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Central injection of des-acyl chicken ghrelin does not affect food intake in chicks

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ABSTRACT

In rodents and goldfish, ghrelin is well known as an orexigenic peptide, and des-acyl ghrelin, which is a ghrelin gene-derived peptide lacking Ser-3 acylation, affects feeding behavior when injected with or without ghrelin. Intracerebroventricular (ICV) injection of ghrelin inhibits food intake in chicks (*Gallus gallus*), but has the opposite effect in rodents and goldfish. The aim of the present study was to investigate the effect of chicken des-acyl ghrelin on feeding in chicks. ICV injection of des-acyl ghrelin alone at doses from 4 to 1000 pmol did not affect food intake in fed and 12-h fasted chicks. Co-injection of des-acyl ghrelin with ghrelin tended to attenuate ghrelin-induced anorexia. In an in vitro study, only the highest concentration (10^{-6} M) of des-acyl ghrelin increased intracellular calcium ion concentration in chicken GHS-R1a-expressing cells. Des-acyl ghrelin (10^{-6} M) slightly but significantly decreased intracellular calcium ion influx induced by 1 or 3 nM ghrelin. The present results demonstrate that des-acyl ghrelin is not positively involved in the central regulation of feeding in chicks. The feeding regulatory network between ghrelin and des-acyl ghrelin in chicks would be different from those in rodents and goldfish.

1. Introduction

Ghrelin is a natural ligand for the growth hormone (GH) secretagogue (GHS) receptor (GHS-R) 1a in vertebrates [8–10,23]. This hormone was first identified as a stimulator of GH release from the pituitary [10], and further studies revealed that ghrelin is a regulator of feeding behavior: central or peripheral injection of ghrelin stimulates food intake in rats [2,7,13,24]. Ghrelin-stimulated feeding has also been demonstrated in goldfish [11,23].

The ghrelin activity is elicited by a form with an octanoyl modification at serine-3 residue (Ser-3), and is lost in a form lacking the acylation (des-acyl ghrelin) [10]. Nevertheless, a few studies have suggested that des-acyl ghrelin affects feeding behavior. Asakawa et al. [2] reported that intracerebroventricular (ICV) or intraperitoneal (IP) injection of des-acyl ghrelin decreases food intake in 16-h fasted mice, but not in fed mice. Chen et al. [3] reported a similar anorexigenic effect of des-acyl ghrelin in rats after IP injection. Furthermore, des-acyl ghrelin-overexpressing mice exhibit a decrease in food intake and body weight at 44 weeks of age [1,2]. On the other hand, Toshinai et al. [22] showed that ICV injection of des-acyl ghrelin stimulates feeding in *ad lib*-fed mice and the orexigenic effect was also observed in GHS-R1a-deficient mice, indicating an independent effect of ghrelin. Taken together, these results indicate the effects of des-acyl ghrelin on food intake are controversial, but it seems that des-acyl ghrelin has some effects on feeding in rodents.

Furthermore, it is likely that des-acyl ghrelin may interact with ghrelin. Ghrelin-stimulated feeding is attenuated by co-administration of des-acyl ghrelin in rats and goldfish while des-acyl ghrelin alone does not show any effect [7,11]. These results suggest the possibility that des-acyl ghrelin functions as a regulator for ghrelin action through a direct or indirect pathway.

We examined feeding regulation by ghrelin in non-mammals, and interestingly, ghrelin inhibits feeding behavior in chicks (*G. gallus*) when injected ICV [6,16,17]; the effect is completely opposite to those in rodents and goldfish. It has been known that the orexigenic effect of ghrelin in rodents and goldfish is mediated by hypothalamic neuropeptide Y (NPY), agouti-related peptide (AgRP) and orexin systems. However, in chicks, ghrelin action is mediated through the corticotropin-releasing hormone (CRH) system [6]. This could suggest a different action of des-acyl ghrelin in chicks.

In the present study, we examined (1) whether des-acyl ghrelin affects feeding in fed and 12-h fasted chicks when injected ICV alone or in combination with ghrelin, (2) whether des-acyl ghrelin interacts with chicken GHS-R1a, and (3) whether nesfatin-1 affects feeding behavior because nesfatin-1, which is a peptide processed from nesfatin/nucleobindin-2, is a candidate for inducing an anorectic effect of des-acyl ghrelin in rats whose feeding is stimulated by ghrelin [7,15].

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2. Materials and methods

2.1. Animals

Day-old male layer chicks (*G. gallus*, Julia, Ninobe Hatchery, Kagawa, Japan) were raised in a room kept at 30 °C with continuous lighting. A commercial diet (crude protein: 24%, metabolizable energy: 3050 kcal/kg, Toyohashi Feed Mills Co. Ltd., Aichi, Japan) and water were available *ad libitum* to the chicks. Chicks were transferred to their individual cages 1 day before each experiment. Before the experiment, body weight was measured and then chicks were distributed into experimental groups so that the average body weight was as uniform as possible between treatment groups. The chicks were maintained in accordance with the recommendations of the National Research Council [14].

2.2. Peptides and ICV injection

Chicken ghrelin and chicken des-acyl ghrelin were synthesized at ASUBIO Pharma Co. Ltd. (custom order; Gunma, Japan) and GenScript Corp. (custom order; NJ, USA), respectively. Rat nesfatin-1 was purchased from Phoenix Pharmaceuticals, Inc. (CA, USA). These peptides were dissolved in 5% mannitol solution containing 0.1% Evans Blue dye, and the vehicle was used for the control treatment. ICV injections were performed according to the method reported previously [4,5]. Briefly, the head of the chick was inserted into an acrylic box with a hole in the top plate. The injection site was 3 mm anterior to the coronal suture, 1 mm lateral from the sagittal suture, and 3 mm deep targeting the left lateral ventricle. Anatomical landmarks were determined visually and by palpation. The peptide solution (10 μl) was injected through the hole using a micro-syringe.

This procedure is quick and does not stress neonatal chicks judging from feeding behavior and release of a stress indicator, corticosterone [5,17]. All injections were made between 8:00 and 9:00 under an *ad libitum* feeding condition unless otherwise noted. Food intake was measured at 30, 60 and 90 min after the injection because the effects of ghrelin and des-acyl ghrelin were observed within 60 min in rats [22]. At the end of each experiment, the chicks were euthanized with an overdose of pentobarbital sodium. The brain was then removed to confirm the accuracy of injection. Any chicks in which the presence of Evans Blue dye in the lateral ventricle was not identified were not used for further analyses. The number of chicks injected successfully is described in each figure legend.

2.3. Effect of chicken ghrelin on feeding

Seven-day-old chicks were ICV injected with 0 (control), 0.4, 2 or 10 pmol ghrelin under an *ad libitum* feeding condition. The doses of ghrelin injected were based on a previous study [17]. A preweighed feeder was given to each chick, and food intake was measured by weighing the feeder to an accuracy of 1 mg at 30, 60 and 90 min after the injection. The ICV injection was also done under food-deprivation condition because the effect on feeding depends on feeding conditions before treatment in chicks [19]. The food deprivation study was performed using 5-day-old chicks which underwent 12-h food deprivation to facilitate feeding.

2.4. Effect of chicken des-acyl ghrelin on feeding

Five-day-old chicks were ICV injected with 0 (control), 4, 20 or 100 pmol des-acyl ghrelin under an *ad libitum* feeding condition. The doses of des-acyl ghrelin were based on a previous study in goldfish [11]. Food intake was measured as described above. A food deprivation study was also performed using 5-day-old chicks which underwent 12-h food deprivation. A higher dose (1000 pmol) of des-acyl ghrelin was examined using fed or 12-h fasted 5-day-old chicks. The dose of des-acyl ghrelin was based on a previous study in rodents [2].

2.5. Interaction of ghrelin with des-acyl ghrelin in feeding

In an *ad libitum* condition, 2 pmol ghrelin, 1000 pmol des-acyl ghrelin plus 2 pmol ghrelin, or 1000 pmol des-acyl ghrelin alone was injected into 5-day-old chicks. Food intake was then measured. A food deprivation study was also performed using 12-h fasted 6-day-old chicks.

2.6. In vitro receptor assay

To test whether des-acyl ghrelin influences binding to GHS-R1a and subsequent signal transduction of GHS-R1a, chicken ghrelin and des-acyl ghrelin were applied to chicken GHS-R1a-expressing cells, and changes in intracellular Ca^{2+} concentration were measured using FLIPR^{tetra} (Molecular Devices, Menlo Park, CA, USA). Chicken GHS-R1a has been identified [19]. The expression vector for chicken GHS-R1a was constructed using pcDNA3.1 v5-His TOPO vector (Invitrogen). The expression vector containing an open-reading frame of the chicken GHS-R1a was transfected into a human embryonic kidney 293 (HEK293) cell line using FuGENE6 reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were plated on poly-D-lysin-coated 96-well black plates and cultured for at least 20 h at 37 °C. Fluo-4 AM (Invitrogen) was then loaded into the cells for 1 h at 37 °C. After washing the plates, cells were treated with ghrelin and des-acyl ghrelin alone or in combination at final concentrations of 10^{-10} to 10^{-6} M using the automated FLIPR system. Chicken motilin [25] was also applied.

2.7. Effect of nesfatin-1 on feeding

Five-day-old chicks were ICV injected with 0 (control), 5 or 25 pmol nesfatin-1 under an *ad libitum* feeding condition. The dose of nesfatin-1 used in this study was based on doses that induced a satiety response in rodents [15]. A food deprivation study was also performed using 12-h fasted chicks.

2.8. Statistical analysis

Feeding data were analyzed with two-way repeated measurement of ANOVA, followed by the Tukey–Kramer test at each time point as a post hoc test. Data are expressed as means ± SEM and statistical significance was set at $P < 0.05$. Data for calcium influx were analyzed by one-way ANOVA, followed by Student's *t*-test at each time point. Data are expressed as means ± SEM and statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of chicken ghrelin on feeding behavior

Fig. 1A and B show the effect of ICV injection of ghrelin on food intake. In *ad lib*-fed chicks, the injection of 2 and 10 pmol ghrelin significantly decreased food intake of fed chicks at 30 min (Fig. 1A). The anorectic effect of 10 pmol ghrelin lasted for 60 min after injection. In 12-h fasted chicks, ghrelin significantly suppressed food intake: the anorectic effects were more potent than those of fed chicks (Fig. 1B), 0.4 pmol ghrelin was effective at 30 min after injection, and the effects of 0.4 and 2 pmol ghrelin