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Modulation of the circadian clock by glucocorticoid receptor isoforms in the H295R cell line

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ABSTRACT

Peripheral clocks are set by different nervous, hormonal and metabolic stimuli, and regulate the circadian expression of several genes. We investigated whether a peripheral clock could be induced in the human adrenocortical cell line H295R and whether glucocorticoid receptor isoforms (GRα and GRß) are involved in this clock system. After synchronization of cells with serum shock, the rhythmic oscillation of clock genes *PER1*, *PER2*, *REV-ERBα*, and *ARNTL* was confirmed. In addition, H295R cells even without serum shock showed rhythmic expression of *PER1*, *PER2*, *CRY1* and *ARNTL*. Glucocorticoid treatment induced a rapid response of *PER1*, *PER2* and *CRY1* in a GRα-dependent manner. Continuous glucocorticoid stimulation after 6 h caused suppression of *REV-ERBα*. Administration of a GR antagonist, RU486, disrupted the circadian oscillation of clock genes and prevented the acute changes in *PER1*, *PER2* and *CRY1* levels. Overexpression of the GRß isoform alone did not alter the expression of the examined clock genes, but did prevent the GRα-related suppression of *REV-ERBα*. These alterations occurred independently from ACTH and CRH. Our data demonstrate that a peripheral clock system is present in a human adrenocortical cell line and that periodic oscillations of clock genes are influenced by glucocorticoids, mainly through GRα.

1. Introduction

Circadian timing of gene expression is an important and evolutionary-conserved regulatory mechanism maintaining physiological processes [1,2] and ensuring the physiological adaptation of the organism to the day/night cycle. The SCN in the hypothalamus is the main circadian synchronizer of the organism [3–5]. Recently it has been shown that beside the central clock in the SCN, almost every tissue in the body possesses a peripheral circadian clock machinery [6]. Peripheral clocks drive the circadian expression of several genes in a tissue-specific manner [2]. The molecular circadian clock machinery consists of two interacting feed-back loops. In the primary loop, positive transcription regulators ARNTL (BMAL1) and CLOCK transcription factors heterodimerize and stimulate the transcription of period (*PER1*, *PER2*, *PER3*) and cryptochrome (*CRY1*, *CRY2*) genes. However, PER and CRY proteins can heterodimerize and, by inhibiting the ARNTL/CLOCK complex, repress their own transcription [7]. The ARNTL/CLOCK complex activates the transcription of nuclear receptors RORα (NR1F1) and *REV-ERBα* (NR1D1), forming the accessory loop of the molecular clock machinery. ARNTL is positively regulated by RORα and negatively regulated by *REV-ERBα* [7]. Post-transcriptional mechanisms have also been demonstrated in the maintenance of the approximately 24 h periodicity of the expression of clock genes [7].

Peripheral clocks are set to external time by different regulatory (neural, hormonal, temperature, metabolic control) pathways [8]. Among these factors glucocorticoids are highly potent agents in the synchronization of peripheral clocks. They are able to modify and reset the circadian clock in many tissues and even in immortalized cell lines [4,9–11]. However, the underlying mechanism of glucocorticoids in the entrainment of peripheral clocks is not understood. Glucocorticoids exert their effect through the GRα isoform. After ligand binding, GRα forms homodimers and regulates transcription directly by binding glucocorticoid responsive elements (GRE) or indirectly by the modulation of other transcription factors. The GRß isoform exerts a dominant negative activity on GRα function. Recently it was shown that GRß may also have a

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GRα-independent transcriptional activity in various cell lines [12–14]. The adrenal gland, through the secretion of cortisol, has an important role in the regulation of peripheral circadian oscillators [4]. Nevertheless, animal experiments demonstrated a functional circadian clock in the adrenal gland [15–17]. The adrenal clock seems to be essential for the daily rhythmic generation of glucocorticoids [18]. In peripheral Cushing's disease, the daily pattern of cortisol secretion is disturbed and steroid production is not under the control of the hypothalamus-hypophysis-adrenal (HPA) axis [19,20]. In adrenal neoplasms, the lack of higher control may give rise to cell-autonomous regulatory mechanisms [20]. In cortisol-producing adrenal adenomas, the expression of both GRα and GRß are increased [19], and glucocorticoid receptor (GR) was recently shown to be involved in the autocrine regulatory feedback of steroid production in adrenocortical cells. Thus, glucocorticoid feedback on the adrenal clock would imply a particular connection between the two systems. Therefore, we aimed to test whether glucocorticoids could regulate the adrenal peripheral clock in the human adrenocortical cell line H295R, and we aimed to identify the possible intervention points of GRα and GRß isoforms in this regulation.

2. Materials and methods

2.1. Cell culture

The H295R cell line was grown in Dulbecco's modified Eagle's medium and Ham's F12 Nutrient Mixture (1:1) supplemented with 15 mM HEPES, 6,25 μg/ml insulin, 6,25 μg/ml transferrin, 6,25 ng/ml selenium, 1,25 mg/ml bovine serum albumin, 5,35 μg/ml linoleic acid and 2,5% Nu-Serum. Cells were cultured in a humidified incubator infused with 5% CO_2 at 37 °C. All compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Circadian experiments

H295R cells were plated at a density of 10^6 cells/well on 6-well tissue culture plates. For serum shock experiments, cells were serum starved for 24 h and incubated with 30% Nu Serum for 2 h then maintained in charcoal stripped Nu-serum-containing medium. To study the effects of GRα, cells were serum starved for 24 h then maintained in charcoal stripped Nu-serum-containing medium and treated with vehicle (0,01% ethanol), 100 nmol DEX, 1 μmol RU486 alone or in combination. In some experiments cells were plated at a density of 10^6 cells/well on 6-well tissue culture plates and on the next day cells were either serum starved or kept in normal growth medium for 24 h, and then maintained in charcoal stripped Nu-serum-containing medium and treated with vehicle (0,01% ethanol), metyrapone (100 μmol) or a combination of metyrapone (100 μmol) and RU486 (1 μmol). Cells were harvested at the indicated time points. All experiments were carried out in triplicate.

2.3. Real-time reverse polymerase chain reaction

Total RNA was isolated with miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 1 μg RNA was reverse transcribed with Invitrogen Superscript VILO reverse transcriptase (LifeTechnologies, Carlsbad, California, USA) in circadian experiments or High-Capacity RNA-to-cDNA Kit (LifeTechnologies) in transfection experiments. Quantitative Real-Time PCR (qRT-PCR) was carried out using predesigned TaqMan Gene Expression Assays (LifeTechnologies): *PER1* [Hs01092603_m1]; *PER2* [Hs00256143_m1]; *CRY1* [Hs00172734_m1]; *ARNTL* [Hs00154147_m1]; *REV-ERBα* [Hs00253876_m1]; *NR3C1* [Hs00353740_m1]; *POMC* [Hs00174947_m1]; *CRH* [s01921237_s1]; and *ACTB* [Hs99999903_m1]. Real-Time reaction was performed on 7500 Fast Real-Time PCR system (Applied Biosystem, Life Technologies, Carlsbad, California, USA) according to the manufacturer's protocol. Gene expression level was normalized to ß-actin (*ACTB*). Fold change (FC) was calculated using the $2^{-\Delta\Delta Ct}$ method. All gene expression measurements were performed in triplicate.

2.4. Transient transfections

Generation of the GRß-pcDNA3.1 plasmid (GRß) was described previously [14]. H295R cells were seeded at a density of 5×10^5 cells/well 24 h before transfection in 6-well plates in antibiotic-free media. On the next day, 2 h prior to transfection, cells were incubated in Opti-MEM serum-free medium (LifeTechnologies) and transfected with 2500 ng empty pcDNA3.1 plasmid as control or GRß plasmid using Lipofectamine3000 reagent (LifeTechnologies). Cells were transfected overnight then media was replaced with charcoal stripped Nu-serum-containing medium and treated either with vehicle or DEX for 6 h. Cells were harvested 48 h post-transfection. Experiments were carried out in triplicate.

2.5. Statistical analysis

Statistical analysis was performed using SPSS Statistics 22 (IBM). Comparison of gene expression data and hormonal measurements between groups were analyzed with ANOVA followed by Tukey's *post hoc* test or Student's *t*-test. To identify rhythmic expression within each group, gene expression was first analyzed with ANOVA to exclude random oscillation, then rhythmic gene expression was further calculated by the cosinor method with an online software program (http://www.circadian.org). Briefly, the estimation of rhythmicity by the cosinor procedure is based on if cosine curves can be fitted by least squares to the data, and if the fitted wave has an amplitude greater than zero (reviewed by Refenetti et al. [21]). A value of $p < 0,05$ was considered to be significant.

3. Results

3.1. Peripheral clock is functional in H295R adrenocortical cells

After synchronization of cells with serum shock, we detected the rhythmic expression of 4 clock genes: *PER1*, *PER2*, *REV-ERBα* and *ARNTL* by cosinor analysis. *CRY1* levels became elevated upon serum shock, but did not show any rhythmic oscillation (Fig. 1). The expression pattern of clock genes was consistent with a regulatory feedback mechanism: *PER1* and *PER2* oscillated in the same phase, whereas *REV-ERBα* and *ARNTL* showed an anti-phase pattern.

3.2. Transcriptional regulation of total GR by glucocorticoids in H295R cells

We evaluated the expression pattern of total *GR* in the H295R cell line. Interestingly, under basal conditions, the *GR* showed rhythmic expression, as confirmed by cosinor analysis, which was interrupted by pharmaceutical treatment with either DEX or RU486 alone or in combination. DEX treatment elevated GR levels at 12 h, but down-regulated its expression after 36 h of GR agonist treatment. In contrast, the non-specific GR antagonist RU486 up-regulated expression of *GR* compared to control and disrupted its rhythmic oscillation (Fig. 2).