration of AhR or Arnt may affect critical genes responsible for cell growth and differentiation.

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