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Modulation of glucocorticoid receptor induction properties by core circadian clock proteins

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ABSTRACT

Glucocorticoid (GC) plays important roles in diverse physiological processes including metabolism and immune functions. While circadian control of GC synthesis and secretion is relatively well appreciated, circadian control of GC action within target tissues remains poorly understood. Here, we demonstrate that CLOCK/BMAL1, the core circadian clock components, reduces maximal GR transactivation (A_{max}) as well as efficacy (EC_{50}) by a novel mechanism that requires binding to DNA and transactivation of target genes. Accordingly, we observe that PER1 and CRY1, the primary targets of CLOCK/BMAL1 action, reduce maximal GR transactivation while not affecting the efficacy. Moreover, we observe hyper-activations of GRE-dependent transcription in BMAL1- or PERs-deficient MEFs. In addition, endogenous GC target genes expression negatively correlates with the CLOCK/BMAL1 activity. Considering that GC sensitivity is widely implicated in human health and diseases, these results provide valuable insights into plethora of GC-related physiology and pathology.

1. Introduction

The rotation of the earth on its axis is a daily phenomenon, which generates alternations of day and night. Accordingly, almost every living thing on the earth is known to actively adapt itself to this periodically changing environment, thereby taking selective advantages. Indeed, a variety of organisms from cyanobacteria to human have evolved to possess endogenous biological clocks. It is now well established that circadian rhythms exist in almost every aspect of physiological processes including sleep-activity pattern, secretion of hormone, body temperature, and metabolism even in the absence of external time cues (Dunlap, 1999; Panda et al., 2002; Dunlap et al., 2004; Refinetti, 2006; Levi and Schibler, 2007; Takahashi et al., 2008; Dibner et al., 2010; Partch et al., 2013).

In mammals, the suprachiasmatic nucleus (SCN), a small region of the anterior hypothalamus located above the optic chiasm and under the third ventricle, acts as a master clock and orchestrates peripheral clocks present in various tissues and organs (van Esseveldt et al., 2000; Levi and Schibler, 2007; Liu et al., 2007; Takahashi et al., 2008; Dibner et al., 2010). At the molecular level, the circadian clocks are operated by the pacemaker within cells. The molecular oscillator consists of circadian clock genes acting in the positive and negative limbs of feedback loops through transcription, post-transcriptional modifications, translation, post-translational modifications, and cellular trafficking (reviewed in Partch et al., 2013). The well-known positive regulator CLOCK/BMAL1 heterodimer activates *pers* and *crys* genes through E-box elements. Subsequently, PERs and CRYs accumulate within the cytoplasm, whose complex enters the nucleus with a timed delay and negatively regulates their own transcription. These processes are cell-autonomous so that the expression levels of clock genes exhibit circadian variations even without any time cues (Nagoshi et al., 2004; Kornmann et al., 2007).

Glucocorticoid (GC), synthesized in and secreted from the adrenal cortex, exerts pluripotent effects on the functions of tissues, organs and the whole body (Vinson, 2009 and references therein). GC elicits numerous metabolic effects including cardiovascular, metabolic, immunologic, and homeostatic functions. Interestingly, the plasma concentration of GC displays a distinct circadian variation in both diurnal and nocturnal animals: the acrophase of GC

Abbreviations: A_{max}, maximal transactivation; bHLH, basic helix-loop-helix motif; BMAL1, brain and muscle arnt-like 1; CLOCK, circadian locomoter output cycle kaput; CRY1, cryptochrome 1; DEX, dexamethasone; EC_{50}, efficacy or half-effective concentration; FKBP5, FK506 binding protein 5; GC, glucocorticoid; GILZ, glucocorticoid-induced leucine zipper; GR, glucocorticoid receptor; NES, nuclear export signal; NLS, nuclear localization signal; PAS, PER-ARNT-SIM domain; PER1, period homolog 1; SG repeat, serine-glycine repeat; TAD, transactivation domain.

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concentration is around dawn in diurnal animals in contrast to dusk in nocturnal animals (Buijs and Kalsbeek, 2001; Foster and Kreitzman, 2004). This indicates that GC is elevated at the time of animal's activity onset. Recently, it has been demonstrated that the rhythm of GC secretion is controlled by both the master clock in the SCN and the peripheral clock within the adrenal gland (Oster et al., 2006; Son et al., 2008; Nader et al., 2010). Thus, the function of GC is expected to be rhythmic, because the synthesis and secretion of GC display a daily variation.

The action of GC within the target cells is mediated by the specific glucocorticoid receptors (GRs). GC enters the cell and binds to the GR. The activated GR homodimer binds to DNA sequences known as glucocorticoid response elements (GREs) to activate transcription of responsive genes (Newton, 2000; Heitzer et al., 2007). During this process, various GR regulators such as coactivators, corepressors and comodulators affect the various aspects of GR-dependent transactivation (reviewed in Simons, 2006, 2008; Quax et al., 2013). Very recently, Lamia et al. (2011) provided an interesting mechanism where CRYs rhythmically repress GR transactivation. Yet, other possible ways of circadian modulation of GC action at the target cell level remain largely unexplored. In the present study, we investigate the modulation of GR transactivation properties by circadian clock genes.

2. Materials and methods

2.1. Chemicals, reagents and plasmids

Dexamethasone (DEX) was from Sigma (Steinheim, Germany). Materials for cell culture were obtained from Invitrogen (NY, USA). Rat GR expression plasmids, GRE*tk*Luc reporter, *Renilla* control plasmids, and various circadian clock genes expression plasmids has been described previously (Cho et al., 2005b; Doi et al., 2007; Kwon et al., 2006; Son et al., 2008; Lee et al., 2012). Other materials, unless indicated otherwise, are all from Sigma.

2.2. Cell culture, transient transfection and dual luciferase assay

NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere incubator (Kendro Laboratory, Langenselbold, Germany) containing 5% CO_2 at 37 °C. The procedure for the generation of mouse embryonic fibroblasts was fully described elsewhere (Cho et al., 2005a). Plasmid DNAs for transfection were prepared using QIAprep® Miniprep kit (QIAGEN GmbH, Germany). For transient transfection, cells were seeded at a density of 5×10^4 cells/well in 24-well culture plates (Corning Inc., Corning, NY). All transfections were performed in triplicates in 24-well plates. After overnight culture, total of 0.4–0.6 μg plasmids containing 0.02 μg of GRE-reporter plasmid, 0.01 μg of *Renilla* internal control plasmid, and other expression plasmids (see figure legends for details) were introduced to the cells using the Lipofetamine™ reagent (Invitrogen, CA, USA) according to the manufacturer's specifications. One day after transfection, cells were treated with increasing concentration of DEX for 24 h as indicated within the figure. Cells were lysed and Dual Luciferase® Reporter (Promega) assay was performed. Luciferase activity was measured in a DLReady Berthold Luminometer (Centro LB960). Dose response curves were drawn as described previously (Cho et al., 2005b; Simons, 2006).

2.3. SDS–PAGE and Western blotting

Anti-GR (Santacruz) and anti-actin (Santacruz or Novus) antibodies were obtained commercially. For Western blotting, proteins were extracted from MEFs by homogenization in 2X SDS-sample buffer (100 mM Tris, 4% SDS, 20% glycerol, pH 6.8) with protease inhibitor cocktail (Roche). Protein concentrations were determined by Lowry method. The extracts containing 30 μg of total protein were resolved on SDS–polyacrylamide gels, transferred to PVDF (Millipore) membranes, and subsequently incubated with primary antibodies diluted in TBS containing 0.3% Tween-20 and 3% BSA (anti-GR, 1:2000; anti-actin, 1:5000 or 1:10000). The antigen–antibody complexes were visualized using horseradish peroxidase-conjugated secondary antibodies and ECL reagents (Amersham Biosciences or Thermo scientific). Stringent 5X washing with TBS were included at each step. Optical densities of immunoreactive bands were quantified by National Institutes of Health ImageJ software (downloaded from http://rsb.info.nih.gov/ij/), and the relative amounts of target proteins were deduced by comparison with the optical band densities from serially diluted reference extracts and after compensation to that of actin internal loading control.

2.4. Animals, entrainment and sampling

All animal experiments were approved and performed under the guidelines of Kyung Hee University Institutional Animal Care and Use Committee. Young adult male mice aged 8–10 weeks were kept and maintained in a light-proof Clean Animal Rack cabinet (Shin Biotech, Seoul, Korea) with constant air ventilation. Lighting schedule was automatically controlled by equipped digital timers and the ambient temperature was maintained at 23 ± 1 °C. Light intensity during the light-phase was maintained at 350–450 lux throughout the area (Park et al., 2012). Animals were entrained to a 12:12 LD photoperiodic cycle with light-on at 0800 h for 2 weeks. Water-soluble dexamethasone phosphate was diluted to 0.3 mg/ml in phosphate-buffered saline (PBS). The animals were intraperitoneally (i.p.) injected with PBS or DEX phosphate (1 mg/kg body weight) at ZT00 or ZT12. Two hours later, the animals were sacrificed by cervical dislocation and liver samples were quickly obtained.

2.5. RNA isolation, reverse transcription, and real-time PCR

Total RNA isolation from cells or tissues was achieved by the single-step acid guanidinium thiocyanate-phenol–chloroform extraction method (Lee et al., 2012). Concentration of RNA in each sample was determined by the Nanodrop (ND-1000, Nanodrop technologies Inc., Wilmington, USA). Each RNA sample diluted to 1 μg/10 μl was incubated with 200 ng of random hexamer at 65 °C for 5 min, and rapidly cooled down on ice for 2 min. Each sample was incubated with 9 μl of reverse transcription mixture (4 μl of RT buffer, 4 μl of 2.5 mM ea of dNTP, 0.5 μl of RNase inhibitor and 0.5 μl of RTase M-MLV) at 37 °C for 1 h. After reverse transcription, samples were incubated at 70 °C for 10 min to inactivate RTase M-MLV. The procedure for real-time PCR using LightCycler has been described previously (Doi et al., 2007). Briefly, aliquots of the cDNA were subjected to real-time PCR by Lightcycler Version 1.5 (Roche, Salt Lake City, USA) in the presence of 2X SYBR premix Ex Taq (Takara, RR041A). Gene expression levels were normalized to TATA box-binding protein (TBP) expression level. Primer sequences used for real-time PCR are summarized in Table 1.

2.6. Statistical analysis

Statistically significant differences were evaluated by Student's *t*-test for comparison between the two groups. Statistical significance was set at $P < 0.05$.