Hypophagic effects of insulin are mediated via NPY$_1$/NPY$_2$ receptors in broiler cockerels

Shiba Yousefvand, Farshid Hamidi, Morteza Zendehdel, and Abbas Parham

Abstract: Neuropeptide Y (NPY) plays a mediatory role in cerebral insulin function by maintaining energy balance. The current study was designed to determine the role of insulin in food intake and its interaction with NPY receptors in 8 experiments using broiler cockerels (4 treatment groups per experiment, except for experiment 8). Chicks received control solution or 2.5, 5, or 10 ng of insulin in experiment 1 and control solution or 1.25, 2.5, or 5 µg of receptor antagonists B5063, SF22, or SML0891 in experiments 2, 3, and 4 through intracerebroventricular (ICV) injection, respectively. In experiments 5, 6, and 7, chicks received ICV injection of B5063, SF22, SML0891, or co-injection of an antagonist + insulin, control solution, and insulin. In experiment 8, blood glucose was measured. Insulin, B5063, and SML0891 decreased food intake, while SF22 led to an increase in food intake. The hypophagic effect of insulin was also reinforced by injection of B560, but ICV injection of SF22 destroyed this hypophagic effect of insulin and increased food intake ($p < 0.05$). However, SML0891 had no effect on decreased food intake induced by insulin ($p > 0.05$). At 30 min postinjection, blood sugar in the control group was higher than that in the insulin group ($p < 0.05$). Therefore, the NPY$_1$ and NPY$_2$ receptors mediate the hypophagic effect of insulin in broiler cockerels.

Key words: hypophagic effects, insulin, broiler cockerels, NPY receptors, ICV injection.

Résumé : Le neuropeptide Y (NPY) joue un rôle de médiateur dans la fonction cérébrale de l’insuline en préservant l’équilibre énergétique. La présente étude a été conçue en vue d’établir le rôle de l’insuline dans la consommation de nourriture et ses interactions avec les récepteurs NPY dans 8 expériences sur des coquelets à rôtir (chacune avec 4 groupes de traitement à l’exception de l’expérience 8). Les poulets ont reçu par injection intracérébroventriculaire (ICV) une solution témoin ou 2,5, 5 ou 10 ng d’insuline dans l’expérience 1, et une solution témoin ou 1,25, 2,5 ou 5 µg de B5063, de SF22 ou de SML0891 dans les expériences 2, 3 et 4, respectivement. Dans les expériences 5, 6 et 7, les poulets ont reçu des injections ICV de B5063, de SF22 et de SML0891, l’injection de chacun de ces produits avec de l’insuline, une solution témoin et de l’insuline. Dans l’expérience 8, nous avons mesuré la glycémie. L’administration d’insuline, de B5063 et de SML0891 a entraîné une diminution de la consommation de nourriture, alors l’administration de SF22 menait à une augmentation de la consommation de nourriture. De plus, l’effet hypophagique de l’insuline était renforcé avec l’injection de B560, mais l’injection ICV de SF22 anéantissait l’effet hypophagique de l’insuline avec une augmentation de la consommation de nourriture ($p < 0.05$). Cependant, le SML0891 n’avait aucun effet sur la diminution de la consommation de nourriture engendrée par l’insuline ($p > 0.05$). Trente minutes après l’injection, la glycémie était plus élevée dans le groupe témoin qu’avec l’insuline ($p < 0.05$). Par conséquent, les récepteurs NPY1 et NPY2 agissent comme médiateurs dans l’effet hypophagique de l’insuline chez les coquelets à rôtir. [Traduit par la Rédaction]

Mots-clés : effets hypophagiques, insulin, coquelets à rôtir, récepteurs NPY, injection ICV.

Introduction

The central regulation of food intake is modulated in different parts of the brain, including the corpus striatum, nucleus tractus solitarius, amygdala, hypothalamus, and arcuate nucleus (Hamidi and Yousefvand 2017). The main hypothalamic area that regulates food intake is the arcuate nucleus, located in the vicinity of the third ventricle of the brain (Zendehdel and Hassanpour 2014).

Insulin in the central nervous system regulates processes such as glucose metabolism (Duarte et al. 2012), energy homeostasis, nerve cell generation, and survival (Plum et al. 2005). The primary target for insulin is the arcuate nucleus in the hypothalamus, a key area of the brain that regulates energy homeostasis (Blevins et al. 2002). In fact, the arcuate nucleus contains a high density of insulin-binding sites (Shiraishi et al. 2008). The arcuate nucleus has two large neuronal populations: neurons containing (1) neuropeptide Y (NPY) / agouti related protein (AgRP) and (2) proopiomelanocortin and cocaine- and amphetamine-regulated transcript (CART) (Zendehdel and Hassanpour 2014; Blevins et al. 2002).

The central melanocortin system in chickens seems to be important in regulating feeding behaviour. Melanocortin neurons are known to be involved in reducing appetite stimulated by insulin, and central injection of insulin reduces food intake in layer chicks. Yet, insulin has no role in regulating food intake in broiler chickens (Shiraishi et al. 2008, 2011b). Obviously, many factors, including bird strain, affect physiological and nutritional status in response to neurotransmitters (Denbow et al. 1983).

NPY is an abundant signaling peptide in the central nervous system of vertebrates (Bromée et al. 2006) and one of the strongest endogenous stimulants of food intake in mammals and birds (Bi et al. 2012). Among NPY receptors, NPY$_1$, NPY$_2$, and NPY$_3$ are involved in the central regulation of food intake (Levens et al. 2004).
Glucose serves different functions in birds, its main one being to produce energy. Chickens are not sensitive to insulin and have a high plasma glucose level, so they require high doses of exogenous insulin to produce metabolic responses to insulin therapy (Ashwell and McMurtry 2003).

So far, no studies have reported on the effects of insulin via NPY receptors on broiler cockerels’ feeding behaviours. Hence, the present study aimed to evaluate the effect of insulin via NPY (NPY1, NPY2, and NPY3) receptors on the food intake of cockerels deprived of food for 3 h (FD3). Owing to the high levels of blood sugar in chickens and their subsequent need to receive a high dose of exogenous insulin to produce hypoglycemia, we investigated the effect of central ICV injection of insulin on the blood sugar of broiler cockerels.

Materials and methods

Animals

The present study was carried out on 324 male broiler chicks (Ross 308) each weighing ~750 g (obtained from Mahan Company, Tehran, Iran). The chicks were kept in flocks until they reached 2 weeks of age, then divided randomly and kept in individual cages. During the study, birds had free access to fresh water and were given a mash diet containing 23% crude protein and 2850 kcal/kg of metabolizable energy (Chineh Company, Tehran, Iran). Chicks were maintained under standard conditions (at 22 ± 1 °C, 50% relative humidity, and continuous lighting) (Olanrewaju et al. 2006). Three hours before ICV injections, the chicks were deprived of food (FD3) but had free access to water. Animal handling and experimental procedures followed the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (USA) as well as the current animal care laws of the Iranian government.

Experimental drugs

The following drugs were used: I5523 (insulin from porcine pancreas), B5063 (selective NPY1 receptor antagonist), SF22 (selective NPY2 receptor antagonist), SML0891 (selective nonpeptide NPY3 receptor antagonist), and Evans blue, all of which were obtained from Sigma (Sigma–Aldrich, St. Louis, Missouri, USA). Xylazine and ketamine were prepared by Alfasan International (Woerden, Netherlands), and Linco-Spectin was obtained from Razak Laboratories (Karaj, Iran). NPY receptor antagonists were dissolved in absolute dimethyl sulfoxide (DMSO), and insulin was dissolved in 0.01 mol/L HCl. All drugs were then diluted in 0.85% saline containing 0.1% Evans blue solution (Mahzoumi et al. 2016).

Surgical preparation

When chicks weighed ~750 g (at 20 days old), stereotactic surgery was performed. Six hours before surgery (stereotactic), chicks were deprived of food (Hamidi and Zendehdel 2016) given access to fresh water. Birds were anesthetized with xylazine (2 mg/g) and ketamine (10 mg/kg) through intramuscular injection (Maiti et al. 2006). A 23-gauge thin-walled stainless steel guide cannula was then stereotactically implanted into the right lateral ventricle. The stereotactic coordinates were AP = 6.7 mm, L = 0.7 mm, and H = 3.5–4 mm below the dura mater with the head oriented as described in Zendehdel et al. (2013). The cannula was secured with 3 stainless steel screws placed in the calvaria surrounding each guide cannula, and then acrylic dental cement (Acropars, Tehran, Iran) was applied to the screws and guide cannula. An orthodontic No. 14 wire (American Orthodontics) trimmed to the exact length of the guide cannula was inserted into the guide cannula while the chicks were not being used for experiments. Linco-Spectin was applied to the incision to prevent infection. The birds were allowed to recover for a minimum of 5 days prior to injection.

Table 1. Treatment procedure in experiment 5.

<table>
<thead>
<tr>
<th>Group</th>
<th>First injection (5 μL)</th>
<th>Second injection (after 15 min) (5 μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control solution</td>
<td>Control solution</td>
</tr>
<tr>
<td>2</td>
<td>Control solution</td>
<td>Insulin (10 ng)</td>
</tr>
<tr>
<td>3</td>
<td>NPY receptor antagonist (1.25 μg)</td>
<td>Control solution</td>
</tr>
<tr>
<td>4</td>
<td>NPY receptor antagonist (1.25 μg)</td>
<td>Insulin (10 ng)</td>
</tr>
</tbody>
</table>

Note: Normal saline was used as the control solution.

Experimental procedures

Seven experiments were carried out in 2 stages. Each stage of the 7 experiment had 4 experimental groups (a control group and 3 treatment groups), while experiment 8 (blood glucose measurement) consisted of 2 experimental groups (a control group and insulin group). The first stage consisted of 4 experiments to determine the effective and subeffective doses of the compounds used, and the second stage involved 3 experiments using compound interactions. A total of 44 birds were used in each of the 7 experiments (n = 11 chicks/group for the experiments, but n = 6 chicks/group for statistical analysis). In experiment 8, 16 chicks were used (n = 8 chicks/group in the experiment, but n = 6 chicks/group for statistical analysis). Injections were performed using a 29-gauge, thin-walled stainless steel injecting cannula that extends 1.0 mm beyond the guide cannula. This injecting cannula was connected through a 60 cm long PE-20 tubing to a 10 μL Hamilton syringe. Injections were performed manually at slow, uniform speeds within 60 s. In the first stage, the volume of each injection was 10 μL. A subsequent 60 s period was allowed to permit the solution to diffuse from the tip of the cannula into the ventricle. Tubing and syringes were kept in 70% ethanol, and the glassware was autoclaved to render materials pyrogen-free. In these experiments, normal saline was used as the control solution. Placement of the guide cannula into the right ventricle was verified by the presence of cerebrospinal fluid and ICV injection of methylene blue followed by slicing of the frozen brain tissue at the end of the experiments. Data only from accurately performed injections (correct planting of cannula) were used in the statistical analysis.

At each stage of the study, only 1 experiment was performed on each bird. After injections in experiments 1–4 (or after the second injections for experiments 5–7), the chicks were immediately returned to their cages and fresh food and water were provided. Cumulative food intake at 30, 60, and 120 min after injection was recorded. All injections were performed between 1100 and 1315. The first stage involved experiments 1–4. The first experiment was designed to evaluate the effect of insulin on the central regulation of food intake and to obtain the effective dose of insulin on food intake in FD3 chicks. Chicks in each group received 2.5, 5, or 10 ng (dissolved in 10 μL saline) of I5523 insulin via ICV injection, and the control group was injected with control solution. Experiment 2 evaluated the effect of ICV injection of B5063 (NPY1 receptor antagonist) on the central regulation of feeding behaviour. The subeffective dose of B5063 on food intake was also obtained. Through ICV injection, FD3 chicks received the control solution or 1.25, 2.5, or 5 μg doses of B5063. Experiments 3 and 4 were performed in parallel to experiment 2, but fasted chicks received the control solution or 1.25, 2.5, or 5 μg doses of SF22 (NPY2 receptor antagonist) or SML0891 (NPY3 receptor antagonist), respectively, to determine their subeffective doses in fasted chicks.

The second stage involved experiments 5–7. In experiment 5, FD3 chicks received ICV injection of the compounds according to Table 1. Experiments 6 and 7 were done in parallel to experiment 5, but fasted chicks received 1.25 ng doses of SF22 and SML0891, respectively. These doses of drugs were calculated based on previous studies (Shiraishi et al. 2008; Stengel et al. 2010).

Experiment 8 consisted of measurement of blood sugar in 2 experimental groups. Preinjection blood samples were collected.

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from all chicks in 2 groups. After the initial blood samples were taken, the control group received 10 μL of control solution and the insulin group received 10 ng of insulin via ICV injection. Thirty minutes after injection, blood samples were collected again from each chicken in both groups. In both steps, blood samples were taken from the jugular vein (Stevens and Ridgway 1966).

Statistical analysis
Cumulative food intake in each experiment was calculated by repeated measurement of 2-way analysis of variance (ANOVA). Data were presented as the means ± standard error of the mean (SEM). Whenever there was a significant difference between the treatments, Tukey’s test (post hoc test) was used to determine any significant difference between the treatments. An independent sample t test was used to compare blood glucose between the control and insulin groups before and after injection. A paired sample t test was used to compare the blood glucose of corresponding groups before and after injection. Results were considered statistically significant at p < 0.05.

Results
We investigated the effects of insulin on cumulative food intake via NPY receptors and the effects of ICV injection of a 10 ng dose of insulin on blood glucose levels in FD3 broiler cockerels. The results are shown in Figs. 1–8.

In experiment 1, ICV injection of insulin (5 and 10 ng doses) resulted in a dose-dependent decrease in cumulative food intake compared with the control group (p < 0.05). However, 2.5 ng of insulin did not affect food intake in comparison with the control group (p > 0.05) (Fig. 1).

In experiment 2, ICV injection of 1.25 μg of B5063 did not influence food intake in FD3 chicks compared with the control group (p > 0.05), whereas 2.5 and 5 μg doses of B5063 reduced food intake in a dose-dependent manner compared with the control group (p < 0.05) (Fig. 2).

In experiment 3, ICV injection of 2.5 and 5 μg doses of SF22 significantly increased food intake in a dose-dependent manner in fasted chicks compared with the control group (p < 0.05), but a 1.25 μg dose of SF22 did not affect food intake compared with the control group (p > 0.05) (Fig. 3).

In experiment 4, the effect of ICV injection of SML0891 on food intake by chicks was evaluated. Administering 2.5 and 5 μg doses of SML0891 dose-dependently decreased cumulative food intake compared with the control group (p < 0.05), while a 1.25 μg dose of
SML0891 had no effect on food intake compared with the control group ($p > 0.05$) (Fig. 4).

On the basis of experiments 1–4, doses of 10 ng of insulin and 1.25 μg of NPY receptor antagonists were selected for experiments 5–8.

In experiment 5, ICV injection of insulin (10 ng) significantly decreased food intake by chicks compared with the control group. The hypophagic effect of insulin was significantly reinforced by the injection of 1.25 μg of B5063 ($p < 0.05$). However, 1.25 μg of B5063 had no effect on food intake compared with the control group ($p > 0.05$) (Fig. 5).

In experiment 6, ICV injection of insulin (10 ng) significantly decreased cumulative food intake by chicks compared with the control group ($p < 0.05$). Injection of SF22 (1.25 μg) + insulin (10 ng) significantly increased food intake by chicks compared with the insulin group at 30, 60, and 120 min postinjection ($p < 0.05$). However, 1.25 μg of SF22 had no effect on food intake compared with the control group ($p > 0.05$) (Fig. 6).

In experiment 7, 1.25 μg of SML0891 did not influence cumulative food intake in fasted chicks compared with the control group ($p > 0.05$). However, ICV injection of insulin (10 ng) significantly decreased food intake compared with the control group ($p < 0.05$). Also, 1.25 μg of SML0891 did not affect the hypophagic effect of insulin (10 ng) in broiler cockerels ($p > 0.05$) (Fig. 7).

Chicks did not exhibit sleepiness or behavioural changes in response to any of the drugs administered during the experiments.

In experiment 8, there was no significant difference in blood sugar between the control and insulin groups before injection ($p > 0.05$), but 30 min after injection, the blood sugar in the control group was significantly higher than that in the insulin group. Blood sugar in both the insulin and control groups at 30 min...
postinjection were significantly higher than those of the 2 groups preinjection (p < 0.05).

**Discussion**

Previous studies have investigated the effects of insulin on the control of feeding behaviour through different neural pathways and via signaling pathways in the liver in mammals and birds. Insulin causes a reduction in central food intake via melanocortin neurons in rats (Benoit et al. 2002). ICV injection of insulin in layer chicks stimulates expression of proopiomelanocortin and inhibits expression of NPY (Shiraishi et al. 2008). In fasted (5 h of hunger) and fully fed chicks, administration of insulin anti-serum causes insulin signals in the liver to switch off, such as AKT phosphorylation (Dupont et al. 2008). Inhibition of the PI3K/AKT pathway inhibits nearly all insulin functions, so this signaling pathway plays an important role in the metabolic functions of insulin (Dupont et al. 2009). The present study provides the first evidence that central insulin and NPY interact via the NPY1 receptor to regulate food intake in broiler cockerels.

According to the results of the first experiment, ICV injection of insulin (at 5 and 10 ng doses) decreased food intake (Fig. 1). In line with the results of our study, ICV injection of insulin in rats and leghorn chicks has an anorexigenic effect (Carvalheira et al. 2003; Honda et al. 2007; Shiraishi et al. 2008, 2011b). Therefore, insulin in both birds and mammals is a hypophagic neuropeptide. But ICV injection of insulin does not affect broiler chicks’ food intake (Shiraishi et al. 2011b). That result contradicts the findings of our study. This contradiction may be due to the different chicken strain investigated in Shiraishi’s study (Chunky) and the one studied in our paper (ROS 308). Obviously, many factors, including bird strain, affect physiological and nutritional status in response to neurotransmitters (Denbow et al. 1983). For instance, ICV injection of insulin reduces food intake in rats fed a high-fat diet versus a low-fat diet (Zendehdel and Hassanpour 2014). Continuously administering insulin detemir (an insulin analogue) for 4 weeks to rats fed a high-fat diet reduced food intake and fat mass in the body (Rojas et al. 2011).

ICV injection of B5063 and SML0891 in experiments 2 and 4, respectively (at 2.5 and 5 μg doses) led to a dose-dependent decrease in food intake in fasted cockerels (Figs. 2 and 4). In the third experiment, 2.5 and 5 μg doses of SF22 significantly increased food intake (Fig. 3). Many researchers have obtained results con-

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**Fig. 5.** Effects of intracerebroventricular injection of control solution, B5063 (NPY1 receptor antagonist; 1.25 μg), insulin (10 ng), and co-injection of B5063 + insulin on cumulative feed intake in broiler cockerels. Data are expressed as means ± SEM. Different letters indicate significant differences between treatments at each time point (p < 0.05).

**Fig. 6.** Effects of intracerebroventricular injection of control solution, SF22 (NPY2 receptor antagonist; 1.25 μg), insulin (10 ng), and co-injection of SF22 + insulin on cumulative feed intake in broiler cockerels. Data are expressed as means ± SEM. Different letters indicate significant differences between treatments at each time point (p < 0.05).
suggest that there is an interaction between insulin and the NPY1 receptor. Various studies confirm our results. Insulin reduces food intake by inhibiting the expression of NPY (Shiraishi et al. 2008). Insulin interacts with NPY neurons, especially in the arcuate nucleus (Levin et al. 1998), and insulin receptors are located in NPY neurons in the arcuate nucleus (Shiraishi et al. 2011). The NPY-expressing neurons act as a vital mediator of insulin’s central role of regulating appetite and energy homeostasis (Yulyaningsih et al. 2014; Steculorum et al. 2016; Loh et al. 2017).

In conclusion, the results of the present study indicate that the hypophagic effect of insulin may be modulated by the NPY1 and NPY2 receptors (not the NPY5 receptor) and central insulin reduces blood sugar in broiler cockerels. Further investigation is required to elucidate the underlying cellular and molecular signaling pathways (such as expression of NPY in the brain and AKT phosphorylation in the liver) in the interconnection between insulin and NPY and its effect on feeding behaviour in broiler cockerels. We plan to investigate the expression of insulin-induced NPY in the brain and AKT phosphorylation in the liver in future studies. Also, with regard to the change in the type of response to insulin

Fig. 7. Effects of intracerebroventricular injection of control solution, SML0891 (NPY5 receptor antagonist; 1.25 μg), insulin (10 ng), and co-injection of SML0891 + insulin on cumulative feed intake in broiler cockerels. Data are expressed as means ± SEM. Different letters indicate significant differences between treatments at each time point (p < 0.05).

Fig. 8. Effect of intracerebroventricular injection of insulin on blood sugar levels in broiler cockerels. Different letters indicate a significant difference between the control and insulin (10 ng) groups before and after injection. Different indices indicate a significant difference between the groups preinjection and postinjection (p < 0.05). Data are expressed as means ± SEM.

In experiment 6, ICV injection of SF22 destroyed the hypophagic effect of insulin and increased food intake (Fig. 6). In fact, the results of this experiment confirm our hypothesis and support the relationship between insulin and the NPY1 receptor. On the other hand, this receptor directly inhibits the release of NPY in the paraventricular nucleus and reduces food intake (Levens et al. 2004).

In experiment 7, ICV injection of SML0891 did not influence the hypophagic effects of insulin (Fig. 7). The results of this study indicate that there is no relationship between insulin and the NPY5 receptor in central regulation of food intake. In line with our result, NPY-induced feeding in mice prominently occurred via the NPY1 receptor, not through the NPY5 receptor (Kanatani et al. 2000).

Chickens are not sensitive to insulin and have a high plasma glucose level, so they require high doses of insulin to produce metabolic responses to insulin therapy (Ashwell and McMurtry 2003). Injection of 40 μg/kg of insulin in 8-day-old chickens (Tokushima et al. 2005) and 80 μg/kg of insulin in pigeons (Sweazea et al. 2006) reduces blood glucose levels. In the current study, 30 min after injection, the control group and insulin group (10 ng insulin) showed a significant increase in blood sugar compared with preinjection levels, which could be due to refeeding of the chicks immediately after injection (Denbow and Cline 2015). Thirty minutes after injection, blood sugar decreased significantly in the insulin group compared with the control group. ICV injection of insulin causes an increase in blood sugar compared with preinjection levels, which could be due to refeeding of the chicks immediately after injection (Denbow and Cline 2015). Thirty minutes after injection, blood sugar decreased significantly in the insulin group compared with the control group. ICV injection of insulin causes an increase in plasma insulin levels (Denbow and Cline 2015), and insulin in the blood inhibits glucose production (Obici et al. 2002). Therefore, in the present study, ICV injection of insulin perhaps causes insulin to enter the bloodstream and decrease plasma sugar levels. Of course, further studies can clarify the exact pathway involved.

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Conflict of interest
The authors declare that there is no conflict of interest associated with this work.

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