Molecular and Cellular Endocrinology 375 (2013) 1–9

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Melatonin receptor activation suppresses adrenocorticotropin production via BMP-4 action by pituitary AtT20 cells

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ARTICLE INFO

Article history:
Received 11 November 2012
Received in revised form 10 May 2013
Accepted 10 May 2013
Available online 20 May 2013

Keywords:
Adrenocorticotropin
Bone morphogenetic protein
Corticotrope
Corticotropin-releasing hormone
Melatonin and ramelteon

ABSTRACT

The role of melatonin, a regulator of circadian rhythm, in adrenocorticotropin (ACTH) production by corticotrope cells has not been elucidated. In this study, we investigated the effect of melatonin on ACTH production in relation to the biological activity of bone morphogenetic protein (BMP)-4 using mouse corticotrope AtT20 cells that express melatonin type-1 (MT1R) but not type-2 (MT2R) receptors. We previously reported that BMP-4 inhibits corticotropin-releasing hormone (CRH)-induced ACTH production and proopiomelanocortin (POMC) transcription by inhibiting MAPK signaling. Both melatonin and an MT1R/MT2R agonist, ramelteon, suppressed CRH-induced ACTH production, POMC transcription and cAMP synthesis. The inhibitory effects of ramelteon on basal and CRH-induced POMC mRNA and ACTH levels were more potent than those of melatonin. Treatment with melatonin or ramelteon in combination with BMP-4 additively suppressed CRH-induced ACTH production. Of note, the level of MT1R expression was upregulated by BMP-4 stimulation. The suppressive effects of melatonin and ramelteon on POMC transcription and cAMP synthesis induced by CRH were not affected by an MT2R antagonist, luzindole. On the other hand, BMP-4-induced Smad1/5/8 phosphorylation and the expression of a BMP target gene, Id-1, were augmented in the presence of melatonin and ramelteon. Considering that the expression levels of BMP receptors, ALK-3/BMPRII, were increased by ramelteon, MT1R action may play an enhancing role in BMP-receptor signaling. Among the MT1R signaling pathways including AKT, ERK and JNK pathways, inhibition of AKT signaling functionally reversed the MT1R effects on both CRH-induced POMC transcription and BMP-4-induced Id-1 transcription. Collectively, MT1R signaling and BMP-4 actions were mutually augmented, leading to fine-tuning of ACTH production by corticotrope cells.

1. Introduction

Melatonin is a lipophilic indoleamine synthesized from serotonin by the pineal gland in conjunction with the suprachiasmatic nucleus (SCN) and various peripheral tissues. The first step of melatonin synthesis is acetylation of serotonin by arylalkylamine-N-acetyltransferase to generate N-acetylserotonin, which is followed by methylation with hydroxyindole-O-methyltransferase (Foulkes et al., 1997). Melatonin participates in the physiologic formation of circadian and seasonal rhythms (Foulkes et al., 1997; Lanoix et al., 2008). Melatonin actions are elicited via two types of G protein-coupled receptors, MT1R and MT2R, which are expressed in the brain and various peripheral tissues (Dubocovich, 2007). In the periphery, melatonin is a transducer of seasonal information that defines the length of the night (Borjigin et al., 1999, 2012; Reiter, 1993). As for the circadian regulation by endogenous hormones, there is an inverse relationship between the fluctuation of circulating melatonin and cortisol. In humans, secretion of melatonin peaks at night and decreases in the daytime. In contrast, plasma cortisol concentration peaks in the early morning and declines during the night. That is, the quiescent part of the cortisol rhythm coincides with the onset of daily melatonin rhythm. It has been shown that the adrenal cortex expresses functional MT1R, which inhibits adrenocorticotropin (ACTH)-stimulated cortisol production in the adrenal gland of capuchin monkeys

Abbreviations: ACTH, adrenocorticotropin; ActRII, activin type II receptor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; BMPRII, BMP type II receptor; CRH, corticotropin-releasing hormone; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; MT1/2R, melatonin type-1/2 receptor; POMC, proopiomelanocortin.

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http://dx.doi.org/10.1016/j.mce.2013.05.010

(Torres-Farfan et al., 2003), rats (Richter et al., 2008) and humans (Campino et al., 2011).

Melatonin also plays an essential role in the formation of reproductive rhythm for seasonal animals by acting on the pars tuberalis of the pituitary, in which melatonin receptors are abundant (Hazlerigg et al., 2001; Pevet et al., 2006). Tsutsui's group demonstrated that melatonin stimulates SCN neurons to release growth hormone (GH) and prolactin (PRL) in the bullfrog pituitary through the expression of GH-releasing peptides (Chowdhury et al., 2008). However, the effect of melatonin on pituitary function has yet to be clarified. Regarding the interrelationship between melatonin secretion and the hypothalamic-pituitary–adrenal (HPA) axis, a specific mode of melatonin secretion in situations of hypercortisolemia including ACTH-dependent and ACTH-independent Cushing's syndrome has been reported (Tomova et al., 2008). Namely, in contrast to a clear circadian pattern, i.e., higher at night and lower in the daytime, of plasma melatonin levels in normal cases, patients with active Cushing's syndrome had disturbed circadian rhythm of melatonin. This finding indicates that excessive cortisol secretion may lead to the abolishment of normal melatonin rhythm. Wu et al. (2006) also demonstrated that the MT1R of melatonin was co-localized with some parvocellular corticotropin-releasing hormone (CRH) neurons in the paraventricular nucleus. Since pituitary MT1R expression has been observed in the pars tuberalis, there seems to be a functional connection among the rhythms of melatonin, ACTH and CRH.

However, the action of melatonin on ACTH production by corticotrope cells has yet to be clarified. Given that melatonin secretion is abnormally lower at night and higher in the daytime in Cushing's patients (Tomova et al., 2008), we assumed that the key circadian factor melatonin is involved in the pathogenesis of disturbed circadian changes of ACTH and cortisol levels shown in Cushing's diseases. In addition, in this study, we focused on another key molecule, bone morphogenetic protein (BMP)-4, that can modulate activity of the undeveloped and differentiated anterior pituitary. The BMP system has been shown to play important roles in initial development of the anterior pituitary (Scully and Rosenfeld, 2002; Suga et al., 2011). BMP-4 is required during the first stage of pituitary organogenesis for proliferation of cells in Rathke's pouch, which gives rise to Pit-1 lineage cells including lactotrope cells. BMP-4 not only governs pituitary organogenesis but also plays a key role in the pathogenesis of differentiated pituitary lineages. For instance, BMP-4 is overexpressed in various lactotrope tumor models including dopamine D2-receptor null mice, estrogen-induced rat prolactinomas and human prolactinomas (Paez-Pereda et al., 2003), in which molecular interaction of BMP-4, Smad4 and estrogen receptor is functionally involved in the regulation of prolactin-promoter activity (Giacomini et al., 2009). Of note, Giacomini and colleagues first reported that BMP-4 expressed in the pituitary inhibits corticotrope cell proliferation and ACTH production by corticotrope cells, wherein BMP-4 expression is augmented by retinoic acid (Giacomini et al., 2006a,b).

In the present study, we investigated the effects of melatonin and an MT1R/MT2R agonist, ramelteon, on ACTH production in relation to the biological activity of BMP-4 using mouse corticotrope AtT20 cells that express functional MT1R. A novel reciprocity of MT1R action and BMP-4 signaling in corticotrope cells, leading to effective control of POMC-ACTH induction, was uncovered in this study.

2. Materials and methods

2.1. Reagents and supplies

Human and rat CRH, melatonin, luzindole, N^6,O^2-dibutyryl adenosine-3′,5′-cyclic monophosphate monosodium salt (BtcAMP), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma–Aldrich Corp. (St. Louis, MO). The MT1R/MT2R agonist ramelteon (Kato et al., 2005) was provided by Takeda Pharmaceutical Co., Ltd. (Osaka, Japan). Recombinant human BMP-4 was purchased from R&D Systems Inc. (Minneapolis, MN), the ERK inhibitor U0126 and p38-MAPK inhibitor SB203580 were from Promega Corp. (Madison, WI), the stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) inhibitor SP600125 was from Biomol Lab. Inc. (Plymouth Meeting, PA), and the AKT inhibitor SH-5 was from Calbiochem (San Diego, CA). Normal rat pituitary tissues were collected from 8-week-old female Wistar rats.

2.2. Measurement of ACTH and cAMP

AtT20/D16v (AtT20) cells were maintained in DMEM supplemented with 10% FCS, penicillin and streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. To assess the effects of melatonin and ramelteon on ACTH synthesis, AtT20 cells (1×10^4 viable cells/well) were precultured in 24-well plates with 10% FCS for 24 h. The medium was then changed to serum-free or 1% serum-containing DMEM, and the cells were subsequently treated with melatonin, ramelteon, luzindole and BMP-4 in the presence or absence of CRH. After 3- to 48-h culture, the supernatants of the culture media were collected and stored at −80 °C until assay. ACTH concentrations in the media were measured by a radioimmunometric assay that enables specific detection of ACTH 1–39 peptide (ACTH IRMA kit; Mitsubishi Chemical, Tokyo, Japan). To assess cellular cAMP synthesis, AtT20 cells (1×10^4 viable cells/well) were cultured in serum-free DMEM containing 0.1 mM of IBMX. After 24-h culture, the conditioned medium was collected and the extracellular contents of cAMP were determined by an enzyme immunoassay (EIA; Assay Designs, Ann Arbor, MI) after acetylation of each sample with assay sensitivity of 0.039 nM.

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

To assess the effects of melatonin and ramelteon on POMC transcription, AtT20 cells (1×10^5 viable cells/well) were precultured in 12-well plates with 10% FCS for 24 h. The medium was then changed to serum-free DMEM, and the cells were subsequently treated with indicated concentrations of BMP-4, melatonin, ramelteon, luzindole alone or in combination with CRH and various inhibitors of intracellular signaling. After 24-h culture, total cellular RNAs were extracted using TRIzol® (Invitrogen Corp., Carlsbad, CA). Expression of MT1R, MT2R and the housekeeping gene ribosomal L19 (RPL19) was detected by RT-PCR. 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. Hot-start PCR was performed using $MgCl_2$ (1.5 mM), dNTP (0.2 mM) and Taq DNA polymerase (2.5 U) (Invitrogen Corp.). PCR primer pairs were selected from different exons of the corresponding genes as follows: 145–165 and 446–466 for MT1 receptor (from GenBank accession NM_008639) and 214–235 and 445–466 (NM_145712) for MT2 receptor. Primer pairs for mouse POMC, Id-1, ALK-3, BMPRII, Smad6/7, BMP-4 and RPL19 were selected as reported previously (Tsukamoto et al., 2010). Aliquots of PCR products were electrophoresed on 1.5% agarose gels and visualized after ethidium bromide staining. For the quantification of POMC, MT1R, MT2R, Id-1, BMP receptors, Smad6/7, BMP-4 and RPL19 mRNA, real-time PCR was performed using the LightCycler-FastStart DNA master SYBR Green I system® (Roche Diagnostic Co., Tokyo, Japan). Accumulated levels of fluorescence for each product were analyzed by the second derivative method after melting-curve analysis (Roche Diagnostic Co.), and then, following assay