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Interaction of pituitary hormones and expression of clock genes modulated by bone morphogenetic protein-4 and melatonin

Naoko Tsukamoto-Yamauchi [a], Tomohiro Terasaka [a], Yasumasa Iwasaki [c], Fumio Otsuka [b, *]

[a] Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kitaku, Okayama 700-8558, Japan
[b] Department of General Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kitaku, Okayama 700-8558, Japan
[c] Department of Endocrinology, Metabolism, and Nephrology, Kochi Medical School, Kochi University, Nankoku 783-8505, Japan

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ABSTRACT

Functional interaction of clock genes and pituitary hormones was investigated by focusing on bone morphogenetic protein (BMP)-4 and melatonin actions in anterior pituitary cells. A significant correlation between the mRNA expression of proopiomelanocortin (POMC) and Per2 was revealed in serial cultures of corticotrope AtT20 cells. Knockdown of Per2 expression by siRNA in AtT20 cells resulted in a significant reduction of POMC mRNA level with or without corticotropin-releasing hormone (CRH) stimulation. Treatments with BMP-4 and melatonin, both of which suppress POMC expression, reduced Per2 mRNA as well as protein levels in AtT20 cells. On the other hand, in lactosomatotrope GH3 cells, an expressional correlation was found between prolactin (PRL) and Clock mRNA levels, which was attenuated in the presence of forskolin treatment. The siRNA-mediated knockdown of Clock expression, but not that of Bmal1, significantly reduced PRL mRNA levels in GH3 cells. Interestingly, Clock mRNA and protein levels did not fluctuate with melatonin, BMP-4 or forskolin treatment, although Bmal1 expression was significantly increased by forskolin treatment. Collectively, a significant correlation between the expression of POMC and Per2 and that between PRL and Clock were uncovered in corticotrope and lactosomatotrope cells, respectively. Per2 expression was inhibited by POMC modulators including melatonin and BMP-4, while Clock expression was steadily maintained. Thus, the effects of melatonin and BMP-4 on clock gene expression may imply differential stability of circadian rhythms of adrenocorticotropin (ACTH) and PRL secreted from the anterior pituitary.

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1. Introduction

The circadian pacemaker is known to regulate physiological rhythm through neurochemical and hormonal transmitters by coordinating the oscillations of peripheral clocks that reside in various organs [1]. As has been shown in peripheral tissues, the pituitary has also been thought to involve the molecular clock that is capable of capturing and sensitizing time autonomously [2,3]. A recent study by Wunderer and colleagues [4] has shown the existence of pituitary clock gene activity in human pituitaries, although it remains inconclusive whether the clock gene activity is functional or not. In this regard, Becquet et al. [5] demonstrated that core-clock genes exhibit a certain expression pattern using rat primary pituitary cells synchronized by forskolin (FSK), indicating the presence of a functional circadian oscillator in the pituitary. These findings suggest that pituitary cells contain the endogenous circadian system. However, the physiological role and the regulatory mechanism of this intrinsic clock in the pituitary have yet to be clarified.

There are various functional regulators such as growth factors and cytokines expressed in the pituitary. Among these, bone morphogenetic proteins (BMPs), which exert various activities on endocrine tissues [6], have been shown to play important roles in the initial development of the anterior pituitary [7]. BMP-4 not only governs pituitary organogenesis but also contributes to the

Abbreviations: ACTH, adrenocorticotropin; BMP, bone morphogenetic protein; CRH, corticotropin-releasing hormone; Cry, cryptochrome; FSK, forskolin; LH, luteinizing hormone; Per, period; POMC, proopiomelanocortin; PRL, prolactin; SCN, suprachiasmatic nucleus.

* Corresponding author. Department of General Medicine, Okayama University of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kitaku, Okayama City 700-8558, Japan. Fax: +81 86 235 7345.
E-mail address: fumiotsu@md.okayama-u.ac.jp (F. Otsuka).

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pathogenesis of differentiated pituitary lineages [8–10]. BMP-4 was found to be overexpressed in lactotrope adenomas derived from rodent models and also in human prolactinomas [11]. It is of note that bioactivity of BMP action was shown in corticotrope cells as a negative regulator of proopiomelanocortin (POMC) expression, adrenocorticotropin (ACTH) secretion and cell proliferation [12–15]. Interestingly, the expression levels of BMP-4 are reduced in human corticotrope adenomas of Cushing's disease compared with the levels in normal pituitary tissues [13].

Melatonin is a lipophilic indoleamine synthesized from serotonin by the pineal gland. Melatonin acts as a hormonal regulator for circadian rhythm in conjunction with the suprachiasmatic nucleus (SCN) and peripheral tissues, in which circulating levels of melatonin are low in the daytime and high at night [3]. However, in Cushing's syndrome, exhibiting a lack of circadian rhythm, the circadian change of melatonin was shown to be out of tune [16]. We previously reported that melatonin action via MT1 receptors augments BMP-4 receptor signaling in corticotrope cells, resulting in effective suppression of POMC transcription and ACTH production [17].

In the present study, we focused on the effects of BMP-4 and melatonin on the expression patterns of key clock genes, including Bmal, Clock, Period (Per) and Cryptochrome (Cry), that are possibly involved in controlling circadian rhythm in the regulatory process of ACTH and prolactin (PRL) production. The differential effects of BMP-4 and melatonin on clock gene expression may reflect the stability or accuracy of circadian fluctuations of ACTH and PRL secretion from the pituitary.

2. Materials and methods

2.1. Reagents and supplies

Human and rat CRH, melatonin and forskolin (FSK) were purchased from Sigma–Aldrich Corp. (St. Louis, MO). Recombinant human BMP-4 was purchased from R&D Systems Inc. (Minneapolis, MN).

2.2. RNA extraction, RT-PCR and quantitative real-time PCR analysis

Mouse pituitary corticotrope AtT20/D16v (AtT20) cells (1×10^5 viable cells) were treated with indicated concentrations of BMP-4 and melatonin alone or in combination with CRH in serum-free DMEM. Rat pituitary lactosomatotrope tumor GH3 cells (1×10^5 viable cells) were treated with indicated concentrations of BMP-4 and melatonin alone or in combination with FSK in serum-free DMEM/F12. After the indicated culture periods, total cellular RNAs were extracted using TRIzol® (Invitrogen Corp., Carlsbad, CA). The extracted RNA (1 μg) was subjected to RT reaction using the First-Strand cDNA Synthesis System® (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U) and deoxynucleotide triphosphate (dNTP; 0.5 mM) at 42 °C for 50 min and 70 °C for 10 min. Primer pairs for mouse POMC, PRL and RPL19 were selected as reported previously [14,15,17,18]. The other primer pairs were selected from different exons of the corresponding genes as follows: 428-448 and 621-642 for Bmal1 (from GenBank accession #AB015203); 1875-1896 and 1975-1996 for Clock (NM_007715); 683-705 and 871-893 for Per2 (NM_011066); and 1638-1660 and 1739-1763 for Cry1 (NM_007771). For the quantification of mRNA levels of target genes, real-time PCR was performed using the LightCycler® Nano Real-Time PCR system (Roche Diagnostic Co., Tokyo, Japan) under optimized annealing conditions at 60–62 °C. The relative expression of each mRNA was calculated by the Δ threshold cycle (Ct) method, in which ΔCt was the value obtained by subtracting the Ct value of RPL19 mRNA from the Ct value of the target mRNA, and the amount of target mRNA relative to RPL19 mRNA was expressed as $2^{-(\Delta Ct)}$. The results were expressed as the ratio of target mRNA to RPL19 mRNA.

2.3. Transient transfection of siRNA

AtT20 and GH3 cells (1×10^5 viable cells) were cultured in 12-well plates in 1 ml of individual growth medium supplemented with 10% FCS without antibiotics. Cells were transiently transfected with 10 μM target gene siRNA (30 pmol/well) including Per2, Bmal1 and Clock, or control siRNA, duplex using the transfection medium and reagents following the protocol of the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA). After 7-h transfection, cells were treated by adding 0.5 ml of normal growth medium containing two times the regular FCS and antibiotics and the cells were incubated for 24 h. After culture for indicated periods, total cellular RNA was isolated. The extracted RNA was subjected to RT reaction, and real-time PCR was performed for the quantification of mRNA levels of siRNA-target genes, POMC and PRL as described.

2.4. Western immunoblot analysis

AtT20 cells and GH3 cells (1×10^5 viable cells/well) were treated with CRH, FSK, BMP-4 and melatonin either alone or in the indicated combinations in serum-free medium. After stimulation for 6 or 24 h, cells were solubilized in 100 μl RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY) containing 1 mM Na_3VO_4, 1 mM NaF, 2% SDS and 4% β-mercaptoethanol. Cells were solubilized by a sonicator in 100 μl RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY) containing 1 mM Na_3VO_4, 1 mM NaF, 2% SDS, and 4% β-mercaptoethanol. The cell lysates were then subjected to SDS-PAGE/immunoblotting analysis using anti-Bmal1, Clock and Per2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-actin antibody (Sigma–Aldrich Co. Ltd.). The integrated signal density of each protein band was analyzed by the C-DiGit® Blot Scanner System (LI-COR Biosciences, NE). For evaluating the target protein levels, ratios of the signal intensities of the target protein/actin were calculated.

2.5. Statistical analysis

Results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. Differences between groups were analyzed for statistical significance using ANOVA with Tukey–Kramer's post hoc test or the unpaired *t*-test, when appropriate, to determine differences, and correlation of each variable was assessed by using simple regression analysis (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). *P* values <0.05 were accepted as statistically significant.

3. Results

First, we investigated the existence of an expressional correlation of POMC and clock genes in mouse corticotrope AtT20 cells. The mRNA levels of POMC and clock genes, including Bmal1, Clock, Per2 and Cry1, were examined in AtT20 cells serially cultured for 6–48 h. As shown in Suppl. Fig. 1, linear regression analysis uncovered a significant correlation between the mRNA expression levels of POMC and Per2 ($R^2 = 0.5$, $^{**}P < 0.01$) in the 24-h culture condition. As shown in Fig. 1A, the correlation between POMC and Per2 mRNA levels for 24 h was apparent ($R^2 = 0.4$, $^{**}P < 0.01$) in the condition of culture without CRH (100 nM) treatment.

To determine the functional role of Per2 expression in corticotrope cells, POMC mRNA levels were evaluated in AtT20 cells in which Per2 expression was repressed by siRNA targeting. As shown

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