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Regulation of corticotropin-releasing factor and urocortin 2/3 mRNA by leptin in hypothalamic N39 cells

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ABSTRACT

Corticotropin-releasing factor (CRF) activates the pituitary-adrenal axis during stress, and shows anorectic effects via CRF type 1 receptors in the hypothalamus. Both urocortin (Ucn) 2 and Ucn3 also act as anorectic neuropeptides via CRF type 2 receptors. Leptin, a product of the obesity gene secreted mainly from adipose tissue, reduces food intake and increases energy expenditure. A possible interaction between leptin and CRF/Ucns has been suggested, as leptin can regulate expression and activation of CRF and Ucns in the hypothalamus. This study aimed to explore the possible function of leptin in the hypothalamus, and its effects in regulating CRF and Ucns. The study identified mRNA expression of the leptin receptor (Ob-R) and its subtypes, CRF, and Ucn2/3 in mouse hypothalamic N39 cells. Leptin stimulated signal transducer and activators of transcription type 3 (STAT3) phosphorylation, directly increased the mRNA levels of both CRF and Ucn2/3 in hypothalamic cells, and increased Ob-Rb mRNA levels. A Janus kinase inhibitor inhibited the leptin-mediated increase in STAT3 phosphorylation, and then the increases in CRF and Ucn2/3 mRNA levels. Leptin may contribute to a stress response or anorectic effect via the regulation of CRF and Ucn2/3 in the hypothalamus.

1. Introduction

Corticotropin-releasing factor (CRF), a major regulatory peptide in the hypothalamic-pituitary-adrenal (HPA) axis [61,62], mediates a variety of physiological functions including the regulation of HPA axis activity during periods of stress [15]. A related compound is Urocortin (Ucn) 1, a 40-amino acid peptide originally cloned from the Edinger-Westphal nucleus, and a member of the CRF family of peptides [63]. Both CRF and Ucn1 contribute to stress responses via G protein-coupled receptors [29,58,62]. The CRF type 1 receptor (CRF_1 receptor) [6,11,65], for which both CRF and Ucn1 have high affinity [62], is predominately expressed in the brain and pituitary gland.

Ucn2 and Ucn3 prohormones were identified in the human genome database and in mouse genomic DNA, respectively [27,35,50], which led to predictions regarding the existence and identity of endogenous peptides [20]. Ucn2 and Ucn3 are more similar to each other than they are to CRF, and both have very high affinity for the CRF type 2 receptor (CRF_2 receptor) but little to none for the CRF_1 receptor [33,38,48,57]. In the mouse brain, Ucn2 is expressed in restricted areas, including the magnocellar division of the paraventricular nucleus (PVN), the arcuate nucleus (ARC), and the locus coeruleus [50]. Ucn3 is expressed in the median preoptic nucleus and ventromedial nucleus (VMH) of the hypothalamus [35].

CRF and Ucn1 show anorectic effects in rodents [25,44,51,56]. Both Ucn2 and Ucn3 also act as anorectic neuropeptides via the CRF_2 receptor [7,19], and Ucn3 additionally regulates glucose-stimulated insulin secretion and energy homeostasis [36]. Ucn3 that innervates the VMH is also a critical molecular mediator for regulating feeding and peripheral energy metabolism [7].

Leptin, a product of the obesity (ob) gene secreted mainly from adipose tissue, regulates various reproductive, neuroendocrine, immune, and metabolic functions [39]. Leptin also regulates energy homeostasis, reduces food intake [4,23,34,46], and increases energy expenditure [34]. The mRNA coding for the long form of the leptin receptor (Ob-Rb) has been found in various brain structures, including nuclei involved in the regulation of energy balance such as the VMH, the PVN, and the ARC [26,41]. Ob-Rb is well-known

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to mediate the effects of leptin through the Janus kinase (JAK)-signal transducer and activators of transcription (STAT) pathway [22], although in the rodent hypothalamus, leptin activates only STAT type 3 (STAT3) [60].

Leptin has been reported to increase CRF secretion from the rat hypothalamus in *in intro* and ex vivo [12,49]. CRF neurons in the PVN and VMH are considered to be an important mediator for leptin that contribute to regulation of feeding and adiposity [40]. Leptin can also influence the activity of Ucn1 neurons in the mouse Edinger-Westphal nucleus [66]; however, little is known about the direct regulation of CRF and Ucns by leptin in the hypothalamus. Belsham et al. have managed to develop cell lines that are representative of the enormous range of hypothalamic cell types [2,14]. N39, developed from primary mouse fetal hypothalamic cultures, is one such homologous neuronal cell line. As they express both CRF_1 and CRF_2 receptors and Ob-R [2,32] these hypothalamic N39 cells have been used to further understand endogenous receptor signal transduction, the possible function of leptin, and the regulation of CRF and Ucns by leptin in the hypothalamus. Cui et al. used these cells to demonstrate that leptin signaling involves the JAK-STAT3 pathway [13]. In the present study, we first examined mRNA expression of Ob-R subtypes as well as the leptin-induced phosphorylation of STAT3. To further elucidate the possible function of leptin and the regulation of CRF and Ucns in the hypothalamus, we then examined the direct effects of leptin on mRNA levels of CRF, Ucns, and Ob-Rb, in hypothalamic N39 cells.

2. Materials and methods

2.1. Materials

Recombinant mouse leptin was purchased from R&D Systems (Minneapolis, MN). JAK inhibitor I was purchased from Calbiochem (San Diego, CA).

2.2. Cell cultures

N39 cells were obtained from CELLutions Biosystems Inc. (Burlington, Ontario, Canada), and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/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 10^4 cells/cm^2 for 4 days before each experiment, and the medium was changed every 48 h. On day 5, 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 or protein 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 leptin for the times indicated in Fig. 2. To examine the dose-dependent effects of leptin, cells were incubated for the indicated times with medium alone (control) or with medium containing increasing concentrations of leptin (10 pM to 1 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. Reverse transcriptase-polymerase chain reaction

RT-PCR was carried out in a programmable thermal controller (Bio-Rad, Hercules, CA) with the following oligonucleotide primers [26]: Ob-R-forward (F) (5′-CAGATTCGATATGGCTTAATGGG-3′), Ob-R-reverse (R) (5′-GTTAAAATTCACAAGGGAAGCG-3′), Ob-Ra-F (5′-ACACTGTTAATTTCACACCAGAG-3′), Ob-Ra-R (5′-AGTCATTCAAACCATTAGTTTAGG-3′), Ob-Rb-F (5′-GTGTGAGCATCTCTCCTGGAG-3′), and Ob-Rb-R (5′-ACCACACCAGACCCTGAAAG-3′). Conditions for the Ob-R and Ob-Rb were 1× (94 °C, 4 min), 40× (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min), and 1× (72 °C, 10 min) and conditions for the Ob-Ra were 1× (94 °C, 4 min), 40× (94 °C, 1 min; 54 °C, 1 min; 72 °C, 1 min), and 1× (72 °C, 10 min). Products were separated by electrophoresis on a 1.2% agarose gel containing ethidium bromide. The expected sizes of PCR products for the Ob-R and Ob-Ra were 473 bp and 237 bp, respectively. The expected size of PCR products for the Ob-Rb was 533 bp.

2.5. Real-time RT-PCR

Total cellular RNA extraction and cDNA synthesis were performed as described earlier [30,31]. The resulting cDNA was then subjected to real-time PCR as follows. The expression levels of mouse CRF, Ucns, and Ob-Rb mRNA were evaluated with quantitative real-time PCR with the following specific sets of primers and probes (Assays-on-Demand Gene Expression Products, Applied Biosystems, Foster City, CA). β2-microglobulin (B2MG) was used as a housekeeping gene to standardize values, as B2MG mRNA levels were not changed during any treatments in this study. Each reaction consisted of 1 × TaqMan universal PCR Master Mix (Applied Biosystems), 1 × Assays-on-Demand Gene Expression Products (Mm01293920 s1 for mouse CRF, Mm00445261 m1 for mouse Ucn1, Mm01227928 s1 for mouse Ucn2, Mm00453206 s1 for mouse Ucn3, Mm00440181 m1 for mouse Ob-Rb, and Mm00437762 m1 for mouse B2MG) and 1 μL of cDNA in a total volume of 25 μL with the following parameters on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems): 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min.

The above assays involved specific sets of primers and a TaqMan probe spanning the exon/exon junction and should not, therefore, have been influenced by DNA contamination. Data were collected and recorded with ABI PRISM 7000 SDS software (Applied Biosystems) and expressed as a function of the threshold cycle (C_T). The amplification efficacies for each gene of interest and the housekeeping gene amplimers were found to be identical when analyzed with diluted samples.

2.6. Western blot analysis

Western blot analysis was performed to examine protein expression of the phosphorylated (p) STAT3/STAT3. Cells were washed twice with phosphate-buffered saline (PBS) and lysed with Laemmli sample buffer. Cell debris was pelleted by centrifugation, and the supernatant was recovered. Samples were boiled and subjected to electrophoresis on a 4–20% gradient polyacrylamide gel, and proteins were transferred to a polyvinylidene fluoride membrane (Daiichi Kagaku, Tokyo, Japan). After blocking with Detector Block® buffer (Kirkegaard & Perry Laboratories, Gaithersburg, MD), the membrane was incubated for 1 h with anti-STAT3 antibody (dilution 1/1000) (Cell Signaling Technology, Beverly, MA), or anti-pSTAT3 antibody (dilution 1/1000) (Cell Signaling Technology), washed with PBS containing 0.05% Tween 20, and incubated with horseradish peroxidase-labeled anti-rabbit immunoglobulin