Involvement of clock gene expression, bone morphogenetic protein and activin in adrenocortical steroidogenesis by human H295R cells

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Abstract. Functional interactions between the levels of clock gene expression and adrenal steroidogenesis were studied in human adrenocortical H295R cells. Fluctuations of Bmal1, Clock, Per2 and Cry1 mRNA levels were found in H295R cells treated with forskolin (FSK) in a serum-free condition. The changes of clock gene expression levels were diverged, with Clock mRNA level being significantly higher than Cry1 and Per2 mRNA levels after 12-h stimulation with FSK. After FSK induction, mRNA levels of StAR and CYP11B2 were highest at 12 hours and CYP17 mRNA level reached a peak at 6 hours, but HSD3B1 mRNA level was transiently decreased at 3 hours. The expression levels of Clock mRNA showed a significant positive correlation with StAR among the interrelationships between mRNA levels of key steroidogenic factors and clock genes. Knockdown of Clock gene by siRNA led to a significant reduction of FSK-induced expression of StAR and CYP17 after 12-h treatment with FSK. BMP-6 and activin, which modulate adrenal steroidogenesis, had inhibitory effects on Clock mRNA expression, whereas treatment with follistatin, a binding protein of activin, increased Clock mRNA levels in the presence of FSK, suggesting an endogenous function of activin in regulation of Clock mRNA expression. Collectively, the results indicated that changes of Clock mRNA expression, being upregulated by FSK and suppressed by BMP-6 and activin, were tightly linked to StAR expression by human adrenocortical cells.

Key words: Activin, Adrenal cortex, Bone morphogenetic protein (BMP), Clock, Steroidogenesis

THE HYPOTHALAMIC–PITUITARY–ADRENAL (HPA) AXIS, which is regulated by hormonal influences of corticosteroids and the negative feedback, is tightly linked to activity of the clock system that produces circadian rhythm [1]. The expression patterns of clock genes are tissue-specific and the expression levels of clock genes and the phasing of fluctuation in adrenal tissues are different in the medulla and each layer of the cortex [2]. It has been shown that corticoid-producing layers of the cortex are the main sites for the circadian pacemaker in the adrenal gland. It also appears that the adrenal clock regulates adrenocorticotropic (ACTH) induction leading to glucocorticoid synthesis. Of interest, the ACTH receptor, its downstream signaling molecules, and various genes implicated in the biosynthesis of glucocorticoids are included in the genes showing a circadian pattern in the adrenal gland [2].

In the presence of major stimulators such as ACTH, angiotensin (Ang) II and potassium, local autocrine/paracrine factors expressed in adrenal tissues can modulate adrenocortical steroidogenesis [3]. Transforming growth factors, fibroblast growth factor and insulin-like growth factors have been indicated to play a regulatory role in the steroidogenesis [3-6]. In our previous studies, the existence of a functional system of bone morphogenetic proteins (BMPs) and activin in adrenocortical cells was revealed [7-11]. BMP-6 was found to be an enhancer for aldosterone production induced by Ang II via activation of MAPK signaling [8-10], but activin was involved in cAMP signaling stimulated by ACTH in adrenocortical cells [7, 12].

Circadian rhythm regulates important physiological functions including blood pressure, renal function, immune response and metabolism [1, 2, 13]. The circadian rhythm regulates adrenocorticotropic (ACTH) induction leading to glucocorticoid synthesis. Of interest, the ACTH receptor, its downstream signaling molecules, and various genes implicated in the biosynthesis of glucocorticoids are included in the genes showing a circadian pattern in the adrenal gland [2].

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The dian clock consists of four core proteins mutually interacting to induce transcription of circadian target genes at the molecular level [13]. The canonical clock genes are Bmal1, Clock, period (Per)1-3 and cryptochrome (Cry)1-2. The proteins of Clock and Bmal1 form a heterodimer interacting with E-boxes to upregulate Clock-related gene transcription including Per and Cry, and the dimerized Per and Cry subsequently repress the transcriptional activity induced by Clock and Bmal1 [13].

The changes of clock gene expression in the adrenal cortex contribute to setting up the circadian rhythm of the HPA axis [1, 2, 13]. Dysfunction of clock gene expression in the adrenal cortex causes secretory dysregulation of corticosteroids. However, the physiological effects of clock-related genes expressed in the adrenal cortex and the functional roles of adrenal growth factors in the regulation of clock gene expression have yet to be elucidated.

In the present study, we investigated the biological actions and functional roles of clock genes, including Bmal, Clock, Per and Cry, in the biochemical process of steroidogenesis in cooperation with the adrenal BMP/activin system using human adrenocortical cells.

Materials and Methods

Reagents and cell culture

Forskolin (FSK) was purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO) and recombinant proteins of human BMP-6, activin A, noggin and follistatin were purchased from R&D Systems Inc. (Minneapolis, MN). Human adrenocortical H295R cells, originating from a human adrenal gland carcinoma cell line, were cultured in DMEM/F12 containing 10% FBS and Insulin-Transferrin-Selenium (ITS-G; Thermo Fisher Scientific, Waltham, MA) with 5% CO₂ at 37°C.

Quantitative real-time PCR analysis

H295R cells (1 × 10⁵ cells/mL) were treated with FSK (1 μM), BMP-6 (10 ng/mL), activin A (10 ng/mL), noggin (30 ng/mL) and follistatin (30 ng/mL) or their combination in serum-free DMEM/F12 in 12-well plates for the indicated periods. Total RNAs of H295R cells were extracted by the method using TRI Reagent® (Thermo Fisher Scientific) and RNA concentrations were evaluated using a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific). Primer pairs were selected from different exons to eliminate PCR products amplified from chromosomal DNA. Primers for steroidalogenic acute regulatory protein (StAR), Cytochrome P450 Family 11 Subfamily B Member 2 (CYP11B2), Cytochrome P450 Family 17 Subfamily A Member 1 (CYP17), and a housekeeping gene, ribosomal protein L19 (RPL19), were prepared on the basis of our previous reports [12, 14]. Other primer sequences were as follows: 359–378 and 561–580 for 3β-hydroxysteroid dehydrogenase type 1 (HSD3B1) (from GenBank accession #NM_000862), 966–985 and 1099–1118 for Bmal1 (AB000812), 2072–2091 and 2262–2281 for Clock (NM_001267843), 1614–1633 and 1870–1889 for Per2 (NM_0022817), and 1143–1162 and 1363–1382 for Cry1 (NM_004075). ReverTra Ace® (TOYOBO Co., LTD., Osaka, Japan) was used for reverse transcription and then quantitative PCR analysis was performed by using the LightCycler® Nano real-time PCR system (Roche Diagnostic Co., Tokyo, Japan). After optimization of each annealing condition and amplification efficiency [14], mRNA levels of target genes were determined by the method using the Δ threshold cycle (Ct). The Ct values of RPL19 were subtracted from those of the target genes to determine the ΔCt value. Each mRNA level of the target gene, normalized by the level of RPL19, was calculated as 2⁻ ΔCt, and then the data were represented as ratios of target genes to RPL19 mRNA.

Transfection for siRNA experiments

H295R cells (5 × 10⁵ cells/mL) were first cultured in 12-well plates containing DMEM/F12 with 10% FBS and ITS-G. The cells were transiently transfected with Clock-specific siRNA or control siRNA duplex (10 μM; 30 pmol/well) for 7-h treatment by using the transfection reagents according to the protocol of the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA) [15, 16]. Then the cells were further incubated in serum-free medium in the presence or absence of FSK (1 μM) for 24 h. The culture medium was then collected and total cellular RNA was isolated using TRI Reagent® (Cosmo Bio Co., Ltd.). The collected RNA was subjected to quantitative RT-PCR to determine the mRNA levels of Clock, StAR, CYP11B2 and CYP17 as mentioned above.

Statistics

Data were obtained from more than three independent experiments with sample triplication. All of the results are shown as means ± SEM. Statistical analysis was performed by ANOVA with Tukey-Kramer’s post hoc test and or unpaired t-test (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). The p values <0.05 were accepted as statistically significant.

Results

First, the changes in clock gene expression were evaluated after stimulation of human adrenocortical H295R cells with FSK (1 μM), which stimulates adenylyl cyclase...
and increases intracellular cAMP leading to induction of steroidogenesis in the adrenal cortex [17], in serum-free conditions for 24 h. As shown in Fig. 1A, changes in the mRNA expression of Cry1 and Per2 showed similar patterns. Cry1 and Per2 mRNA levels were increased at 3 h and 6 h after FSK (1 μM) stimulation, respectively, and then the expression levels of both Cry1 and Per2 decreased to the lowest level at 12 h after FSK stimulation. The change in the expression of Clock mRNA was significantly different from the changes in expression of Cry1 and Per2. Clock expression levels attained a moderate peak after 12-h treatment with FSK (1 μM; Fig. 1A).

Changes in mRNA levels for steroidogenetic factors and enzymes were also examined. Transcription of the CYP11B2 gene is potently induced by Ang II, leading to

Fig. 1 Serial changes of clock genes and steroidogenetic enzyme expression induced by FSK in human adrenocortical cells. A) H295R cells (1 × 10^5 cells/mL) were treated with FSK (1 μM) in serum-free DMEM/F12 for 24 h. At time points from 3 to 24 h, total RNAs were extracted, and then the expression levels of Clock, Bmal1, Per2 and Cry1 genes were standardized by RPL19 levels and expressed as fold changes. Results are shown as means ± SEM and were analyzed by the unpaired t-test: * p < 0.05 between the indicated groups. B) H295R cells (1 × 10^5 cells/mL) were treated with FSK (1 μM) in a serum-free medium for 24 h. At the indicated time, total RNAs were extracted and the expression levels of StAR, CYP11B2, CYP17, and HSD3B1 gene mRNAs were standardized by RPL19 levels and expressed as fold changes. Results are shown as means ± SEM and were analyzed by ANOVA: * p < 0.05 vs. basal groups.
enhanced production of aldosterone [18, 19]. Transcription of the CYP17 gene, encoding 17α-hydroxylase-C17,20-lyase (P450c17) for androgen and corticoid biosynthesis, is activated by ACTH-cAMP signaling [20]. A group of enzymes of 3β-hydroxysteroid dehydrogenase (HSD3B), which metabolize pregnenolone into progesterone, are required for the synthesis of glucocorticoids, mineralocorticoids and sex steroids, and they were found to be overexpressed in Cry-null mice [21]. As shown in Fig. 1B, StAR and CYP11B2 mRNA levels were significantly increased in a time-dependent manner, reaching the highest levels at 12 h after FSK stimulation, and CYP17 mRNA was also increased with a peak at 6 h after the stimulation. HSD3B1 mRNA levels were transiently reduced at 3 h and then increased for 24 h after FSK stimulation.

The expressional interrelationship between clock genes and steroidogenic enzymes in H295R cells was examined. The mRNA levels of StAR, CYP11B2 and CYP17 and clock genes including Clock, Bmal1, Per2 and Cry1 were determined in H295R cells treated with FSK (1 μM) for 24 h. As shown in Fig. 2, linear regression analysis among these factors showed that the most significant correlation was between the mRNA expression levels of StAR and Clock ($r^2 = 0.57, *p < 0.05$). Correlations between mRNA expression levels of StAR and Bmal1 ($r^2 = 0.27, **p < 0.01; n = 43$), mRNA expression levels of CYP11B2 and Clock ($r^2 = 0.18, **p < 0.01; n = 44$), mRNA expression levels of CYP17 and Bmal1 ($r^2 = 0.19, **p < 0.01; n = 43$), mRNA expression levels of HSD3B1 and Clock ($r^2 = 0.14, *p < 0.05; n = 44$), and mRNA expression levels of HSD3B1 and Per2 ($r^2 = 0.17, **p < 0.01; n = 42$) in FSK-treated H295R cells were also significant.

To elucidate the role of Clock expression in adrenocortical steroidogenesis, steroidogenetic activity was investigated in H295R cells in which Clock expression was greatly reduced by using the siRNA-targeting method. As shown in Fig. 3, Clock mRNA levels were significantly suppressed by 94% and 76% after 12-h and 24-h treatment, respectively, in FSK (1 μM)-induced conditions. The Clock suppression resulted in significant reductions of StAR mRNA by 71% and CYP17 mRNA by 48%, but not that of CYP11B2 or HSD3B1, in H295R cells treated with FSK (1 μM) for 12 h, suggesting that Clock expression is functionally related to StAR and
CYP17 expression (Fig. 3).

To clarify the functional interrelationship between the adrenal BMP/activin system and Clock gene expression, the effects of BMP-6 and activin on Clock mRNA expression were evaluated in H295R cells cultured for 24 h. As shown in Fig. 4A, BMP-6 (10 ng/mL) significantly increased the level of CYP11B2 mRNA expression induced by FSK (1 μM), while the effects of BMP-6 on mRNA levels of StAR, CYP17 and HSD3B1 were relatively weak. Different from the effects of BMP-6, activin (10 ng/mL) treatment resulted in a reduction in the levels of StAR and CYP17 mRNA expression induced by FSK (1 μM). On the other hand, as shown in Fig. 4B, it was revealed that both treatments with BMP-6 and activin (10 ng/mL) significantly suppressed the expression of Clock mRNA in the absence or presence of FSK (1 μM) in H295R cells cultured for 24 h, suggesting a functional feedback system between the BMP/activin system and Clock gene expression in adrenocortical cells. To know the involvement of endogenous BMP-6 and activin in the regulation of Clock expression, binding proteins of noggin and follistatin (30 ng/mL) for BMPs and activins, respectively, were utilized. Noggin is known to bind and antagonize various BMPs with higher affinities for BMP-2 and -4, while follistatin, which predominantly binds activin, can also affect some BMPs such as BMP-2, -4, and -7 [22, 23]. As shown in Fig. 4B, treatments with noggin and follistatin had no effect on Clock mRNA levels in the FSK-free condition, whereas treatment with follistatin increased Clock mRNA levels in the presence of FSK, suggesting that endogenous activin is likely to act for regulating Clock expression in the setting of FSK-induced steroidogenesis.

Discussion

Functional roles of the molecular clock in the adrenal gland have been shown to be associated with steroidogenesis involving the glucocorticoid receptor (GR) [24], aldosterone production [25], and salt-sensitive hypertension [26]. A recent excellent work by Doi et al. has proven that Cry1/2 regulates aldosterone production through increased expression of the adrenal rate-limiting enzyme 3β-hydroxysteroid dehydrogenase (HSD) [21]. Richards et al. showed in an in vitro study that Per1 regulates the expression of 3βHSD and also confirmed it in an in vivo study by demonstrating that reduction of Per1 decreased levels of plasma aldosterone via impaired expression of 3βHSD in mice [27]. These results support the hypothesis that Cry expression and Per expression play a key role in the regulation of aldosterone synthesis through enzymatic activation of 3βHSD.

Nagy et al. showed the presence of a peripheral clock in H295R cells [24]. In their study, rhythmic oscillation of clock genes was confirmed after synchronizing cells by serum shock. Also, H295R cells were found to show rhythmic expression of Per1, Per2, Cry1 and Bmal1 even without serum shock. In addition to their results, we
revealed a differential pattern of expressional changes in Bmal1, Clock, Per2 and Cry1 in a serum-free condition of H295R cells stimulated by FSK. Among these genes, the pattern of Clock expression was significantly different from those of Bmal1, Per2 and Cry in FSK-treated H295R cells, suggesting the presence of a functional link between Clock expression and FSK-induced steroidogenesis in adrenocortical cells.

It has been shown that clock-dependent steroidogenesis in the adrenal gland and the central mechanism regulating glucocorticoid release mutually cooperate [28]. It has also been shown that the adrenal gland possesses its own clock and that the peripheral clock is tightly linked to steroidogenesis driven by StAR activity [29]. It was further shown by an in vivo examination with adrenal-specific knockdown of the Bmal1 gene that the adrenal peripheral clock plays a critical role in harmonizing the circadian condition by generating rhythmic production of glucocorticoids [29]. In our study, it was revealed that Clock mRNA expression was closely linked to adrenal steroidogenesis by regulating StAR and CYP11B2 expression in human adrenocortical cells (Fig. 5). The expression of steroidogenetic factors and enzymes including StAR, CYP11B2 and CYP17 was readily induced by FSK stimulation in H295R cells. The expression level of Clock mRNA showed a significant positive correlation with StAR among the interrelationships between the expression levels of clock genes and steroidogenic factors. Suppression of Clock gene expression resulted in reduction of FSK-induced expression of StAR and CYP17, suggesting that the molecular clock is tightly linked to activation of steroidogenesis by adrenocortical cells.

A functional BMP/activin system complete with ligands including BMP-6 and activins, type I and type II receptors, and the binding protein exists in H295R cells [7]. Here we found that BMP-6, which enhances adrenal steroidogenesis by activating CYP11B2 expression induced by FSK, had an inhibitory effect on Clock mRNA expression by H295R cells. Of interest, activin regulates aldosterone synthesis by modulating the ACTH-cAMP cascade [7, 12], whereas BMP-6 induces Ang II-induced aldosterone production by activating Smad signaling [8, 11]. The bioavailability of BMP-6 in the adrenal cortex may be involved in the occurrence of cellular breakthrough from aldosterone suppression under the condition of chronic treatment with Ang II type-1 receptor blockers [9]. Based on the results of experiments using an anti-BMP-6 antibody, endogenous BMP-6 is likely to be an inducer of aldosterone production in vivo [10].

Fig. 4 Interaction of the BMP/activin system and Clock expression in adrenocortical cells. A) H295R cells (1 × 10^5 cells/mL) were treated with BMP-6 or activin (10 ng/mL) in a serum-free condition in the presence of FSK (1 μM). After 24-h culture, total RNAs were extracted and the expression levels of target gene mRNA were standardized by RPL19 levels and expressed as fold changes. Results are shown as means ± SEM. The results were analyzed by ANOVA: * p < 0.05 between the indicated groups and vs. control groups. B) Cells (1 × 10^5 cells/mL) were treated with BMP-6, activin (10 ng/mL), noggin and follistatin (30 ng/mL) in a serum-free condition in the presence and absence of FSK (1 μM) for 24 h. Total RNAs were then extracted and the expression levels of Clock mRNA were standardized by RPL19 levels and expressed as fold changes. Results are shown as means ± SEM. The results were analyzed by the unpaired t-test: * p < 0.05 vs. control groups.
suppressive effects of BMP-6 and activin on Clock mRNA expression may indicate the existence of a feedback loop to repress adrenal steroidogenesis via suppression of Clock activity. The results obtained by using the binding proteins noggin and follistatin further suggested that endogenous activin rather than BMP-6 is functionally active for regulating Clock expression in FSK-induced conditions of H295R cells.

As for interaction of the clock gene and steroidogenesis, we investigated the involvement of clock gene expression in ovarian steroidogenesis using human granulosa cells in an earlier study [16]. In that study, clock gene expression showed a strong positive correlation with aromatase mRNA level but was negatively correlated with 3βHSD mRNA level. Moreover, expression of Clock gene was found to be enhanced by BMP-7 and was functionally linked to estradiol induction and progesterone reduction by human granulosa cells [16]. Thus, it is possible that the local BMP system plays a modulatory role in expression control of clock genes that is functionally linked to hormonal regulation.

Collectively, our results revealed that the expression levels of Clock, being upregulated by FSK and suppressed by BMP-6 and activin, are functionally linked to StAR expression by human adrenocortical cells. It has gradually been revealed that the BMP system can be a fine regulator of fundamental endocrine activity [30]. Further research on the interrelationships between expression of clock genes and steroidogenetic factors will expand our understanding of the pathophysiology of hormonal rhythm modulated by local BMP and activin signaling and novel clinical targets for the regulation and fine-tuning of adrenal steroidogenesis.

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Disclosure Statement

The authors have nothing to disclose.

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