Central Insulin Suppresses Feeding Behavior via Melanocortins in Chicks

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Running title: Insulin and Food Intake in Chicks

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Abstract

Growing evidence suggests that insulin interacts with both orexigenic and anorexigenic peptides in the brain in the control of feeding behavior in mammals. However, the action of central insulin in chicks has not yet been identified. In the present study, we investigated the effects of central injection of insulin on feeding behavior in chicks. Intracerebroventricular (ICV) administration of insulin, at doses that do not influence peripheral glucose levels, significantly inhibited food intake in chicks. Central injection of insulin in chicks also significantly increased expression of pro-opiomelanocorin (POMC) mRNA, and decreased that of neuropeptide Y (NPY) mRNA. Finally, co-injection of the melanocortin antagonist (SHU9119 or HS014) prevented the reduction in food intake caused by ICV administration of insulin. These data suggest that insulin functions in chicks as an appetite-suppressive peptide in the central nervous system, and that the central melanocortin system mediates this anorexic effect of insulin, as in mammals.

Key words: insulin; melanocorins; POMC; food intake; chick

1. Introduction

Numerous studies have been developed in support of the hypothesis that pancreatic insulin, in addition to its role as a regulator of plasma glucose, also functions as an adiposity signal to the central nervous system in mammals [1, 2]. This signal in the brain induces both short- and long-term effects on food intake regulation and body weight [3, 4, 5, 6]. The primary target of this hormone seems to be the hypothalamus including the arcuate nucleus (ARC), which is a key brain area for regulating energy homeostasis [7, 8]. In fact, the ARC has a large density of insulin-specific binding sites, and has a large population of neurons that have been shown to express insulin receptor mRNA by in situ hybridization [9].

Recent research has focused on the mechanisms underlying the anorexigenic effects of insulin. Central administration of insulin during food deprivation in rats prevents the fasting-induced increase in hypothalamic levels of the orexigenic neuropeptide Y (NPY) mRNA in the ARC [10]. Moreover, central injection of insulin increases the anorexigenic peptide α-melanocyte stimulating hormone (α-MSH) precursor pro-opiomelanocortin (POMC), and the anorexic effect of insulin is also blocked by a melanocortin antagonist [11]. Taken together, these findings suggest that the anorexigenic effects of insulin in the central nervous system are associated with changes in the expression of hypothalamic neuropeptides.

In birds, the involvement of central insulin in the regulation of feeding behavior is uncertain but insulin seems to act as a central adiposity signal for regulating feeding behavior and energy balance, similar to mammals. The insulin receptor is present in the chicken brain [12], and the chicken insulin receptor gene has been cloned [13]. In addition, insulin secretion is clearly correlated with food intake in birds [14]. There is, however, no information on whether central insulin affects food intake and/or the expression of hypothalamic neuropeptides in chickens. Thus, the purpose of the

present experiments was to determine if insulin could act within the central nervous system of neonatal chicks to control ingestive behavior, and interacts with both the orexigenic and anorexigenic peptidergic systems in ways similar to mammals.

2. Materials and methods

2.1. Animals

Day-old male Single Comb White Leghorn (SCWL) chicks (Akita Co. Ltd, Hiroshima, Japan) were maintained in a room with 24-h lighting and at a temperature of 30°C. They were given free access to a commercial starter diet (Nichiwa Sangyo Co. Ltd., Kobe, Japan) and water during the pre-experimental period. They were distributed into experimental groups based on their body weight so that the average body weight was as uniform as possible for each treatment. The birds were reared individually in experimental cages and had ad libitum access to food up to the time of experiments. The handling of birds was performed in accordance with the regulations of the Animal Experiment Committee of Hiroshima University.

2.2. Drugs and intracerebroventricular (ICV) infusion protocols

Porcine insulins were purchased from MP Biomedicals, Inc. (Auroa, OH, USA). SHU9119 (non-selective melanocortin-3 and 4 (MC3/4-R) receptor antagonist) was obtained from Phoenix Pharmaceuticals (Belmont, CA, USA). HS014 (selective melanocortin-4 (MC4-R) receptor antagonist) was purchased from Sigma (St. Louis, MO, USA). The drugs were dissolved in a 0.1% Evans Blue solution, which was prepared in 0.85% saline. Saline containing Evans Blue was used as a control. After being deprived of food for 3 h, the birds (3- or 4-day-old) were ICV injected with the solutions (10 μl) using a microsyringe according to the methods used by Davis et al. [15]. Each chick was injected once only with a dose of either drug(s) or saline. At the end of the experiments, birds were sacrificed by decapitation, followed by brain

sectioning to identify the location of the drug injection. Data were deleted for individuals in which the presence of Evans Blue dye in the lateral ventricle was not verified. The number of birds used for data analysis is shown in each figure.

2.3. Experimental designs

2.3.1. Effect of ICV insulin on food intake

Chicks were injected with three levels (0, 2 and 20 ng) of insulin. Food intake was measured at 30, 60 and 120 min after injection, and determined by measuring the reduction of diet from a pre-weighed feeder. The weight of feeders was measured using an electric digital balance of precision ± 1 mg.

2.3.2. Effect of ICV insulin on POMC, NPY and AGRP mRNA levels in chick brain

Birds were injected with two levels (0 and 20 ng) of insulin. At 15, 30 and 60 min after the injection, the chicks were killed and the brainstems including hypothalamus were collected. RNA was isolated from the dissected tissue using Trizol reagent (invitrogen, CA, USA) according to the manufacturer's instructions. To rule out the possibility that PCR products would result from the amplification of genomic DNA contaminating the RNA sample, RNA samples were treated with DNase I using the DNA-free kit (Ambion, Austin, USA). Total RNA (480 ng) was reverse transcribed at 42°C for 15 min in 10 μl of 1 × PrimeScript buffer containing 50 μM random primers and Prime Script RT Enzyme Mix I (Takara, Tokyo, Japan). The reaction product was subjected to Q-PCR performed according to the user instructions for the Light Cycler system (Roche Applied Sience, IN, USA). Briefly, following a denaturation step at 95°C for 10 s, PCR was carried out with a thermal protocol consisting of 95°C for 5 s and 60°C for 20 s in 20 μl buffer containing 1 × SYBR Premix EX Taq (Takara, Tokyo, Japan) and 0.2 μM of each primer. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous

control. The primers used were as follows: POMC, 5'-aacagcaagtgccaggacc-3' (sense) and 5'-atcacgtacttgcggatgct-3' (antisense). AGRP, 5'-aggccagacttggatcagatg-3' (sense) and 5'-actccaggaggcggacac-3' (antisense). NPY, 5'-ggcactacatcaacctcatc-3' (sense) and 5'-ctgtgctttccctcaacaa-3' (antisense). GAPDH, 5'-gccgtcctctctggcaaa-3' (sense) and 5'-tgtaaaccatgtagttcagatcgatga-3' (antisense). For the data analysis, the average threshold cycle (C_T) of each set of triplicates was calculated. To normalize the data, the ΔC_T was calculated for each sample by subtracting the average C_T of GAPDH from the average C_T of the gene of interest. For relative quantitation, the ΔC_T was averaged for the defined control group and was then subtracted from the ΔC_T of each experimental sample to generate the $\Delta\Delta C_T$. The $\Delta\Delta C_T$ was then used to calculate the approximate fold difference, $2^{\Delta\Delta CT}$. The results were expressed as the gene of interest mRNA/GAPDH mRNA ratio. Each PCR run included a no template and replicates of control and unknown samples.

2.3.3. Effect of ICV melanocortin antagonists on insulin-induced anorexia

Chicks were injected with saline, insulin (20 ng), melanocortin antagonist (SHU9119 or HS014) or insulin co-injected with melanocortin antagonist. The doses of both antagonists applied here were decided according to the preliminary trials, in which they did not affect feeding behavior in chicks. Food intake was measured at 30, 60 and 120 min after injection.

2.4. Statistical analysis

The data were analyzed using the commercially available package, StatView (Version 5, SAS Institute, Cary, USA, 1998). For analysis of feeding data, comparisons between means were made using the Tukey-Kramer test. For comparisons between means of each mRNA level, a student-t test was done at each time point. Differences were considered to be significant when P was less than 0.05. Results are presented as means ± SEM.

3. Results

3.1. Effect of ICV insulin on food intake

The ICV injection of insulin significantly inhibited food consumption in fasted chicks when compared with control ($P<0.001$) (Fig. 1). The anorexic effect by high dose of insulin (20 ng) lasted at least for 120 min. However, both doses of insulin did not affect the level of plasma glucose at each time point when compared with control (unpublished data).

3.2. Effect of ICV insulin on POMC, NPY and AGRP mRNA levels in chick brain

As depicted in Figure 2, POMC expression in the insulin-treated chicks was significantly higher than that of the level in the saline chicks at 15 min post-injection ($P<0.05$), but not significantly different at 30 and 60 min post-injection ($P>0.1$). Although levels of NPY mRNA were not affected by ICV injection of insulin at 15 and 60 min post-injection ($P>0.1$), NPY expression in the insulin-treated chicks was significantly decreased at 30 min post-injection ($P<0.001$). AGRP expressions were not affected by ICV injection of insulin at each time point when compared with control ($P>0.1$).

3.3. Effect of ICV melanocortin antagonists on insulin-induced anorexia

Both antagonists (SHU9119: 2.0 pmol, HS014: 0.5 pmol) in the absence of insulin had no effect on food intake for 120 min post-administration ($P>0.05$) (Figs. 3 and 4). Insulin by itself caused a significant reduction of food intake during each experimental period ($P<0.001$), and this was completely reversed by the presence of each antagonist ($P<0.01$).

4. Discussion

The present results show that ICV injection of insulin produced anorexic

response in chicks (Figs. 1, 3 and 4), are in good agreement with other reports in mammals [3, 11, 16]. Thus, these results suggest that endogenous insulin participates in the regulation of feeding behavior in chicks, similar to mammals. However, the effective dose in this study was markedly higher than that in mammals [3, 11, 16]. Chickens have blood glucose concentrations that are twice as high as in mammals and have a marked resistance to the peripheral insulin injection [14, 17]. Simon et al. [18] suggested that the insulin resistance in chickens is associated with lower insulin receptor numbers in liver tissues compared with mammals. It is reasonable to think that the number of insulin receptors in chick brains is also less than that in mammalian species.

POMC expression was stimulated and NPY expression was suppressed by ICV injection of insulin in chick brains (Fig. 2). Hence, the possible mechanism involved in the anorexigenic effects of insulin in chicks may be through activation of the central melanocortin system and decreased activity of the NPY system, as in mammals [2, 11, 19]. However, there was the increase of POMC expression (15 min post-injection) before the decrease of NPY expression (30 min post-injection). The reason assumed for this delay response is as follows: the adiposity signals directly depolarizes the POMC neurons while simultaneously hyperpolarizing the somata of NPY neurons [2, 20]. Then, it might be necessary to synthesize the POMC-derived melanocortins promptly but the NPY expression might gradually decrease.

Although the differences in both POMC and NPY expression were not detected at the 60-min point, the anorexic effect of insulin was maintained until 120 min post-injection (Fig. 2). It seemed that the anorexigenic system mediated by second-order neuronal signals, such as corticotropin-releasing hormone in the paraventricular nucleus [21] might be still active. In fact, ICV injection of insulin (20 ng) significantly increased plasma corticosteron concentration at 60 min post-injection

(unpublished data).

In the case of AGRP mRNA, we found that central insulin did not affect the level of that at each time point (Fig. 2). The reason for this is not clear, but some hypotheses are proposed. Firstly, it is likely that insulin may have little influence on AGRP expression. It is reported that anorexic doses of insulin tended to reduce hypothalamic AGRP mRNA in mice but the difference was not significant [16], and NPY/AGRP neurons are not necessary for the orexigenic response to glucose deficiency [22]. Secondly, the central AGRP system in chicks seems to be different from that in mammals. Expression AGRP in chickens is similar to that in the transgenic obesity mice with hyperinsulinemia and hyperglycemia [23, 24].

To this end, we determined the ability of melanocortin receptor antagonists to block the anorexic actions of centrally administered insulin (Figs. 3 and 4). Both non-specific and specific antagonists were able to significantly attenuate the reduction in food intake elicited by insulin administration. Therefore, insulin appears to exert its central catabolic action by acting through melanocortin receptors, similar to mammals [11]. Although MC3-R and MC4-R, which have potential roles in the control of food intake and body weight in mammals, are highly expressed in mammal brains [25, 26], MC3-R expression is not detected by RT-PCR in the chicken brain [27, 28]. The anorexigenic action of central insulin in birds seems to be mediated by MC4-R. Consistent with this assumption, ICV injection of HS014 stimulated food consumption in ad libitum doves [29].

Further research on the central melanocortin or NPY/AGRP system is needed to understand the role of central insulin in the regulation of appetite in avian species. However, the results described here suggest that the central melanocortin system is an important downstream target for the effects of insulin to regulate food intake in neonatal chicks.

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Legends

Fig. 1. Cumulative food intake of fasted chicks injected ICV with saline or one of two doses of insulin (2 or 20 ng). Values are means ± SEM of the number of chicks in parentheses. Means with different letters are significantly different at $P<0.05$.

Fig. 2. Effect of central insulin on POMC (upper panel), NPY (middle panel) and AGRP (lower panel) mRNA in chick brainstem as determined by real-time quantitative PCR. The number of chicks used were as follows: 15 min control, 6; 15 min insulin, 6; 30 min control, 5; 30 min insulin, 5; 60 min control, 5; 60 min insulin, 5, respectively. Values represent means ± SEM. *$P<0.05$, compared with saline control at each time point.

Fig. 3. Cumulative food intake of fasted chicks injected ICV with saline, insulin (20 ng), SHU9119 (SHU: 2.0 pmol) or insulin co-injected with SHU. Values are means ± SEM of the number of chicks in parentheses. Means with different letters are significantly different at $P<0.05$.

Fig. 4. Cumulative food intake of fasted chicks injected ICV with saline, insulin (20 ng), HS014 (HS: 0.5 pmol) or insulin co-injected with HS. Values are means ± SEM of the number of chicks in parentheses. Means with different letters are significantly different at $P<0.05$.

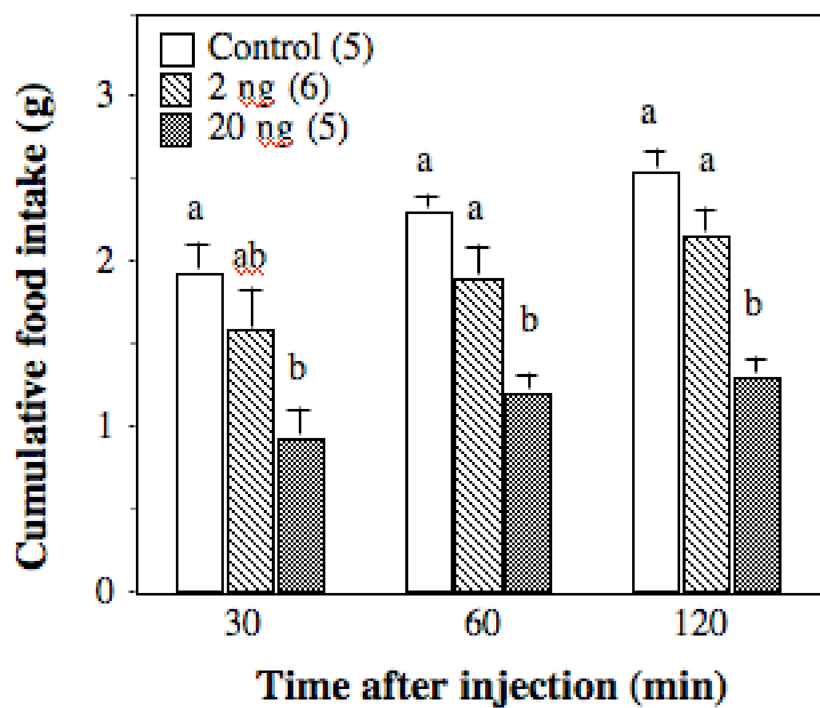

Figure 1

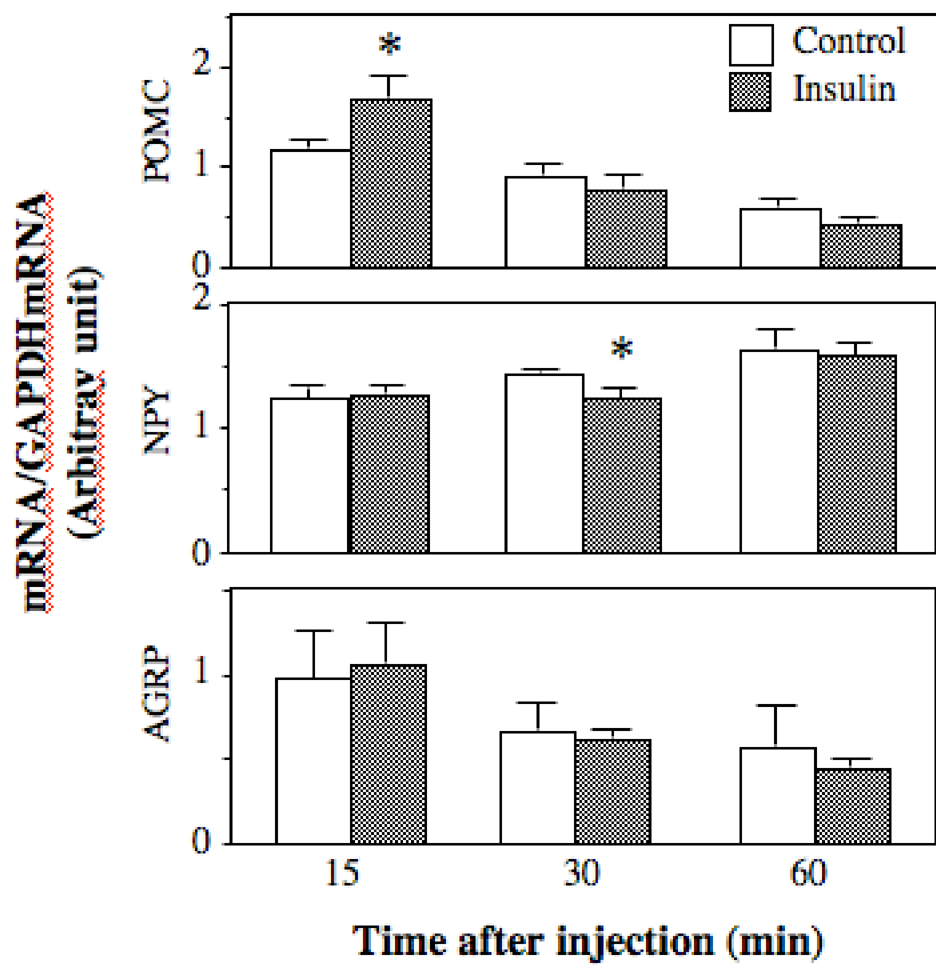

Figure 2

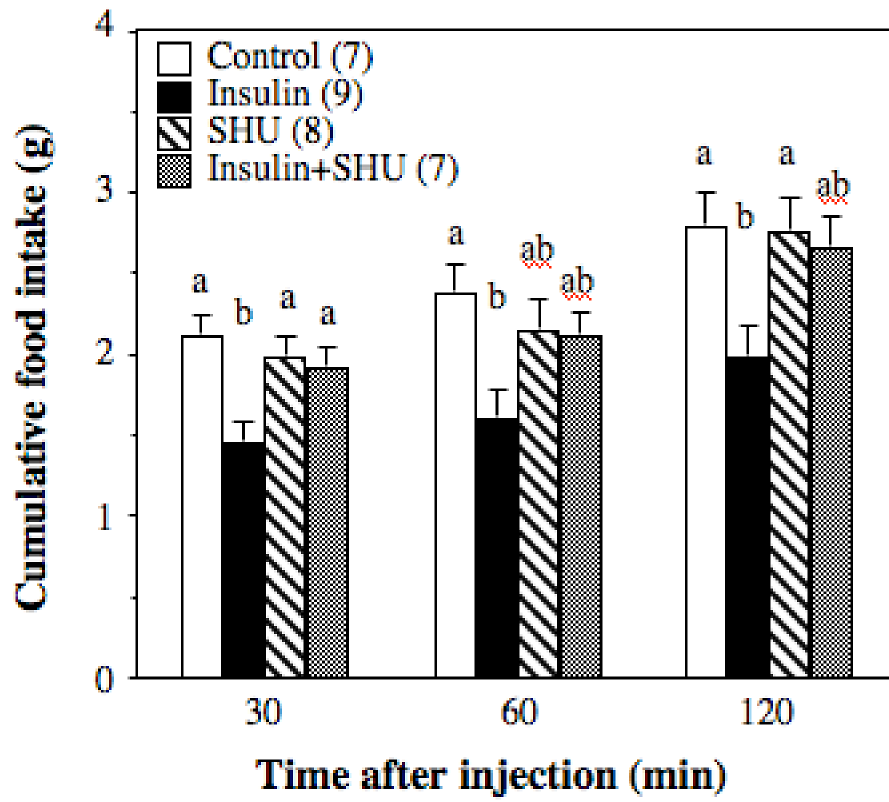

Figure 3

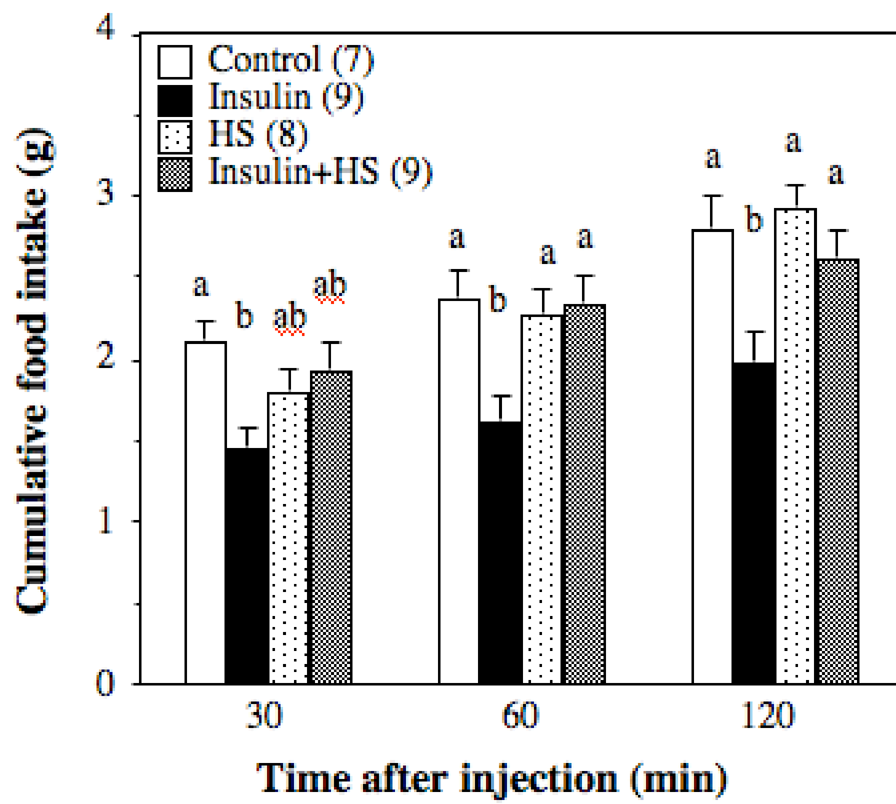

Figure 4