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Aryl hydrocarbon receptor-mediated *Cyp1a1* expression is modulated in a CLOCK-dependent circadian manner

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ABSTRACT

The expression of genes involved in xenobiotic detoxification is under the control of the circadian clock. The aryl hydrocarbon receptor (AhR) is one of the transcription factors responsible for the induction of detoxification enzymes in response to xenobiotic toxins, and the expression of AhR has been suggested to be regulated by a circadian oscillator. In this study, we investigated whether toxin-mediated activation of the AhR signaling pathway was modulated by CLOCK protein, a key component of the mammalian circadian clock. The expression of AhR and its DNA binding ability in the lungs of wild-type mice showed significant 24-h oscillation. *Clock* mutant (*Clk/Clk*) mice, producing CLOCK protein deficient in transcriptional activity, failed to show significant oscillation in the expression of AhR. The mRNA levels of *AhR* in the lungs of *Clk/Clk* mice were significantly lower than in wild-type mice. A single intraperitoneal injection of benzo[α]pyrene, a ligand of AhR, induced the expression of *Cyp1a1* in the lungs of wild-type mice, but the induction varied depending on the benzo[α]pyrene injection time. The dosing time-dependency of benzo[α]pyrene-induced *Cyp1a1* expression was also modulated by *Clock* gene mutation. These findings suggest that CLOCK protein affects the toxin-induced expression of detoxification enzymes through modulating the activity of AhR. Our present findings provide a molecular link between the circadian clock and xenobiotic detoxification.

1. Introduction

Most living organisms from bacteria to humans have a biological clock and exhibit a circadian rhythm in behavioral and physiological functions such as body temperature, blood pressure and the sleep-wake cycle (Um et al., 2011; Doi et al., 2010; Franken and Dijk, 2009). In mammals, the master clock controlling circadian rhythms is located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Silver et al., 1996; Shigeyoshi et al., 1997). Furthermore, biological clocks also exist in peripheral tissues and function as local clocks that are synchronized with the master clock via hormonal and/or neuronal signals (McNamara et al., 2001; Terazono et al., 2003). Recent studies have identified that a basic mechanism of the biological clock consists of interlocked transcription-translation feedback loops. The feedback loop is composed of the basic helix–loop–helix transcription factors CLOCK and BMAL1. These factors form heterodimers and drive transcription of *Period* (*Per1*, *Per2*), and *Cryptochrome* (*Cry1*, *Cry2*) genes via their response element E-box (CACGTG) (Gekakis et al., 1998; Ueda et al., 2005). Once PER and CRY proteins have reached critical concentrations, they attenuate CLOCK/BMAL1 transactivation, thereby generating 24-h rhythms in their own transcription (Kume et al., 1999; Griffin et al., 1999). Similar to *Per* and *Cry*, E-box located in the promoter region of other genes is also recognized by CLOCK/BMAL1 heterodimers (Ripperger et al., 2000; Jin et al., 1999). Hence, this molecular clock regulates 24-h rhythms in output physiology through the periodic expression of clock-controlled genes.

Circadian clock-controlled output pathways include those that control the expression of many enzymes and regulators involved in xenobiotic detoxification (Zhang et al., 2009). *Clock* mutant (*Clk/Clk*) mice have a point mutation causing the deletion of exon 19 of the *Clock* gene, thus synthesizing mutant CLOCK protein (CLOCKΔ19) deficient in transcriptional activity and exhibiting abnormal circadian gene expression (Gekakis et al., 1998). The activities of efflux transporters and DNA repair enzymes are down-regulated in *Clk/Clk* mice (Kim et al., 2009; Murakami et al., 2008). PAR-domain basic leucine zipper (PAR bZip) proteins albumin D-site binding protein (DBP), thyrotroph embryonic factor (TEF), and hepatic leukemia factor (HLF) also act as mediators of circadian clock-controlled xenobiotic detoxification (Gachon et al., 2006). PAR bZip triple knock-out mice are hypersusceptible to xenobiotic compounds, and the deficiency in detoxification is attributed to the downregulation of genes involved in cytochrome P450 (CYP)-dependent metabolism, enzymatic conjugation, and efflux transport.

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Aryl hydrocarbon receptor (AhR) is one of the transcription factors containing a basic helix-loop-helix-Per/Arnt/Sim (bHLH-PAS) domain (Kikuchi et al., 2003). AhR signaling is responsible for the induction of enzymes in response to stimulation with xenobiotic compounds. Upon binding xenobiotic compounds, AhR translocates into the nucleus and recognizes an enhancer DNA element designated the xenobiotic responsive element (XRE; TNGCGTG) sequence located in the promoter region of the target gene, resulting in the induction of xenobiotic-related genes such as *CYP1A1*, *1A2*, and *1B1* (Kawajiri and Fujii-Kuriyama, 2007). In fact, these P450 enzymes are potently induced by several ligands of AhR, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo[α]pyrene (Sagredo et al., 2006; Monteiro et al., 2008).

It has been reported that the expression of AhR in the rodent liver, lung and SCN shows circadian oscillation (Richardson et al., 1998; Mukai et al., 2008), but the mechanism remains to be clarified. In this study, we explored whether the expression of AhR was under the control of CLOCK protein and also investigated how the circadian oscillation of AhR signaling modulated the benzo[α]pyrene-induced *Cyp1a1* expression in the lungs of mice.

2. Materials and methods

2.1. Materials

Benzo[α]pyrene (Alfa Aesar, MA, USA) was dissolved in olive oil (Nacalai Tesque Inc., Kyoto, Japan) and injected intraperitoneally (i.p.) in a volume of 0.1 mL/10 g body weight.

2.2. Animals and treatment

Clock mutant (*Clk/Clk*) mice (C57BL/6J-ClockmlJt/J) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were backcrossed using a Jcl: ICR background of more than eight generations, and genotypes were determined using PCR. All mice were housed in a light-controlled room (light on from 07:00 to 19:00) and adapted to this light/dark cycle for 2 weeks before the experiments at room temperature of 24 ± 1 °C and humidity of 60 ± 10% with food and water ad libitum. Both wild-type and *Clk/Clk* mice were injected with 0.01 mg/kg (i.p.) of benzo[α]pyrene or olive oil at 09:00, or 21:00. Animals were treated in accordance with the guidelines stipulated by the Animal Care and Use Committee of Kyushu University.

2.3. Cell culture and transfection

Mouse embryonic fibroblasts (MEFs) were isolated from 10.5-day-old embryos of wild-type or *Clk/Clk* mice. Heads and livers were removed and the remaining tissues were minced. The minced tissue was digested in 0.25% trypsin-EDTA for 15 min. After digestion, cells were filtered and the filtrate was centrifuged at 8500 × *g* for 3 min. The pellets were resuspended and used for experiments. MEFs were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich Japan, Tokyo, Japan) supplemented with 10% FBS (FBS; SAFC Biosciences, Kansas City, MI) and 0.5% penicillin-streptomycin (Invitrogen, Carlsbad, A, CA) at 37 °C in a humidified 5% CO_2 atmosphere. Twenty-four hours before transfection, MEFs were placed on 24-well plates at a density of 2×10^4 cells per well in DMEM containing 10% FBS and 0.5% penicillin-streptomycin. MEFs were transfected with CLOCK expression vectors using Lipofectamine-LTX reagents (Invitrogen).

2.4. Construction of CLOCK expression vector

CLOCK expression vector was prepared as follows: the coding regions of the transcriptional regulators were obtained by RT-PCR and used after their sequences had been confirmed. All coding regions were ligated into the pcDNA 3.1 vector (Invitrogen).

2.5. RT-PCR analysis

Total RNA was extracted from mouse lung or MEFs using RNAiso (Takara Bio Inc., Shiga, Japan). Complementary DNA (cDNA) was prepared via reverse transcription of total RNA using a ReverTra Ace® qPCR RT kit (Toyobo Co. Ltd., Osaka, Japan). Diluted cDNA samples were analyzed by real-time PCR. Real-time PCR was performed using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo) and the 7500 Real-time PCR system (Applied Biosystems, Foster City, CA). The sequences of the primers were as follows: *Cyp1a1*, 5′-CCTCTTTGGAGCTGGGTTTG-3′ and 5′-GCTGTGGGGGATGGTGAA-3′; *AhR*, 5′-AATCCCACATCCGCATGATT-3′ and 5′-TTTGCAAGAAGCCGGAAAAC-3; *β-actin*, 5′-CCAGGGTGTGATGGTGGGAA-3′ and 5′-TTCACGGTTGGCCTTAGGGT-3′.

2.6. Western blotting

For preparation of microsomes, mouse lung was homogenized in 0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 9000 × *g* for 10 min and the collected supernatant was re-centrifuged at 105,000 × *g*, for 60 min. The pellet obtained by re-centrifugation was used as a microsomal fraction for analysis of Cyp1a1 protein levels. Samples (20 μg protein) were separated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were reacted with antibodies against Cyp1a1 or ACTIN (Santa Cruz Biotechnology, Santa Cruz, CA). Specific antigen/antibody complexes were made visible using peroxidase-conjugated secondary antibodies and Chemi-Lumi One (Nacalai Tesque Inc., Kyoto, Japan).

2.7. Determination of Cyp1a activity

The microsomal fraction was used for determination of Cyp1a activity, which was assessed using a P450-Glo CYP1A Assay Kit (Promega, Madison, WI). In this assay system, CYP enzymes act on a luminogenic substrate to produce a luciferin product that generates light with the luciferin detection reagent, so that enzymatic activity is provided in terms of relative light units (RLU). The protein concentration in the microsomal fraction was determined using Lowry's method (DC protein assay; Bio-Rad, Hercules, CA). The enzymatic activity of Cyp1a was expressed as RLU/mg protein/min.

2.8. Electrophoretic mobility shift assay

^{32}P-labeled, double stranded oligonucleotides were used as a probe. The probe contains XRE found on the 5′-flanking region of the mouse *Cyp1a1* gene (−996 to −977). The sequence of the probe is as follows (underlining indicates XRE); 5′-GGAGTTGCGTGAGAAGAGCC-3′. Nuclear protein (5 μg) prepared from mouse lung was incubated with the probe in binding buffer (8 mmol/L *N*-2-hydroxyl piperazime-*N*′-2-ethane sulfonic acid (pH 7.9), 40 mmol/L KCl, 0.4 mmol/L $MgCl_2$, 6.8% glycerol, 0.1 μg/μL bovine serum albumin, 0.8 mmol/L dithiothreitol, 0.2 mmol/L phenylmethylsulfonyl fluoride, 0.1 μg/μL poly dI-dC) in the presence or absence of anti-AhR antibodies N19; Santa Cruz Biotechnology) for 10 min at 4 °C. Protein-DNA complexes were run on a 4% polyacrylamide gel and detected by autoradiography.

2.9. Statistical analysis

The statistical significance of the differences among groups was analyzed by analysis of variance and Tukey's multiple comparison test. A 5% level of probability was considered significant.

3. Results

3.1. 24-h oscillation of AhR signaling in lungs of mice

As reported previously (Richardson et al., 1998), the mRNA levels of *AhR* in the lungs of wild-type mice showed significant 24-h oscillation with peak levels from the late light phase to the early dark phase ($p < 0.05$; Fig. 1A). Electrophoretic mobility shift assay using mouse lung nuclear extracts and XRE containing probes suggested the presence of a circadian XRE binding protein in the lungs of wild-type mice (Fig. 1B). The temporal change in the abundance of protein–DNA complexes was attributable to that of AhR proteins because the bands of the complexes were supershifted when nuclear extracts were incubated with XRE probe in the presence of antibodies against AhR (Fig. 1C). The mRNA levels of *Cyp1a1*, a target gene of AhR, also fluctuated rhythmically with a period length about 24 h ($p < 0.05$; Fig. 2A). The rhythmic phase of *Cyp1a1* mRNA corresponded to the oscillation of AhR binding to XRE probes. Protein levels of Cyp1a1 and its enzymatic activity in the microsomal fraction of the lungs of wild-type mice also showed significant 24-h oscillation (Fig. 2B and C), but the phase of Cyp1a1 protein oscillation was delayed by about 4 h relative to its mRNA rhythm.

3.2. Decreased expression of AhR in Clk/Clk mice

To explore whether the expression of AhR is under the control of the circadian clock, we investigated the mRNA levels of *AhR* in the lungs of *Clk/Clk* mice. As shown in Fig. 3A, the mRNA levels of *AhR* in the lungs of *Clk/Clk* mice failed to show significant time-dependent variation. As compared to wild-type mice, mRNA levels of AhR in