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Central administration of insulin and refeeding lead to Akt and ERK phosphorylation in the chicken medulla

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Abstract

The purpose of this study was to investigate whether medullary cellular signaling pathways contribute to feeding regulation in chickens. Fasting inhibited the phosphorylated protein and its rates of ERK but not Akt in the chicken medulla, while refeeding promoted Akt and ERK. Intraperitoneal administration of sulfate cholecystokinin 8 did not affect medullary Akt and ERK phosphorylation in chickens. Intracerebroventricular administration of insulin significantly induced the phosphorylation of Akt and ERK in the chicken medulla. These findings suggest that the medullary Akt and ERK pathways are involved in the appetite-suppressive pathway of insulin in chickens.

Keywords: Akt, chickens, ERK, food intake, hypothalamus, medulla

1. INTRODUCTION

Accumulating evidence in mammals has demonstrated that hypothalamic cellular signaling pathways such as Akt signaling, mTOR signaling, JAK/STAT signaling, and AMPK signaling contribute to a control of food intake [1-5]. These pathways are activated by peripheral peptides, such as insulin and leptin [3-5], and nutrients, such as glucose and leucine [3,4,5]. Additionally, the medullary Erk signaling plays a key role in regulating food intake [6]. Previous studies in chickens have indicated that hypothlamic Akt, mTOR, and AMPK signaling are involved in the regulation of food intake [7,8]. Central injection of JAK inhibitors has been reported to decrease food intake in chickens [9]. However, it remains unclear whether central ERK signaling contributes to feeding regulation in chickens.

Several gut peptides such as cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), and peptide YY (PYY) suppress food intake via the afferent vagal nerve which terminates in the nucleus tractus solitarii (NTS) [10-12]. A previous study in rats reported that intraperitoneal (IP) administration of sulfated cholecystokinin 8 (CCK8s) significantly increased the level of phosphorylated ERK in the NTS, and a fourth ventricular injection of ERK inhibitor attenuated CCK8s-induced ERK phosphorylation and inhibition of food intake [13]. Peripheral administration of CCK8s significantly decreased food intake in chickens [14-16]. Additionally, vagotomy abolished CCK8s-induced suppression of food intake in chickens [17]. These findings suggest that peripheral CCK8s inhibits food intake via activation of ERK signaling in the medulla oblongata in chickens, similar to that in mammals.

Previous studies in mammals have revealed that insulin inhibits food intake and activates hypothalamic PI3K/Akt signaling [1,5]. Recent studies have shown that insulin also activates ERK signaling in the dorsal vagal complex (DVC), which contains the NTS,

dorsal motor nucleus of the vagus, and area postrema (AP), to decrease food intake [6,18,19]. Interestingly, DVC injection of a small amount of insulin significantly elevates the phosphorylation of ERK but not Akt in DVC [6], and DVC injection of ERK inhibitor, but not PI3K inhibitor, abolishes the anorectic effect of insulin [19]. These findings suggest that insulin inhibits food intake via hypothalamic PI3K/Akt signaling and medullary (specifically DVC) ERK signaling. We previously demonstrated that intracerebroventricular (ICV) administration of insulin inhibits food intake and activates hypothalamic Akt-mediated signaling in chickens [8,20]. However, it has not yet been investigated whether insulin activates medullary Akt and ERK signaling in chickens.

In the present study, we investigated whether the medullary Akt and ERK signaling pathways are involved in the regulation of food intake in chicks.

2. Materials and Methods

2.1 Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee and were performed according to the Kobe University Animal Experimental Regulation. Newly hatched male layer (White Leghorn) chicks were purchased from a local hatchery (Japan Layer K. K., 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 (Nichiwa Sangyo Co. Ltd., Kobe, Japan).

2.2 Experimental design

2.2.1 Effects of feeding conditions on phosphorylation of Akt and ERK in chicken medulla

oblongata.

Seven-day-old chicks were weighed and allocated to three groups based on their body weight (six birds per group). One group was given food *ad libitum*, and the other groups were deprived of food for 24 h. Then, the *ad libitum* group and one fasting group (8-day old) were euthanized, and the other fasting group was refed for 1 h before being euthanized. The dorsomedial region of the medulla oblongata was excised based on reference to a stereotaxic atlas drawn by Kuenzel and Masson [21], immediately frozen in liquid nitrogen, and stored at -80 °C until use in the western blot analysis.

2.2.2 Effects of peripheral CCK8s administration on food intake and medullary ERK phosphorylation in chickens.

Eight-day-old chicks were divided into two groups based on their body weight (eight birds per group). CCK8s (Peptide Institute, Inc., Osaka, Japan) was dissolved in a phosphate-buffered solution. CCK8s (60 nmol/kg) or vehicle (as a control) was intraperitoneally administered at a volume of 3 mL/kg after 3 h of fasting. Food intake was measured at 30 and 60 min after administration.

Nine-day-old chicks were divided into two groups based on their body weight (six birds per group). CCK8s or vehicle was intraperitoneally administered as described above. At 5 min post-administration, the chicks were euthanized and the medulla oblongata was excised for western blot analysis.

2.2.3 Effects of central insulin administration on phosphorylation of Akt and ERK in chicken medulla oblongata.

Nine-day-old chicks were divided into two groups based on their body weight (eight birds per group). Porcine insulin (MP Biomedicals Inc., Aurora, OH, USA) was dissolved in a

saline solution containing 0.1% Evans blue and 43.5 μM hydrochloric acid. Insulin (100 pmol/chick) or vehicle (as a control) was administered intracerebroventricularly at a volume of 10 μL after 3 h of fasting, according to the method of Davis et al [22]. Thirty minutes after administration, the chicks were euthanized by decapitation and the medulla oblongata was excised for western blot analysis. Successful injections were verified by observing the presence of Evans blue dye in the lateral ventricle, as shown in a previous study [23]. Five successfully injected samples were randomly selected for western blot analysis.

2.3 Western blot analysis

Western blot analysis was performed as previously reported [8,20]. Frozen tissue samples were ultrasonicated in a lysis buffer containing 150 mM sodium chloride, 10 mM tris(hydroxymethyl)aminomethane, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid, 1% Triton X-100, 0.5% NP-40, 100mM sodium fluoride, 23mM sodium phosphate, 2mM sodium orthovanadate, and protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). 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 using the Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). The lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting using HorizeBlot (ATTO Co., Tokyo, Japan) according to the manufacturer's instructions. Bands were detected using Chemi-Lumi one Super (Nacalai Tesque, Inc., Kyoto, Japan), visualized with the LumiCube (Liponics Inc., Tokyo, Japan), and quantified using CS Analyzer software (ATTO Co., Tokyo, Japan). Anti-Akt (#9272), anti-phospho-Akt (Thr308) (#13038), anti-p44/42 MAPK (ERK1/2) (#4695), anti-phospho- p44/42 MAPK

(ERK1/2) (Thr202/Tyr204) (#9101), anti-β-actin (#8457), anti-α-tubulin (#2125), and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (#7074) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin and anti-α-tubulin antibodies were used as loading controls.

2.4 Statistical analysis

Dunnett's test was performed to analyze the differences among the three groups using StatView version 5 (SAS Institute, Cary, NC, USA). In comparing the two groups, a *t*-test was performed using Excel 2016 (Microsoft, Redmond, WA, USA).

3. Results and Discussion

Refeeding significantly increased the phosphorylated protein content and phosphorylation rate of Akt in chicken medulla compared to fasting (Fig 1). Similarly, the phosphorylated ERK protein content was significantly increased by refeeding (Fig 1). Although no significant difference was observed on analysis by Dunnett's test, a *t*-test showed that refeeding tended to increase the phosphorylation rate of ERK (Fig 1, $P = 0.07$). These findings suggest that the medullary Akt and ERK signaling pathways physiologically contribute to satiety in chickens.

No significant change was observed in the phosphorylated protein content and the phosphorylation rate of Akt in chicken medulla between the *ad libitum* feeding and fasting groups (Fig 1). In contrast, according to the results of the *t*-test, fasting tended to decrease Erk (Fig 1, $P = 0.053$, 0.050). These findings suggest that medullary Erk signaling is involved in not only satiety signals but also hunger signals in chickens.

IP administration of 60 nmol/kg CCK8s significantly inhibited cumulative food intake in

chicks (Fig 2A), consistent with a previous study [16]. However, the same dose of CCK8s did not affect Akt and ERK phosphorylation in the chicken medulla oblongata (Fig 2B). In an additional experiment to excise the medulla oblongata at 15 min after IP administration of CCK8s, no significant change was observed in Akt and ERK phosphorylation (unpublished data). Therefore, it is likely that medullary Erk signaling is not involved in peripheral CCK8s-induced suppression of feeding in chickens, unlike in rats [13], even though peripheral CCK8s suppresses food intake via the vagal afferent neurons in chickens [17]. In addition, peripheral CCK8s does not appear to be involved in refeeding-induced phosphorylation of medullary Akt and ERK signaling.

Intracerebroventricular administration of 100 pmol/chick insulin significantly upregulated Akt and ERK phosphorylation in chicken medulla (Fig 3). A previous study showed that ICV administration of 50 pmol/chick insulin significantly decreased food intake in chickens [8]. Therefore, these findings suggest that, in addition to the hypothalamic Akt signaling pathway [8], the medullary Akt and ERK signaling pathways are involved in insulin-induced suppression of food intake in chickens.

Previous studies have shown that plasma insulin concentration is decreased by 24 h of fasting in 4-day-old chicks [24] and increased by 1 h of refeeding after 24 h of fasting in 8-day-old chicks [8]. In the present study, the phosphorylation of both Akt and ERK in chicken medulla was responsive to the central administration of insulin (Fig 3) and feeding conditions (Fig 1). These finding suggest that elevation of plasma insulin concentration activates medullary Akt and ERK signaling in response to feeding in chickens.

Amylin is co-secreted with insulin from pancreatic β-cells in response to nutrient stimuli and reduces food intake in mammals [25]. Subcutaneous injection of amylin induces ERK phosphorylation in the AP, NTS, and lateral parabrachial nucleus [26]. Interestingly,

previous studies in birds showed that central and peripheral administration of amylin decreased feed intake and increased the number of c-fos immunoreactive AP and NTS cells [27]. Although it remains unclear whether amylin is co-secreted with insulin in response to nutrient stimuli in chickens, these findings raise the hypothesis that amylin is involved in the phosphorylation of ERK increased by refeeding in chicken medulla oblongata.

Glucagon-like peptide-1 and PYY inhibit food intake in mammals [10-12] and chickens [28]. In addition, GLP-2 has been suggested to function as an anorexigenic hormone in chickens [29]. Recent studies in chickens have shown that short-term fasting significantly decreases PYY expression in the pancreas [30] and jejunum [31]. Additionally, we confirmed that ileum preproglucagon mRNA levels were significantly increased by refeeding in 21-day-old chickens (unpublished data). These findings indicate that these gut hormones contribute to the postprandial suppression of appetite in chickens. GLP-1 and PYY directly activate the nodose ganglion neurons of the vagal afferent nerves in rodents [32,33]. Therefore, further studies are required to clarify whether peripheral GLP-1, GLP-2, and PYY activate medullary Akt and ERK phosphorylation via the vagal afferent nerves in response to food intake in both mammals and chickens.

4. Conclusion

The results of the present study showed that central insulin injection and refeeding upregulated the phosphorylation of Akt and ERK in chicken medulla, suggesting that both signaling pathways contribute to the appetite suppression effect of insulin in chickens.

Acknowledgements

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301 **Legends**

302 **Figure 1. Effects of feeding conditions on protein levels of the medullary Akt and**
303 **ERK in chickens**

304 Data are expressed as mean ± SEM of six birds in each group. Dunnett's test was used to
305 analyze the differences between groups. *Significance with respect to the 24 h-fasting
306 group. Ad, ad libitum feeding group; F, 24-h fasting group; R, 1-h refeeding after 24-h
307 fasting.

308

309 **Figure 2. Effects of peripheral injection of sulfate cholecystokinin 8 on food intake**
310 **and medullary Akt and ERK phosphorylation in chickens**

311 Data are expressed as mean ± SEM of eight (A) and six (B) in each group. The *t*-test was
312 used to analyze the differences between groups. **Significance with respect to the vehicle
313 group ($P < 0.01$).

314

315 **Figure 3. Effects of central insulin injection on medullary Akt and ERK**
316 **phosphorylation in chickens**

317 Data are expressed as mean ± SEM of five birds in each group. The *t*-test was used to
318 analyze the differences between groups. *Significance with respect to the vehicle group
319 (*, $P < 0.05$).

Figure1

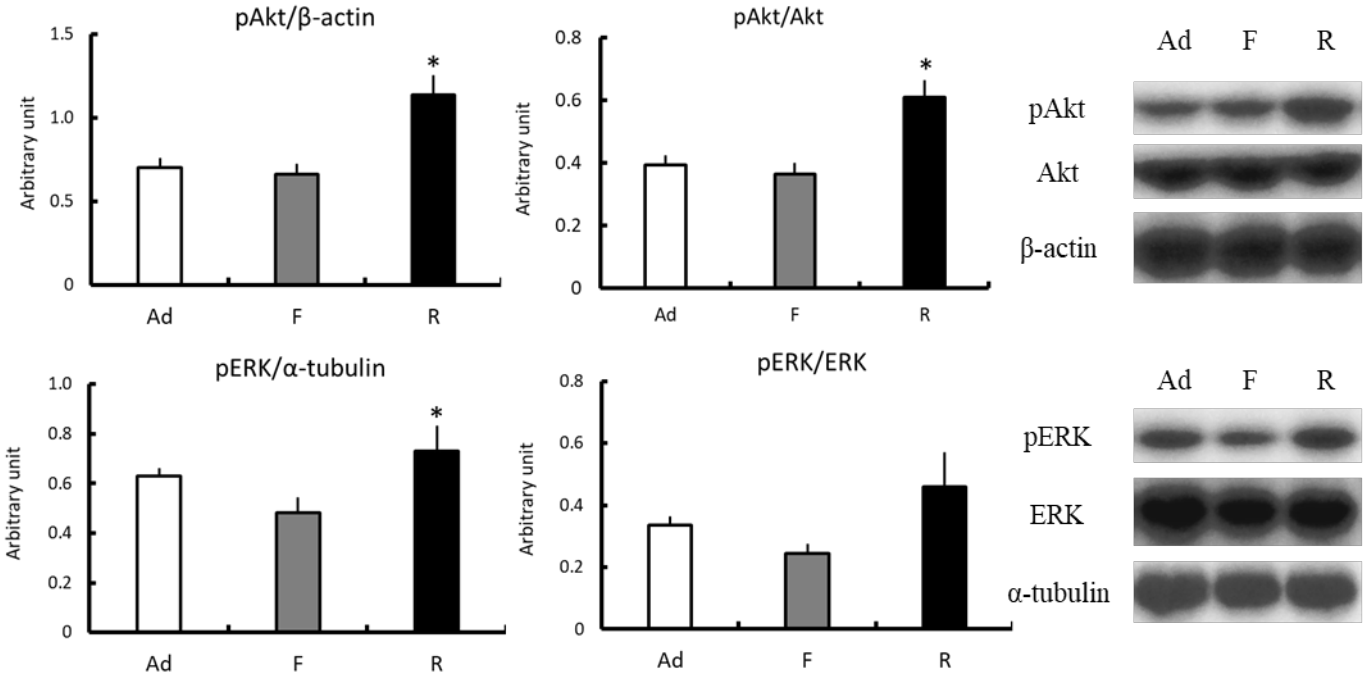
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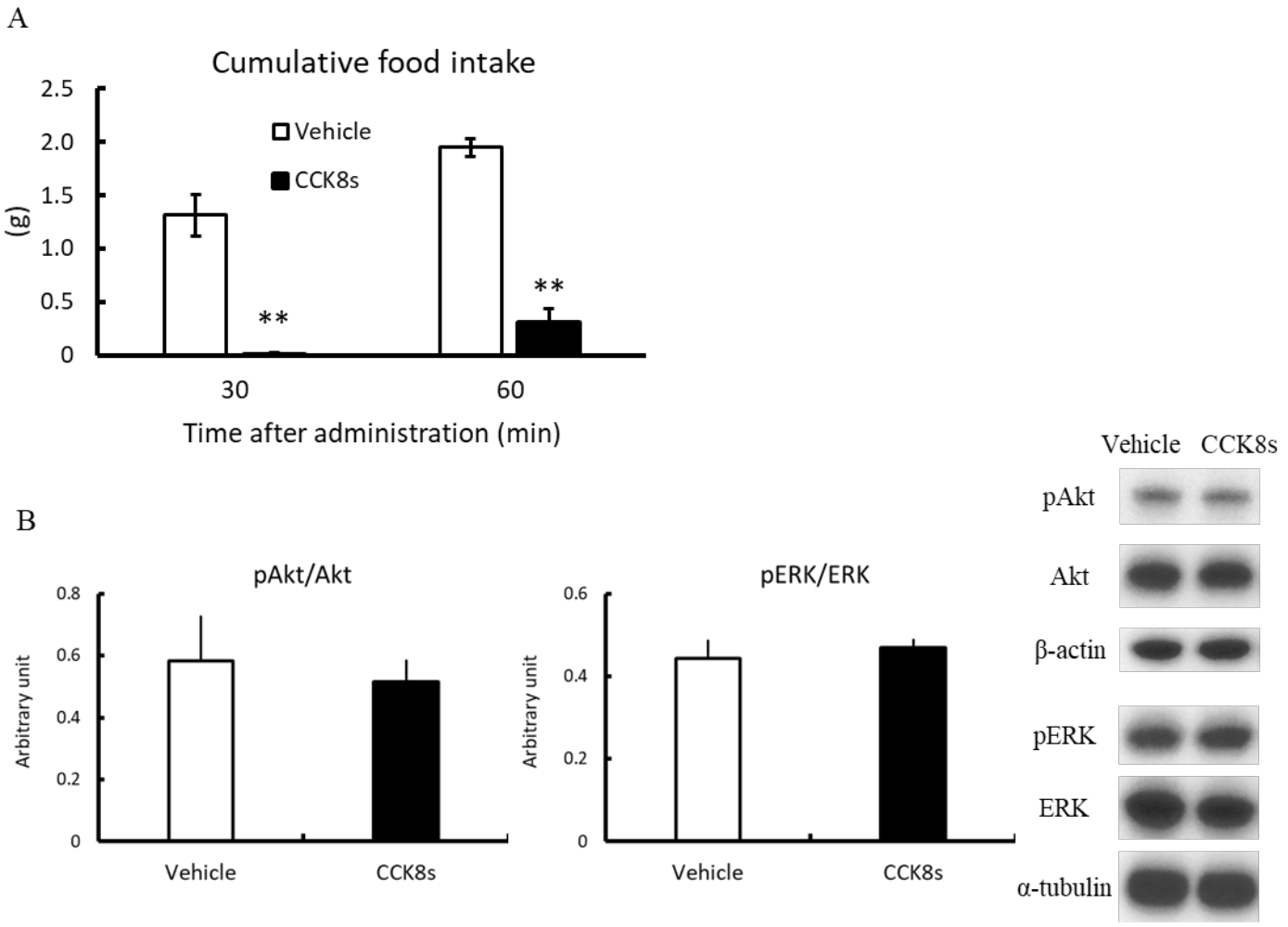
Figure 2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CCK8s
pERK/ERK
Arbitrary unit
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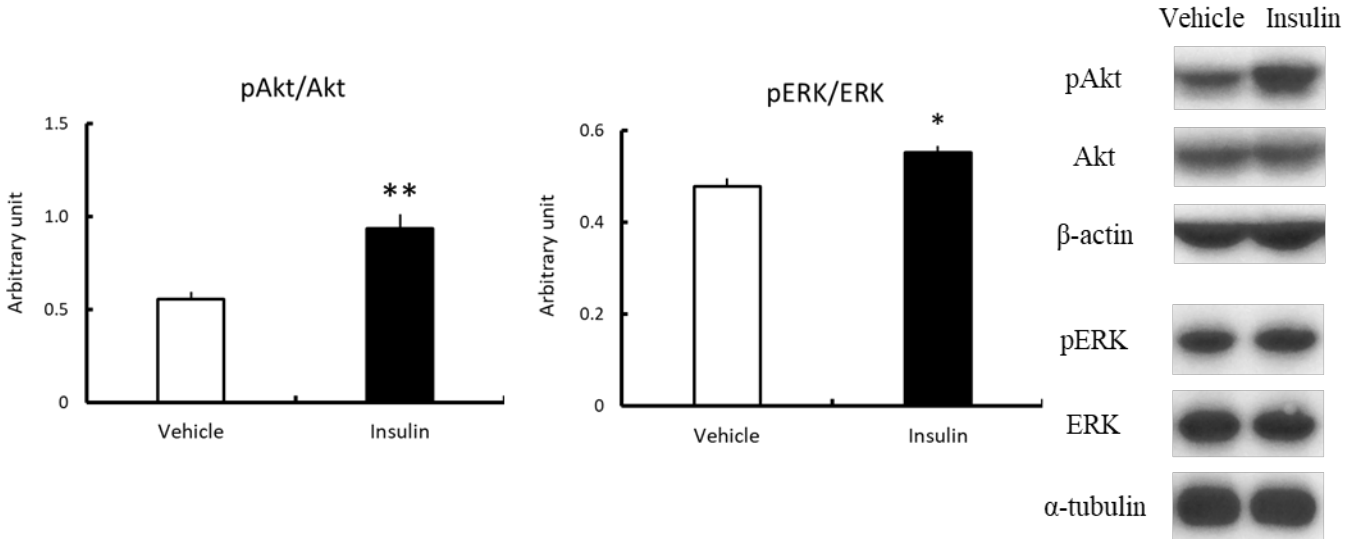
Figure 3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