Regulatory role of AMP-activated protein kinase in corticotropin releasing hormone secreting neurons in the paraventricular hypothalamus in social stress-induced alteration of food selection behavior

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INTRODUCTION

Psychological stress stimulates intake of sweet foods or high carbohydrate diets in human as well as animals. The hypothalamus is responsible for the control of feeding behavior, and the paraventricular hypothalamus (PVH) plays a key role in macronutrient selection between carbohydrate and fat. Injection of an orexigenic peptide, neuropeptide Y (NPY), into the PVH preferentially stimulates carbohydrate intake. Our laboratory recently revealed that AMP-activated protein kinase (AMPK) in a subset of corticotropin releasing hormone (CRH) expressing neurons in the PVH (PVH-CRH neurons) increased a high-carbohydrate diet (HCD) selection over a high-fat diet (HFD) selection in mice after overnight fasting or NPY injection into the PVH. CRH plays important roles in the regulation of stress-related behavior as well as adrenocorticotropin (ACTH) secretion from the pituitary and glucocorticoid secretion from adrenal gland [Hypothalamic-Pituitary-Adrenal (HPA) axis]. AMPK is an evolutionarily conserved serine/threonine protein kinase. AMPK is activated in response to increasing intracellular AMP/ATP ratio and Thr172-phosphorylation of catalytic α-subunit (α1 and α2) of AMPK. AMPK promotes catabolic pathways whereas it inhibits anabolic pathways. AMPK in mediobasal hypothalamus regulates food intake by responding to hormonal and nutrient signals. However, the role of AMPK in PVH-CRH

neurons in stress-induced change in food selection behavior remains elusive. The objective of present study is to unravel whether AMPK in PVH-CRH neurons regulates food selection behavior between a HCD and HFD in mice response to stress.

METHODS

Animals

Male C57BL/6J mice were obtained from Nihon SLC (Hamamatsu, Japan), and B6(Cg)-*Crh*$^{tm1(cre)Zjh}$/J knock-in mice (CRH-Cre mice) were from Jackson Laboratories (Bar harbor, ME). Cre recombinase (Cre) was expressed under the control of endogenous *Crh* promoter/enhancer elements, due to the gene insertion of internal ribosome entry site and *Cre* cassette at the 3' UTR of CRH gene. Male ICR mice were obtained from Nihon SLC and used as an aggressor in social defeat stress, as described below. All animal experiments were performed in accordance with institutional guidelines for the care and handling of experimental animals, and were approved by the Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences.

Virus infection and administration of agents into the PVH

A bilateral guide cannula was pre-implanted in the PVH of mice. Lentivirus expressing short hairpin RNA (shRNA) for CRH (shCRH) was injected into the PVH of C57BL/6J mice. Lentivirus expressing shRNA for α1- and α2 subunits of AMPK (shAMPKs$^{f/f}$) or constitutively active form of AMPK (CA-AMPK$^{f/f}$) in a Cre-dependent manner was injected into the PVH of CRH-Cre mice. Adeno associated virus (AAV) expressing inhibitory DREADD (Designer Receptors Exclusively Activated by Designer Drugs), hM4Di, in a Cre-dependent manner (AAV-hM4Di$^{f/f}$) was injected into the PVH of CRH-Cre mice. Clozapine-N-oxide (CNO), activator of hM4Di, was infused into the PVH of AAV-hM4Di$^{f/f}$ infected CRH-Cre mice. NPY Y1 receptor (Y1R) selective antagonist BIBP3226 was injected into the PVH of C57BL/6J mice. All recombinant DNA experiments were approved by the relevant committee of the National Institute for Physiological Sciences, and were performed under biosafety level 2 containment for lentivirus and level 1 for AAV.

Social defeat stress

An experimental mouse was introduced in the home cage of an ICR mouse (aggressor) for 5 minutes. All experimental mice displayed subordinate posturing within the 5 min-period. The cage was then divided into two equal compartments by a stainless steel partition, and

animals were allowed to sensory contact in the same cage for 24 hours.

Measurement of food selection and locomotor activity

Food selection experiments were performed with the combination of HCD1 (D11071504M: 57% of calories from sucrose) and HFD (D12492: 55% of calories from lard), and that of HCD2 (D1171501: 50% of calories from starch) and HFD (D12492), respectively. Constituents other than carbohydrate and fat were same among HCDs and HFD. These diets were from Research Diet (New Brunswick, NJ). Food selection and locomotor activity were monitored with the use of multifaceted feeding and activity monitoring system (MFD-100M; Shinfactory, Fukuoka, Japan).

Measurement of mRNA expression

The abundance of mRNAs for CRH, α1AMPK, α2AMPK, and NPY was determined by real time quantitative polymerase chain reaction (RT-qPCR) analysis (StepOne Real-Time PCR system, Life technologies) with SYBR *Premix Ex Taq* (Takara, Shiga, Japan).

Measurement of AMPK phosphorylation

Thr172-phosphorylated AMPK (p-AMPK) was measured by immunoblot and immunohistochemical analysis with specific antibody for p-AMPK (Cat. 2535, Cell Signaling Technology, Danvers, MA).

Statistics

Data are presented as means ± s.e.m. Statistical comparisons among multiple groups were performed by analysis of variance (ANOVA) followed by Tukey-Kramer's post hoc test. Statistical analysis between 2 groups was performed by unpaired or paired Student's *t* test (two-tailed). A P value of < 0.05 was considered statistically significant.

RESULTS

Social defeat stress increased CRH mRNA expression in the PVH and plasma corticosterone concentration, and decreased locomotor activity. All mice chose HFD over HCDs when fed ad libitum. In contrast, social defeat stress increased selection of HCDs (HCD1 or HCD2) and decreased that of HFD in two-diet choice experiments. Total calorie intake did not change. Inhibition of CRH expression in the PVH by shCRH blunted the enhancement of carbohydrate selection and the increased CRH mRNA expression and plasma corticosterone

concentrations after social defeat stress. Specific inhibition of neuronal activity of PVH-CRH neurons by DREADD system also suppressed stress-induced increase in carbohydrate selection. Immunoblot and immnohistochemical analysis revealed that phosphorylation of AMPK in a subset of PVH-CRH neurons was increased by social defeat stress. Specific inhibition of AMPK expression in PVH-CRH neurons by shAMPKs$^{f/f}$ abolished the social defeat stress-induced change in food selection. In contrast, it did not affect change in CRH mRNA expression, plasma corticosterone concentration, or locomotor activity after social defeat stress. Expression of CA-AMPK$^{f/f}$ in PVH-CRH neurons increased selection of HCD selection and decreased that of HFD, similar to those after social defeat stress. However, it did not affect stress markers: CRH mRNA expression, plasma corticosterone concentration, and locomotor activity. Social defeat stress significantly increased mRNA expression of NPY in the dorsomedial hypothalamus (DMH) but not arcuate hypothalamus (ARH). Administration of Y1R antagonist BIBP3226 into the PVH partially suppressed stress-induced carbohydrate selection.

DISCUSSION

My data indicate that AMPK in PVH-CRH neurons plays a key role in regulation of food

selection behavior regarding the choice between fat and carbohydrate diet after social defeat stress. I found that social defeat stress activated AMPK in PVH-CRH neurons and enhanced carbohydrate selection in a two-diet choice experiment between a HCD and HFD. This alteration of food selection was completely suppressed by inhibition of AMPK expression in PVH-CRH neurons. Furthermore, carbohydrate selection was induced by expression of CA-AMPK in PVH-CRH neurons. These findings clearly show that AMPK activation in PVH-CRH neurons is necessary and sufficient for alteration of food selection behavior in response to social defeat stress. In contrast, AMPK in CRH neurons in the PVH did not affect total calorie intake in two-diet choice experiments.

I found that stress-induced carbohydrate selection is mediated by action of CRH and activation of CRH neurons in the PVH. Inhibition of CRH expression or its neuronal activity blunted stress-induced change in food selection behavior. Recent study in our laboratory suggested that activation of AMPK increases $[Ca^{2+}]_i$ in a subset of PVH-CRH neurons. AMPK activation thus likely increases CRH secretion from PVH-CRH neurons. I propose that social defeat stress enhances carbohydrate selection by activation of AMPK in a subset of PVH-CRH neurons and enhancement of CRH secretion.

My results also showed that social defeat stress-induced activation of HPA axis is

independent of AMPK in PVH-CRH neurons. Inhibition of AMPK expression or CA-AMPK expression in PVH-CRH neurons did not change CRH mRNA amount in the PVH or plasma corticosterone levels. CRH neurons in the PVH is unlikely involved in the regulation of locomotor activity. Previous studies suggest that other brain regions such as amygdala regulate locomotor activity after stress.

NPY is a possible candidate for stress-induced activation of PVH-CRH neurons. Present results showed that social defeat stress increased NPY mRNA expression in the DMH but not ARH and that administration of Y1R antagonist into the PVH significantly inhibited social defeat stress-induced carbohydrate selection. Previous study showed that expression of c-Fos in the PVH by psychological stress was suppressed by inhibition of neuronal activity of the DMH. Recent study in our laboratory also showed that NPY injection into the PVH activates AMPK in the PVH. These results suggest that NPY activates CRH neurons in the PVH via AMPK activation in response to stress.

In conclusion, my findings show that AMPK in PVH-CRH neurons is a principal regulator of social defeat stress-induced alteration of food selection behavior. This study provides an important insight into the regulation of food selection under stress condition and a new model of stress-induced carbohydrate craving.