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LEPTIN CAN SUPPRESS THE TRANSCRIPTION OF CORTICOTROPIN-RELEASING FACTOR IN THE IMMORTALIZED HYPOTHALAMIC NEURON

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Abstract Corticotropin-releasing factor (CRF) is primary regulator of hypothalamus-pituitary-adrenal (HPA) axis, which is activated by various types of stress. Starvation is also a potent activating factor of CRF. Because the peripheral energy store reflects on plasma levels of leptin, secreted from adipose tissue, it is presumed that leptin may be involved in the CRF activity during starvation. Indeed, the attenuation of CRF or HPA axis activity by leptin treatment has been demonstrated in several reports. While many studies indicate the leptins involvement in CRF activity, its precise regulation has not been elucidated. The aim of this study is to determine the impact of leptin on the hypothalamic CRF neuron. To exclude the other hypothalamic factors, associated with leptins central effect, we employed the in vitro study using immortalized CRF expressing neuron (IVB cell). Since the mRNA expression of long-form leptin receptor was detected clearly in IVB cell with RT-PCR, we next investigated the impact of leptin on this cell. IVB cell was treated with leptin in different concentrations and periods, then, the mRNA expression and the promoter activity of CRF were analyzed with real-time PCR and luciferase assay, respectively. In result, leptin suppressed CRF promoter activity with dose- and time- dependent manner in IVB cell. This indicates that leptin can suppress the CRF transcription directly in hypothalamus. Our result might explain the mechanism partially how leptin attenuate the activation of CRF or HPA axis induced by stress or starvation.

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Key words: corticotrophin-releasing factor (CRF); leptin; immortalized hypothalamic neuron

Introduction

Corticotropin-releasing factor (CRF) is primary regulator of hypothalamus-pituitary-adrenal (HPA) axis, which is activated by various types of stress. Starvation is also a potent activating factor of CRF[1,2]. Because the peripheral energy store reflects on plasma levels of leptin, secreted from adipose tissue, it is presumed that leptin may be involved in the CRF activity during starvation. Indeed, several studies have demonstrated the leptin treatment could attenuate the activation of CRF and HPA axis induced by stress or starvation[3]. In addition, leptin's receptor is distributed not only in the hypothalamic arcuate nucleus, where is the primary center of energy homeostasis, but also in the hypothalamic paraventricular nucleus, where CRF neurons are localized predominantly[4]. While many studies indicate the leptin's involvement in CRF activity, its precise regulation has not been elucidated.

The aim of this study is to determine the impact of leptin on the hypothalamic CRF neuron. To exclude the other hypothalamic factors, such as NPY and αMSH, associated with leptin's central effect, we employed the *in vitro* study using immortalized CRF expressing neuron (IV-B cell)[5]. First of all, we demonstrate the expression of leptin's receptor as well as CRF in this neuron. Then, we evaluate the effect of leptin on CRF expression and the involvement of STAT3 pathway in this intrasellular signaling.

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Methods

Cell culture

Immortalized hypothalamic CRF neuron (IV-B cell) was kindly provided from Dr. J.Kaschow. IV-B cell was derived from the embryonic day 19 rat pup hypothalamus. Rat hypothalamic neurons were immortalized by retrovirus-mediated transfer of the SV40 large T antigen gene. Then, CRF expressing neurons were screened by the monitoring of CRF peptide with RIA. According previous reports, IV-B cell were expressing arginine vasopressin (AVP), glucocorticoid receptor (GR), as well as CRF, suggesting parvocellular CRF neuron which was critical for HPA axis modulation. IV-B cells were maintained in culture in DMEM containing 10% FBS at 37℃.

Experimental procedure

IV-B cells were plated onto 6 well dishes. After washing grown cells with serum free media, cells were incubated in leptin-containing media with several concentrations (0, 10, 100, 1000 ng/ml). For the effect of leptin treatment to CRF mRNA expression, cells were incubated for 6 hours. Using RNeasy Kit (Quiagen), total RNA was extracted from these incubated cells in each well. Real-time PCR were performed to analysis CRF mRNA expression in each sample. For the effect of leptin treatment to CRF transcriptional activity, cells were incubated for 15 minutes. Promoter activity of CRF gene was analyzed by CRF luciferase assay.

OBRb mRNA expression in IV-B cell

Complementary DNA was synthesized from total RNA extracted from IV-B cells with Superscript III (Invitrogen). To identify the expression of OBRb mRNA in IV-B cell, PCR was carried out with following primers. OBRb-forward : TTTCCTCTTGTGTCCTGCTG, OBRb-reverse : AGAAGTGAGACCATAGCTGC.

Real-time PCR analysis

Complementary DNA was synthesized from total RNA extracted from IV-B cells with Superscript III (Invitrogen). Real-time PCR using CYBER Green (BioRad) was performed with following primers.

CRF-forward: TGGATCTCACCTTCCACCTTC TG,

CRF-reverse: CCGATAATCTCCATCAGTTTC CTG,

HPRT-forward: CCTGCTGGATTACATTAAAG CACTG,

HPRT-reverse: GTCAAGGGCATATCCAACAA CAAAC

CRF luciferase assay

The construct, pCRF-Luc, which is the promoter region of CRF gene (-498 → +115) connected with luciferase reporter was transfected into IV-B cell. Following the leptin-treatment, this transfected cell was homogenized in lysis buffer (Promega), and its luminescent was analyzed with luminometer.

Statistical analysis

All data was analyzed by repeated measurea ANOVA followed by Fisher's least significant different test (LSD). Results are shown as mean ±S.E.M. Differences were considered to be statistically significant at $p<0.01$.

Results and discussion

To confirm the existence of leptin receptor on CRF neuron, we assessed whether the mRNA of leptin receptor was expressed in immortalized hypothalamic CRF expressing neuron (IV-B cell). RT-PCR could detect it as well as CRF mRNA clearly in IV-B cell (Figure1).

This indicates that IV-B cell was received some influence by leptin. Therefore, we next evaluate the impact of leptin treatment on the CRF mRNA expression and the transcriptional activity of this gene. CRF mRNA expression

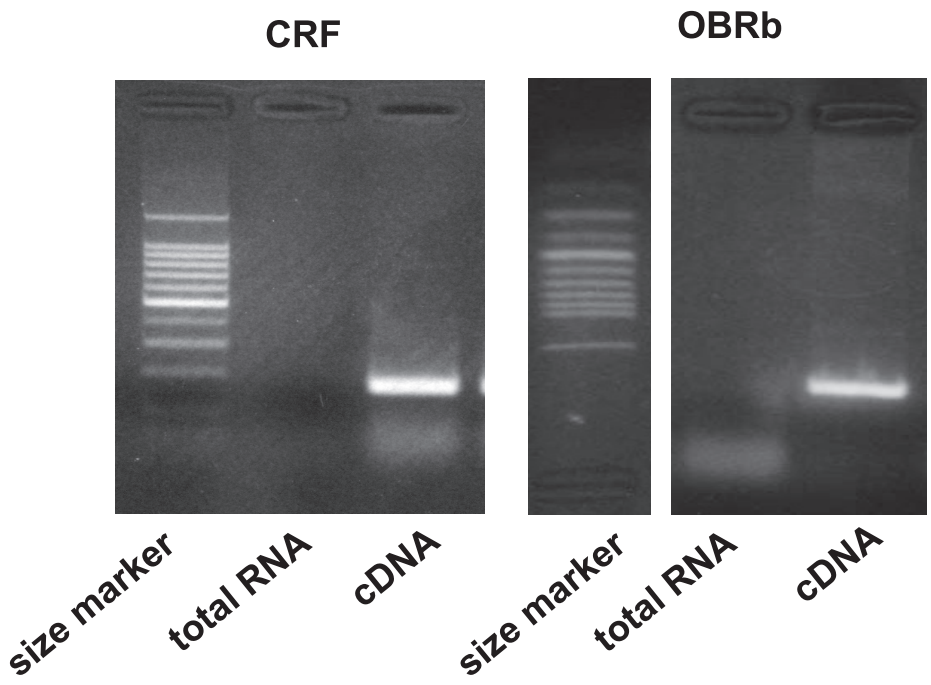

Figure 1 Messenger RNA expressions of CRF and leptin receptor (OBRb) in IV-B cell were identified by RT-PCR.

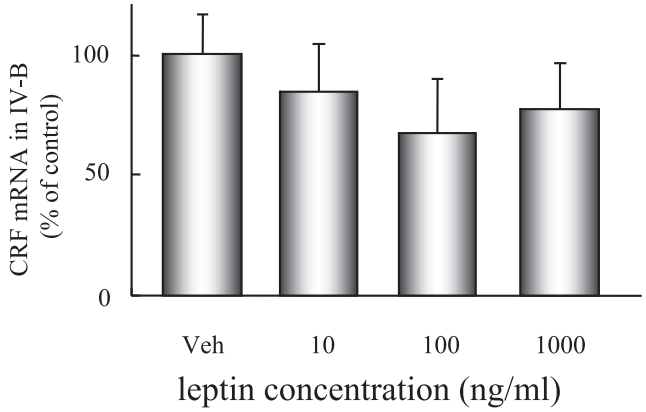

Figure 2 Immortalized CRF neuron (IV-B) was treated with leptin at several concentrations (0-10^3 ng/ml). Following 6 hours incubation, CRF mRNA expression was quantified with real-time PCR. (* $p<0.01$. vs Veh ; ANOVA, Fisher LSD)

was not influenced even by the treatment with 1000 ng/ml of leptin (Figure 2), while the transcriptional activity of CRF gene was suppressed significantly by leptin treatment with time and dose dependent manner (Figure 3).

It is not clear why these results were not consistent. To understand the involvement of leptin in CRF expression furthermore, we may have to evaluate CRF mRNA following longer or shorter incubation, or to evaluate the post transcriptional regulation of CRF gene.

We, furthermore, evaluate the intracellular

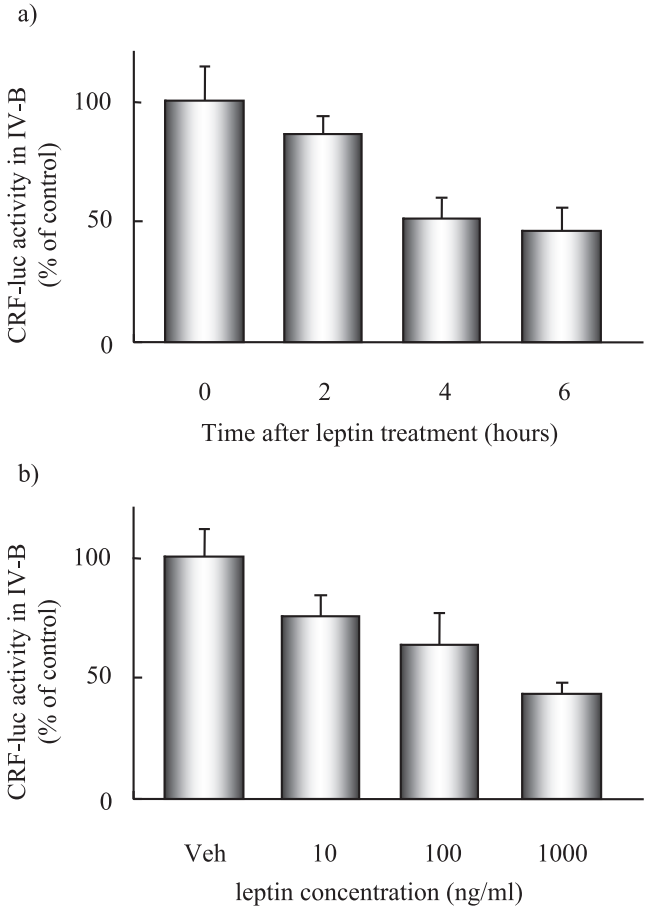

Figure 3 a) IV-B cell was treated with leptin at several concentrations (0-10^3 ng/ml). Following 6 hours incubation, CRF promoter activity was quantified with luciferase assay. (* $p<0.01$. vs Veh ; ANOVA, Fisher LSD) b) IV-B cell was treated with leptin (10^3 ng/ml). Time course of the leptin's effect on CRF promoter activity, over 6 hours, was assessed with luciferase assay. (* $p<0.01$. vs before treatment ; ANOVA, Fisher LSD)

signaling of leptin in IV-B cell. Since leptin-signaling is mediated via STAT-3 pathway in many leptin-responsive hypothalamic neurons, we evaluated the activity of this signaling in IV-B cell treated with leptin. In our study, the phosphorylation of intracellular STAT3 is not activated by leptin treatment in IV-B cell (Figure 4).

The phosphorylation of STAT3 is the critical step in the feeding regulation of leptin. Leptin binding to leptin receptor activates the associated Jak2 tyrosine kinase, which in turn phosphorylates Tyr985 and Tyr1138 of the intracellular tail of leptin receptor (Figure 5).

Phosphorylated Tyr1138 binds STAT3, resulting in its tyrosine phosphorylation and subsequent nuclear translocation and transcriptional activation, mediating the translation of several genes including POMC. On the other hand, phosphorylated Tyr985 recruits SHP-2, which becomes phosphorylated, recruits GRB2 and activates the ERK signaling pathway, resulting in the supression of NPY. While the Tyr1138 → STAT3 pathway is central for melanocortin action, especially feeding regulation, Tyr985 → ERK pathway and other signals predominate in the control of NPY-mediated physiology, such as reproductive function. In also hypothalamic CRF neuron, leptin might modulate CRF expression, and consequent HPA axis activity, via ERK

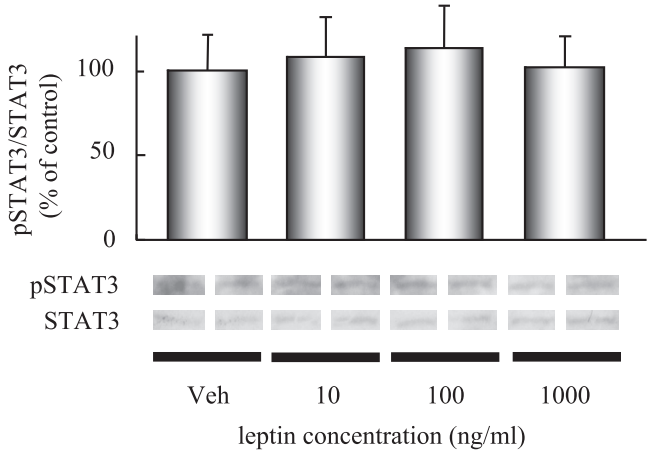

Figure 4 IV-B cell was treated with leptin at several concentrations (0-10^3 ng/ml). Following 15 minutes incubation, phosphorylated STAT3 and intact STAT3 were quantified with Western Blotting. The ratios of pSTAT3 to STAT3 were compared in each other groups. (* $p<0.01$. vs Veh ; ANOVA, Fisher LSD)

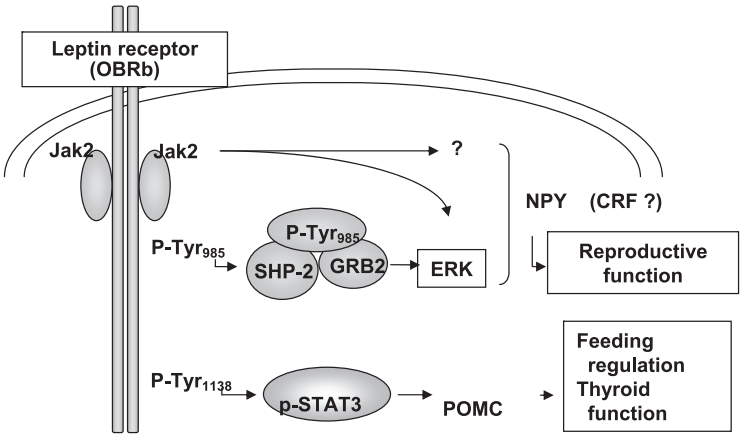

Figure 5 The intracellular signaling downstream of leptin recptor.

pathway or other signals. Further studies will be required to precise intracellular mechanism.

In this study, we clarify that leptin is associated with the CRF expression, at least in transcripional level, in immortalized hypothalamic CRF neuron. This suggests leptin's contribution to the modulation of HPA axis activity in starvation.

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