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Research article

Hypothalamic Akt-mediated signaling regulates food intake in chicks

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ABSTRACT

The central anorexigenic mechanism seems to be similar in mammals and chicks, because the appetite-suppressive action of a number of peptide hormones is similar in both species. Accumulating evidence in mammals has revealed that hypothalamic Akt-mediated signaling factors (for instance, mTOR and FOXO1) are significantly involved in the regulation of food intake. However, the role of hypothalamic Akt in feeding regulation is yet to be determined in chickens. In this study, we showed that pAkt (Thr308)/Akt, pFOXO1/FOXO1, and pS6 levels were significantly increased in the hypothalami of chicks refed 1 h after a 24 h-fast in correlation to increases in the plasma concentrations of insulin, one of the activators of the Akt-mediated signaling pathways. In addition, central administration of insulin increased the phosphorylation of Akt, FOXO1, and S6 in chicken hypothalami. Furthermore, intracerebroventricular injections of both phosphoinositide 3-kinase inhibitor LY294002 and mTOR inhibitor rapamyacin enhanced the food intake of chicks. These findings suggest that hypothalamic Akt-mediated signaling pathways contribute to the regulation of food intake in chicks.

1. Introduction

Several studies have found that hypothalamic Akt-mediated signaling pathways play critical roles in the regulation of food intake and energy metabolism in mammals [1–6]. Central injections of the inhibitor of phosphoinositide 3- kinases (PI3 K, an upstream regulator of Akt phosphorylation) prevent insulin-induced anorexia and weight loss [7]. Similarly, intracerebroventricular (ICV) administrations of rapamycin, inhibitor of the mechanistic target of rapamycin (mTOR), blocks insulin-, leucine-, or leptin-induced suppression of food intake and body weight changes in rodents [3,6], and refeeding increases phosphorylation of mTOR downstream factors (S6K1 and S6) in the arcuate and paraventricular nuclei of rats [3]. The injection of adenovirus expressing constitutively active S6K1 to the mediobasal hypothalami of rats decreased the expression of orexigenic neuropeptides, such as neuropeptide Y (NPY), and agouti-related peptides (AgRP) [8]. *In vivo* studies in mice revealed that the inhibition of forkhead box-containing proteins of the O family 1 (FOXO1, which is phosphorylated and inactivated by Akt) in the hypothalamus decreased food intake and body weight, whereas its activation increased both [4]. In addition, *in vitro* and *in vivo* studies showed that FOXO1 stimulated the transcription of NPY and AgRP, but suppressed that of proopiomelanocortin (POMC, the precursor of alpha-melanocyte stimulating hormone, an anorexigenic peptide) [4,5]. All these findings suggest that hypothalamic Akt-mediated signaling pathways including the Akt/FOXO1 and Akt/mTOR pathways are important for regulating food intake by modulating the expression of neuropeptides in mammals.

Insulin is known to be a primary hormone for regulating glucose, lipid, and protein metabolism through the activation of various signaling pathways, including Akt signaling, in peripheral tissues (*e.g.* adipose tissue, liver, and muscle). Additionally, the central action of insulin has been documented in mammals over the past two decades. For example, mice with a neuron-specific disruption of the insulin receptor gene developed diet-sensitive obesity with increases in body fat and the plasma levels of triglycerides, leptin, and insulin [9]. Central and peripheral administration of insulin suppressed food intake in diabetic rats [10–12]. Intracerebroventricular administration of insulin decreases NPY expression and increases POMC in rats [10,11]. In addition, intraperitoneal administration of insulin enhanced phosphorylated Akt content in the mediobasal hypothalami of rats [7]. These findings indicate that insulin regulates the expression of appetite-related neuropeptides through Akt-mediated signaling resulting in the inhibition of food intake in mammals.

Similarly, insulin acts as an anorexigenic hormone in chickens. Central or peripheral administration of insulin is reported to suppress food intake [13–18]. Past immunohistochemical studies have shown that the insulin receptor is expressed in the chicken hypothalamus and co-localized with α-MSH and NPY in the infundibular nuclei [19]. In

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addition, central insulin injections increased POMC expression, but decreased NPY expression [13,15]. However, it remains unclear whether the hypothalamic Akt-mediated signaling pathway contributes to the regulation of food intake in chickens. In addition, it is unclear whether hypothalamic Akt-mediated signaling responds to insulin and feeding conditions in chicken, because the regulation of Akt phosphorylation is markedly different depending on the tissues in chickens. For example, in chicken liver and skeletal muscle, Akt phosphorylation is significantly increased upon refeeding and injecting with insulin [20,21], while conversely, it is significantly decreased upon fasting and injecting with anti-insulin serum [22]. Interestingly, no significant change is observed in Akt phosphorylation in chicken adipose tissue after fasting and injecting with anti-insulin serum [23]. Thus, it is necessary to examine whether hypothalamic Akt-mediated signaling is regulated by insulin injection and refeeding in chicks.

In this study, we investigated whether the hypothalamic Akt-mediated signaling pathway was involved in the central regulation of food intake in chicks.

2. Materials and methods

2.1. Animals and procedures

All animal procedures were approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimental Regulation. Day-old male layer (White Leghorn) chicks were purchased from a local hatchery (Ghen Corporation, Gifu, Japan) and maintained in a room with an automatically controlled 23 h light/1 h dark cycle (23:00–24:00 dark). They were given free access to water and a commercial chicken starter diet (23.5% crude protein and 3050 kcal/kg, Feed One. Co. Ltd., Kanagawa, Japan). The animal experiments described below were started at 10:00.

For experiment 1, 7-d-old chicks were weighed and placed in one of two cages, based on their body weight (six birds in each cage). In a previous study, 30–60 min of refeeding after 24 h-fasting dramatically increases the plasma insulin concentration [18]. Therefore, we employed the same feeding conditions in this study. After 24 h of fasting, chicks were refed 0 or 1 h prior to euthanasia by decapitation. Their blood was collected from the carotid artery. Plasma was separated immediately by centrifugation at 3000 × *g* for 10 min at 4 °C, and the plasma concentrations of insulin were measured using a commercial kit (Rat Insulin ELISA KIT (TMB), Shibayagi, Gunma, Japan), according to the manufacturer's instruction. In a previous study, we had confirmed that the data measured by the Rat insulin kit used in this study was strongly correlated with those measured by the commercial Chicken insulin ELISA kit (MyBiosource, San Diego, CA, USA) [24]. The hypothalamus was excised based on reference to a stereotaxic atlas drawn by Kuenzel and Masson [25], immediately frozen in liquid nitrogen, and stored at −80 °C until use in the western blot analyses.

For experiment 2, 9-d-old chicks were divided into two groups based on body weight (eight birds in each group). Porcine insulin was dissolved in a saline solution containing 0.1% Evans blue. Either insulin (50 pmol; MP Biomedicals Inc., Aurora, OH, USA) or vehicle (as a control) was intracerebroventricularly administered after 3 h of fasting, as in previous studies [13,15,16,18,26]. An hour after administration, the chicks were euthanized by decapitation, and their hypothalami were excised for western blot analysis. Verification of injections was done by observation of the presence of Evans blue dye in the lateral ventricle. Four successfully injected samples in each group were randomly selected for western blot analysis.

For experiment 3, 8-d-old chicks were weighed and divided into three cages based on body weight (eight birds in each cage). LY294002 (PI3 K inhibitor) or rapamycin (mTOR inhibitor) was dissolved in dimethyl sulfoxide and further diluted in saline solution containing 0.1% Evans blue, 5% polyethylene glycol 400, and 5% Tween-80 before injection, as previously reported [27]. After 3 h of fasting, either LY294002 (4 nmol), rapamycin (25 μg), or vehicle (as a control) was intracerebroventricularly administered as described in experiment 2, following which the chicks were reared individually in experimental cages. An hour after administration, the chicks were fed. Food intake was measured 30, 60, and 120 min after feed provision, and then the chicks were euthanized by decapitation to verify the injection. In previous studies, chicks were reared individually in experimental cages up to the time of experiments [16,18]. We confirmed in the preliminary study that 2 h of cumulative food intake in chicks reared and administered vehicle as described above was comparable to that in 4 h-individually-reared and fasted layer chicks, indicating that differences of experimental conditions between the present study and previous ones did not affect the cumulative food intake in chicks.

2.2. Western blot analysis

Western blot analysis was performed as previously reported [24]. Frozen hypothalami samples were ultrasonicated in a lysis buffer. Homogenates were centrifuged at 17,900 × *g* for 15 min at 4 °C, and the supernatants were stored at −80 °C. Protein concentrations were determined by the Lowry method. Muscle lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using the HorizeBlot (ATTO Co., Tokyo, Japan) according to the manufacturer's instructions. Bands were detected by Chemi-Lumi one Super (Nacalai Tesque, Inc., Kyoto, Japan), visualized with the LumiCube (Liponics Inc., Tokyo, Japan), and quantified using the CS Analyzer software (ATTO Co., Tokyo, Japan). Anti-Akt (#9272), anti-phospho-Akt (pAkt) (Thr308) (#9275), anti-pAkt (Ser473) (#9271), anti-S6 ribosomal protein (S6) (#2217), anti-phospho-S6 ribosomal protein (pS6) (Ser240/244) (#5364), anti-FOXO1 (#9454), anti-phospho-FOXO1 (pFOXO1) (Ser256) (#9461), anti-β-actin (#4967), and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (#7074) were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.3. Statistical analysis

One-way analysis of variance followed by Dunnett's (experiment 3) or Student's *t*-test (experiment 1 and 2) was performed to analyze the difference between groups. All statistical analyses were performed using Excel 2013 (Microsoft, USA) with the Statcel 3 add-in software (OMS, Tokyo, Japan).

3. Results and discussion

First, we examined whether hypothalamic Akt-mediated signaling pathways are regulated postprandially in chickens. Refeeding significantly ($P < 0.05$) increased the levels of pAkt (Thr308) and pS6, and the phosphorylation of Akt (Thr308) and FOXO1 (Fig. 1). With the corresponding activation of Akt/mTOR and inactivation of FOXO1 in the hypothalami, plasma insulin concentrations significantly increased in chicks after 1 h of refeeding (958 ± 245% of 24 h-fasting group; $P < 0.05$). These results suggest that hypothalamic Akt/mTOR signaling and FOXO1 were activated/inactivated postprandially, possibly in response to the elevation of blood insulin levels in chicks.

Leptin, as well as insulin, suppresses food intake and induces PI3 K and mTOR activation in the central nervous system of mammals [3,8,28]. In contrast, ICV injection of a synthetic partial leptin peptide of chicken does not affect food intake in chicks [29], and the leptin activity in chicken serum is undetectable [30]. These findings suggest that leptin is not involved in feeding regulation in chicks, and raise the possibility that insulin, and not leptin, is a major regulator of the hypothalamic Akt-mediated signaling pathways in chicks. Therefore, we next examined whether insulin regulates the Akt-mediated signaling pathways in chicken hypothalami. In the preliminary study, an ICV administration of 50 pmol of insulin significantly decreased 1 h of