Neuroscience Letters 660 (2017) 63–67

Contents lists available at ScienceDirect

Neuroscience Letters

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

Research paper

Regulation of gonadotropins by urocortin 2 in gonadotropic tumor LβT2 cells

Kazunori Kageyama[a,*], Shingo Murasawa[a], Kanako Niioka[a], Fumio Otsuka[b], Hiroko Yagi[a], Makoto Daimon[a]

[a] Department of Endocrinology and Metabolism, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori, 036-8562, 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

ARTICLE INFO

Keywords:
Corticotropin-releasing factor
Urocortin
Pituitary
Stress
Gonadotropin

ABSTRACT

A close interaction has been shown between the hypothalamo-pituitary-gonadal axis and the hypothalamic-pituitary-adrenal axis. Urocortin 2 (Ucn2) has a very high affinity for the corticotropin-releasing factor (CRF) type 2 (CRF_2) receptor. Pituitary Ucn2 regulates expression and secretion of gonadotropins in response to stress. The CRF_2 receptor in the pituitary contributes to the modulation of gonadotropins. To explore the possible function of Ucn2 and the CRF_2 receptor in pituitary gonadotropic tumor cells, we examined the direct regulation of gonadotropins by Ucn2 in a representative pituitary gonadotropic tumor, mouse LβT2 cells.

LβT2 cells were found to express CRF_1 receptor and CRF_2 receptor mRNA. Ucn2 decreased CRF_1 receptor mRNA levels, while it increased CRF_2 receptor mRNA levels. Ucn2 directly decreased the mRNA levels of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in LβT2 cells. Ucn2 also decreased gonadotropin-releasing hormone receptor (GnRHR) mRNA levels. A selective CRF_2 receptor antagonist suppressed the Ucn2-induced decreases in LH, FSH, and GnRHR mRNA levels. Ucn2 acts on gonadotrophs expressing the CRF_2 receptor, and inhibits the production of gonadotropins in the pituitary gonadotropic tumor cells. (177 words)

1. Introduction

Corticotropin-releasing factor (CRF) is a major regulatory peptide in the hypothalamic-pituitary-adrenal (HPA) axis [25,26]. CRF mediates a variety of physiological functions including the regulation of HPA axis activity during periods of stress [4]. CRF contributes to stress responses via G protein-coupled receptors [26], and has higher affinity for the CRF type 1 (CRF_1) receptor [1,3,27] than the CRF type 2 (CRF_2) receptor. Urocortin 2 (Ucn2) has a very high affinity for the CRF_2 receptor but little to none for the CRF_1 receptor [7,22]. In the mouse brain, Ucn2 is expressed in restricted areas, including the magnocellular division of the paraventricular nucleus, the arcuate nucleus, and the locus coeruleus [22]. Ucn2 also acts as an anorectic neuropeptide via the CRF_2 receptor [2,5].

A close interaction exists between the hypothalamo-pituitary-gonadal (HPG) axis and the HPA axis [8,11]. Gonadotropin-releasing hormone (GnRH) is involved in the regulation of gonadotropins under various stress responses [17], and gonadotropin-inhibitory hormone can directly interact with GnRH to regulate the reproduction of vertebrates [24]. Stress profoundly inhibits reproductive function by suppressing the pulsatile release of GnRH and consequently luteinizing hormone (LH), at least in part via the CRF system as well as through the GABAergic system [18]. CRF is involved in the suppression of the HPG axis, especially the GnRH pulse generator [13,14], and it has been suggested that the CRF_1 receptor in the hypothalamus plays a major role in modulating GnRH in response to stressors [15]. The CRF_2 receptor in the hypothalamus is also involved in the modulation of pulsatile LH secretion [5,13,16]. Ucn2 is expressed and regulated in rat pituitary corticotrophs [19]. The CRF_2 receptor localizes in gonadotrophs of the anterior pituitary [12]. Both *in vivo* and *in vitro* studies have shown that Ucn2 decreases expression of gonadotropins in the rat pituitary [20,21].

Although Ucn2 has potent effects on the gonadal system in the normal rat pituitary, the possible roles of Ucn2 and CRF receptors have yet to be fully elucidated in gonadotropic tumors. To further understand the possible function of Ucn2 in pituitary gonadotropic tumors, we examined the direct regulation of gonadotropins by Ucn2 in a representative pituitary gonadotropic tumor, mouse LβT2 cells. We then determined the roles of the specific CRF_2 receptor in Ucn2-induced changes in gene expression of gonadotropins.

* Corresponding author.
E-mail address: kkageyama@hkg.odn.ne.jp (K. Kageyama).

http://dx.doi.org/10.1016/j.neulet.2017.08.052
Received 14 July 2017; Received in revised form 7 August 2017; Accepted 22 August 2017
Available online 26 August 2017
0304-3940/

2. Materials and methods

2.1. Materials

Mouse Ucn2 was purchased from the Peptide Institute (Osaka, Japan). Antisauvagine-30 (AS-30) was synthesized by Asahi Techno Glass Corp. (Chiba, Japan).

2.2. Cell culture

LβT2 cells were provided by Dr. Pamela L. Mellon, University of California, San Diego. Cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were plated at 1×10^5 cells/cm^2 for 3 days before each experiment, and the medium was changed 2 days after plating. To remove the effect of factors contained in FBS, the cells were washed and then starved overnight using DMEM supplemented with 0.2% bovine serum albumin prior to each experiment. At the end of each experiment, total cellular RNA was collected and stored at −80 °C until the assay was performed. All treatments were performed in triplicate.

2.3. RNA extraction

Cells were incubated with medium alone (control) or with medium containing Ucn2 for the times indicated in Figs. 1–4. To examine the dose-dependent effects of Ucn2, cells were incubated for the indicated times with medium alone (control) or with medium containing increasing concentrations of Ucn2 (1–100 nM). At the end of each experiment, total cellular RNA was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from total RNA (0.5 μg) using random hexamers as primers with the SuperScript First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions.

2.4. Quantitative real-time RT-PCR

Total cellular RNA extraction and cDNA synthesis were performed as previously described [9,10]. The resulting cDNA was analyzed by real-time PCR as described below. The expression of mouse LH (NM_008497.2), FSH (NM_008045.2), and GnRHR (NM_010323.1) mRNA was evaluated using quantitative real-time PCR with specific primer and probe sets (Assays-on-Demand Gene Expression Products; Applied Biosystems, Foster City, CA). β2-Microglobulin (B2MG) was used as a reference gene to standardize expression levels; B2MG mRNA levels were not significantly altered by any of the treatments used in this study. Each real-time PCR reaction consisted of 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 1 × Assays-on-Demand Gene Expression Products (Mm00656868_g1 for mouse LH, Mm00433361 m1 for mouse FSH, Mm00439143 m1 for mouse GnRHR, and Mm00437762_m1 for mouse B2MG), and 500 ng of cDNA in a total volume of 25 μL. Amplification was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the following cycle parameters: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Specific sets of primers and a TaqMan probe spanning the exon–exon junction were used in the real-time PCR assays and the results should not, therefore, have been affected by DNA contamination. Data were collected and recorded using the ABI PRISM 7000 SDS software (Applied Biosystems) and are expressed as a function of the threshold cycle (C_T). The amplification efficiency of each gene of interest and the reference gene were found to be identical when analyzed using diluted samples.

Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. In brief, for each sample assayed, the C_T values for the gene of interest and reference gene were determined. The C_T of the gene of interest was then corrected by subtracting the C_T of the housekeeping gene (ΔC_T) for each sample. Untreated control samples were used as reference samples, and the ΔC_T of all experimental samples was reduced by the average ΔC_T of the control samples ($\Delta\Delta C_T$). Finally, the abundance of the experimental mRNA relative to the control mRNA was calculated using the formula $2^{-\Delta\Delta CT}$.

2.5. Statistical analysis

Each experiment was performed at least three times. Samples were provided in triplicate for each group of experiments. Each value is expressed as the mean ± standard error of the mean (SEM). Homogeneity of variance and data distribution were examined using analysis of variance (ANOVA), followed by Fisher's protected least-significant difference *post-hoc* test. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Effects of ucn2 on CRF_1 receptor and CRF_2 receptor mRNA levels

LβT2 cells were incubated with Ucn2 to determine the effects on the

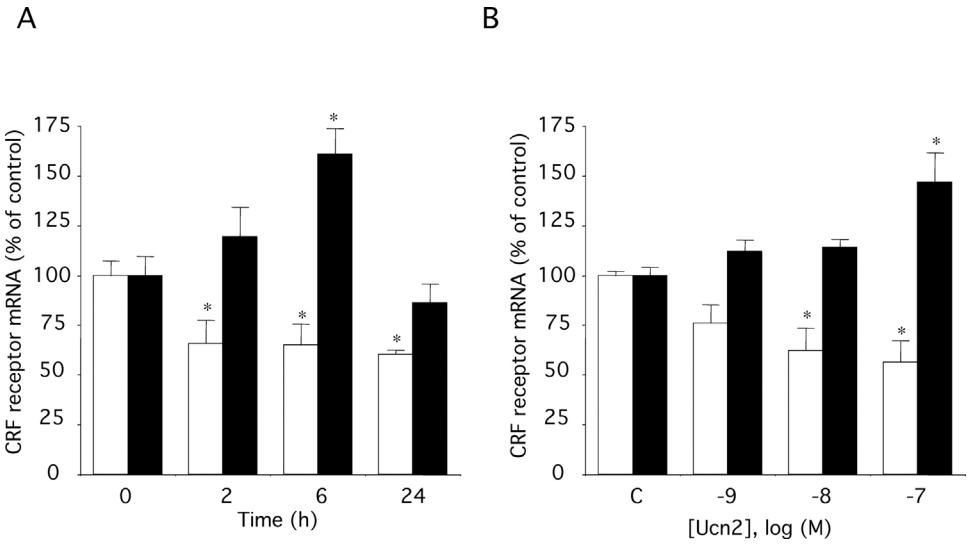

Fig. 1. Effects of Ucn2 on CRF receptors' mRNA levels in LβT2 cells. Open columns show CRF_1 receptors, and closed columns show CRF_2 receptors. Control cells (C) were treated with medium alone. Cells were treated in triplicate and the average of three independent experiments is shown. Statistical analysis was performed using one-way ANOVA, followed by *post-hoc* tests. *$P < 0.05$ (compared with control). (A) Time-dependent effects of Ucn2 on CRF receptors' mRNA levels: cells were incubated with medium containing 100 nM Ucn2. (B) Dose-dependent effects of Ucn2 on CRF receptors' mRNA levels: to examine dose-dependent effects of Ucn2 on CRF_1 receptors and CRF_2 receptors, cells were incubated for 24 h and 6 h, respectively, with medium containing from 1 to 100 nM Ucn2.