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Chicken ghrelin and growth hormone-releasing peptide-2 inhibit food intake of neonatal chicks

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Abstract

Ghrelin is an endogenous ligand for the growth hormone secretagogue (GHS) receptor. Ghrelin stimulates feeding in rats, however, intracerebroventricular (i.c.v.) injection of rat ghrelin inhibits feeding of neonatal chicks. In the present study, the effect of i.c.v. injection of different ghrelins including chicken and bullfrog ghrelin, and synthetic GH-releasing peptide (GHRP) on feeding of neonatal chicks was investigated. Chicken ghrelin strongly suppressed feeding. To compare the inhibitory effect, chicken and rat ghrelin were examined. The suppressive effect of feeding by chicken and rat ghrelin was almost identical. Bullfrog ghrelin contains a change in the acylated amino acid from Ser to Thr, strongly suppressed feeding. The i.c.v. injection of GHRP-2 (KP-102), a synthetic GHS, also inhibited feeding. These results indicate that the chicken GHS receptor is affected by several forms of GHS, and that food intake of neonatal chicks is inhibited by GHS receptor agonists.

Keywords: Ghrelin; Intracerebroventricular injection; Neonatal (chick); Feeding

1. Introduction

Ghrelin, recently identified from rat stomach as an endogenous ligand for the growth hormone secretagogue (GHS) receptor, is a 28-amino acid endocrine peptide, and is involved in the regulation of growth hormone (GH) release. Ghrelin has a unique octanoylation at the Ser residue at the third position of the N terminus, and this octanoyl moiety is essential for ghrelin activity (Kojima et al., 1999). Ghrelin mRNA is expressed predominantly in the stomach, but is also expressed in the brain and other tissues at low levels. Therefore, ghrelin may also be involved in other physiological functions.

In rats, intracerebroventricular (i.c.v.) injection of rat ghrelin stimulates GH release, feeding, and also causes obesity (Tschöp et al., 2000; Nakazato et al., 2001; Wren et al., 2001). In neonatal chicks, however, i.c.v. administration of rat ghrelin strongly inhibits feeding under both ad libitum and fasting conditions (Furuse et al., 2001). Furthermore, hyperactivity is observed less than 30 min after i.c.v. injection (Saito et al., in press), and sleep-like behavior thereafter (Tachibana et al., 2001).

In non-mammalian species, ghrelin has only recently been identified from the chicken and bullfrog (Kaiya et al., 2001, 2002) (Fig. 1). Chicken ghrelin is a 26-amino acid peptide and, like in mammals, has an *n*-octanoylated or *n*-decanoylated serine in the third position. The intravenous injection of chicken ghrelin increases plasma GH and corticosterone levels in chicks. Bullfrog ghrelin stimulates GH and prolactin secretion from bullfrog pituitary cells. The acylated amino acid of bullfrog ghrelin is Thr instead of Ser. When comparing the amino acid sequence of rat and chicken ghrelin, the N-terminal seven amino acid residues are identical, but the remainder of the sequence is largely different. Thus, central administration of rat

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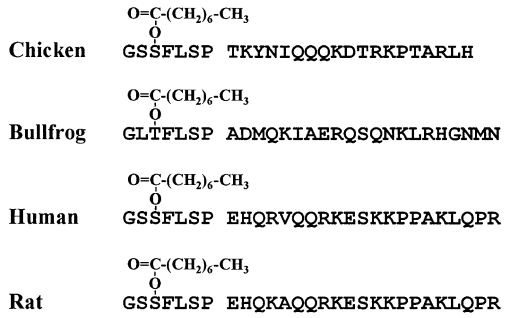

Fig. 1. Amino acid sequence of ghrelin in various species.

ghrelin may act as an antagonist of the chicken GHS receptor and suppress feeding as reported by Furuse et al. (2001).

Peptidyl and non-peptidyl GHS stimulates GH secretion in vivo and in vitro in various species (Cheng et al., 1993; Hashizume et al., 2001; Hhung et al., 2001; Roh et al., 1997a,b; Torsello et al., 1998; Wu et al., 1996). GH-releasing peptide (GHRP)-2 (KP-102; D-Ala-D-βNal-Ala-Trp-D-Phe-Lys-NH_2), one of the peptidyl GHS, stimulates GH release by direct action on both pituitary somatotrophs and the hypothalamus. GHRP also increases feeding in rats (Okada et al., 1996; Shibasaki et al., 1998; Kuriyama et al., 2000). The effect of GHRP-2 on feeding behavior in the chicken, however, has not been investigated. The purpose of the present study was to clarify the central effect of different ghrelins including homologous chicken peptide and GHRP-2 on food intake in neonatal chicks.

2. Materials and methods

2.1. Animals

Day-old male broiler chicks (Cobb) were purchased from a local hatchery (Mori Hatchery, Fukuoka, Japan). The chicks were housed in a windowless temperature-controlled room (28 °C), and provided continuous light. They had free access to water and a commercial starter diet (Toyohashi Feeds and Mill, Aichi, Japan). On the day prior to injections, birds were placed in individual cages. Three-day-old chicks were used. Experimental procedure followed the guidance for Animal Experiments in Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government.

2.2. ICV injection

Rat ghrelin was purchased from Peptide Institute (Osaka, Japan). Chicken and bullfrog ghrelin were synthesized at Suntory Institute for Medicinal Research and Development (Gunma, Japan). GHRP-2 was a gift from Kaken Pharmaceutical (Tokyo, Japan). In this study, we used rat, chicken and bullfrog ghrelin with an octanoyl moiety. All peptides were dissolved in 0.85% saline containing 0.1% Evans Blue, and injected i.c.v. in a volume of 10 μl using a microsyringe as described by Davis et al. (1979). The control group was given saline containing 0.1% Evans Blue.

2.3. Feeding experiments

Before each experiment, birds were weighed and distributed into experimental groups based on their body weight. The average weight between groups was as uniform as possible. The birds were deprived of food for 3 h before each experiment, but given free access to water. They were given food for 2 h after i.c.v. injections. Food intake was measured at 30, 60 and 120 min post-injection. At the end of the feeding experiment, the chicks were sacrificed by injection of urethane overdose. Only data from birds confirmed to have Evans Blue dye present in the lateral ventricle were used.

Rat ghrelin has previously been shown to potently inhibit feeding of chicks (Furuse et al., 2001). Thus, we investigated whether homologous chicken ghrelin inhibits feeding. To compare the effect of chicken ghrelin with that of rat ghrelin, equal doses of previous rat ghrelin (0.38, 0.75 and 1.5 nmol) of chicken ghrelin was injected i.c.v.

In next experiment, the effects of more lower doses (0.05 and 0.1 nmol) of chicken and rat ghrelin on food intake was examined.

In mammalian ghrelin, it has been demonstrated that the N-terminal pentapeptide acts as the active core of ghrelin (Bednarek et al., 2000; Matsumoto et al., 2001). The amino acid sequence in the N-terminal 1–7 residues of mammalian and avian ghrelin are identical, and these ghrelins contain an acylated serine. However, bullfrog ghrelin has a different sequence at the N-terminus (Gly-Leu-Thr (*n*-octanoyl)-Phe-Leu segment), and the acylated amino acid at the 3 position is changed to Thr instead of Ser as in mammalian and avian ghrelins. Thus, we investigated the effect of differences in the N-terminal amino acid sequence of ghrelin on food intake. Saline, 0.38, 0.75 and 1.5 nmol of bullfrog ghrelin were examined. To examine the effect of GHS on food intake in chickens, a peptidyl GHS, GHRP-2 was used at doses of 0.38, 0.75 and 1.5 nmol.

2.4. Statistical analysis

In the feeding experiments, data were analyzed by two-way ANOVA with repeated measurements using a commercially available package, StatView (Version 5, SAS Institute, Cary, USA, 1998). Comparisons between means were made using Duncan's multiple range test. To comparison of inhibitory effect between ghrelin and GHRP-2, data was evaluated using the Mann–Whitney *U*-test. The results are presented as means ± S.E.M.

3. Results

The i.c.v. administration of chicken ghrelin potently inhibited feeding through 2 h post-injection ($F(3,31) = 13.354$, $P < 0.0001$) in a dose-dependent manner (Fig. 2A). Cumulative food intake through 2 h increased in the control group, but not in chicks injected with chicken ghrelin ($F(6,62) = 2.549$, $P < 0.05$), thus causing a significant treatment by time interaction. This indicated that the inhibitory effect of chicken ghrelin on feeding continued through 2 h post-injection. While the lower doses of chicken ghrelin (0.05 and 0.1 nmol) also significantly inhibited ($F(2,18) = 19.634$, $P < 0.0001$) food intake (Fig. 2B), food intake gradually returned to the control level by 120 min after injection in both these groups.

Chicken and rat ghrelin significantly inhibited feeding to a similar degree ($F(4,27) = 3.381$, $P < 0.05$) (Fig. 3). There was no difference in the effect between chicken and rat ghrelin ($F(3,22) = 0.186$, $P > 0.05$).

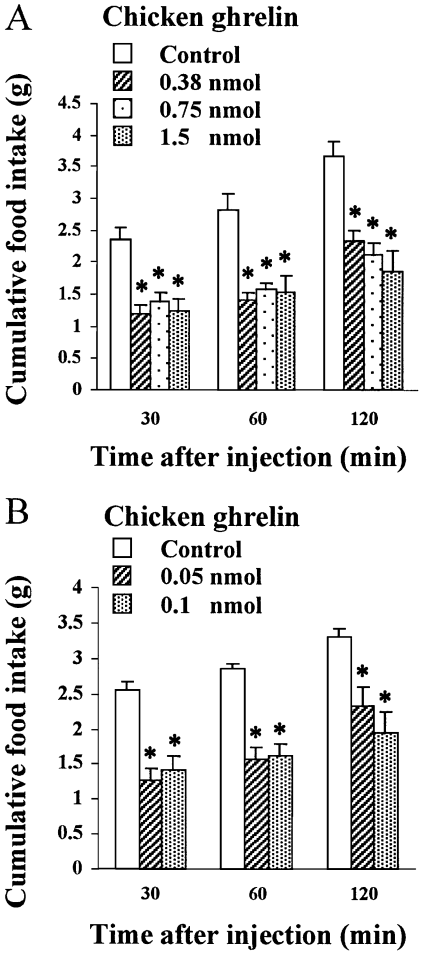

Fig. 2. Cumulative food intake during 2 h after intracerebroventricular injection of chicken ghrelin at (A) the same dose of previous rat ghrelin and (B) more lower dose in neonatal chicks. Before injection, 3-h fasting was made. Values are means ± S.E.M. Significant difference from the control (saline) at each time point represented $^{*}P < 0.01$. The number of chicks used for this experiment was as follows: (A) control, 9; 0.38 nmol, 9; 0.75 nmol, 9; and 1.5 nmol, 8 and (B) control, 8; 0.05 nmol, 6; 0.1 nmol, 7.

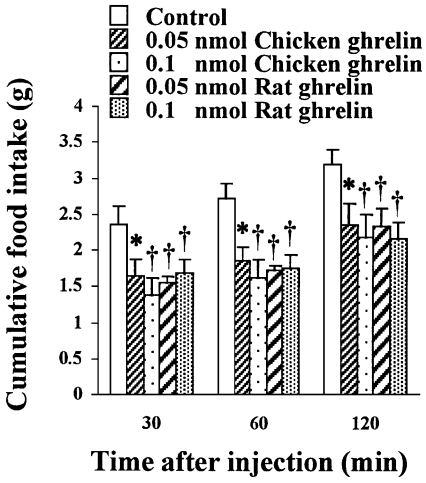

Fig. 3. Cumulative food intake during 2 h after intracerebroventricular injection of chicken and rat ghrelin in neonatal chicks. Before injection, 3-h fasting was made. Values are means ± S.E.M. Significant difference from the control (saline) at each time point represented $^{*}P < 0.05$ and $^{\dagger}P < 0.01$. The number of chicks used for administration of chicken and rat ghrelin was as follows: control, 6; chicken ghrelin 0.05 nmol, 6; chicken ghrelin 0.1 nmol, 8; rat ghrelin 0.05 nmol, 5; and rat ghrelin 0.1 nmol, 7.

Fig. 4 shows the effect of bullfrog ghrelin on food intake. Food intake was inhibited in a dose-dependent manner ($F(2,56) = 7.834$, $P < 0.0001$) by bullfrog ghrelin through 2 h post-injection ($F(3,28) = 28.341$, $P < 0.0001$). The magnitude of inhibitory effect was similar to that of chicken and rat ghrelin.

GHRP-2 also inhibited feeding ($F(3,28) = 4.760$, $P < 0.001$) but the effect was not as robust as with the ghrelins (Fig. 5). The injection of GHRP-2 also caused a

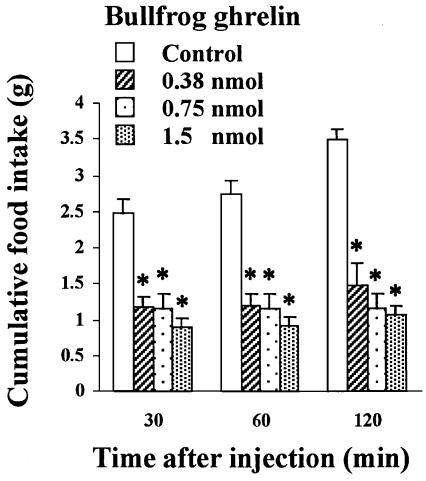

Fig. 4. Cumulative food intake during 2 h after intracerebroventricular injection of bullfrog ghrelin in neonatal chicks. Before injection, 3-h fasting was made. Values are means ± S.E.M. Significant difference from the control (saline) at each time point represented $^{*}P < 0.01$. The number of chicks used for this experiment was as follows: control, 10; 0.38 nmol, 8; 0.75 nmol, 5; and 1.5 nmol, 9.

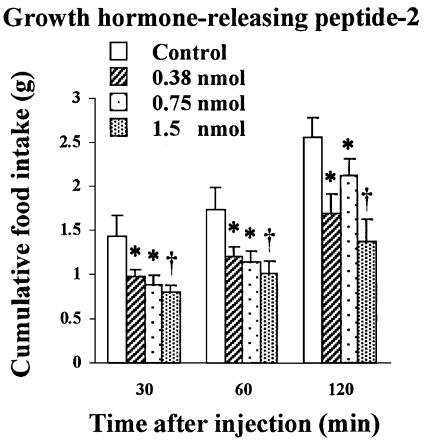

Fig. 5. Cumulative food intake during 2 h after intracerebroventricular injection of growth hormone-releasing peptide-2 in neonatal chicks. Before injection, 3-h fasting was made. Values are means ± S.E.M. Significant difference from the control (saline) at each time point represented * $P<0.05$ and † $P<0.01$. The number of chicks used for this experiment was as follows: control, 7; 0.38 nmol, 9; 0.75 nmol, 7; and 1.5 nmol, 9.

significant time by treatment interaction ($F(6,56)=2.513$, $P<0.05$), but the effect was weaker compared with the same dose of ghrelins.

4. Discussion

Central administration of rat ghrelin strongly suppresses food intake of chicks (Furuse et al., 2001). This was opposite of the effect seen in rats in which the i.c.v. injection of rat ghrelin stimulates feeding (Tschöp et al., 2000; Nakazato et al., 2001; Wren et al., 2001). Since the amino acid sequence of rat ghrelin is quite different from chicken ghrelin except for the N-terminal seven residues, it was possible that rat ghrelin was acting as an antagonist of the chicken GHS receptor and thus inhibiting the effect of endogenous chicken ghrelin. In the present study, however, the i.c.v. injection of chicken ghrelin strongly inhibited food intake, which is similar the effect of rat ghrelin (Furuse et al., 2001). Therefore, these results indicate that rat ghrelin did not act as an antagonist of the chicken GHS receptor, and the central action of ghrelin on feeding in the chick is species-specific. Chicken ghrelin stimulates intracellular Ca concentrations ($[Ca^{2+}]_i$) in CHO-GHSR62 cells expressing the rat ghrelin receptor, and increases plasma GH concentration in rats with a comparable potency to rat ghrelin (Kaiya et al., 2002). Although the overall amino acid sequence of chicken and rat ghrelin differ substantially, they have a similar potency in suppressing food intake of neonatal chicks in the present study. Chicken and rat ghrelin have the same sequence at the N-terminal seven residues, and it is believed that this segment is important in determining the efficacy of ghrelin (Bednarek et al., 2000; Matsumoto et al., 2001). The comparison of the effect of chicken and rat ghrelin on suppressing food intake supports this hypothesis.

The amino acid sequence of bullfrog ghrelin is quite different from chicken and rat ghrelin. The N-terminal sequence of bullfrog ghrelin is changed from Ser^2–Ser^3 in mammalian ghrelins to Leu^2–Thr^3, and the Thr instead of Ser residue is acylated. Bullfrog ghrelin increased $[Ca^{2+}]_i$ in CHO-GHSR62 cells and plasma GH levels in rats, but the potency was weak compared to that of rat ghrelin (Kaiya et al., 2001). Therefore, it was expected that bullfrog ghrelin would not affect or only weakly affect feeding behavior in the chick. In this study, however, bullfrog ghrelin inhibited food intake with similar potency to chicken and rat ghrelin. It seems that the difference in the N-terminal sequence does not influence feeding in the chick. Similarly, intravenous injection of human or chicken ghrelin produced similar changes in plasma GH levels in chicks (Kaiya et al., 2002). In the evolutional order, since the chicken is located between the rat and bullfrog, chicken GHS receptor may be able to accept several sequences of ghrelin.

Octanoic acid is a potent inhibitory factor of feeding in chicks. Food intake of chicks was significantly reduced by diets containing triacylglycerol composed by octanoic acid (Furuse et al., 1993). Since a common feature among ghrelins is the modification of octanoic acid at the third amino acid, it is possible that this lipid may influence the inhibitory effect. However, GHRP-2, which does not contain octanoic acid, also inhibited food intake with a comparable potency to ghrelins. Furthermore, we compared the inhibitory effect of GHRP-2 and ghrelins on food intake in neonatal chicks (Table 1). The results show that the inhibitory effect of chicken and bullfrog ghrelin on food intake is more potent than that of GHRP-2. Furthermore, these results indicate that the inhibitory effect of chicken ghrelin, the homologous ghrelin of chicks, is weaker than that of bullfrog ghrelin. Therefore, there is a possibility that chick GHS receptor is similar to bullfrog, though chicken ghrelin

Table 1
Comparison of inhibitory effect of feeding (% of control) in neonatal chicks treated with chicken ghrelin, bullfrog ghrelin or GHRP-2

Dose (nmol)	Treat	Time after injection (min)		
		30	60	120
0.38	Chicken ghrelin	48.9 ± 5.5	50.6 ± 4.6[a]	36.8 ± 5.1
	Bullfrog ghrelin	53.0 ± 6.2	56.5 ± 5.9[a]	57.7 ± 9.2[a,b]
	GHRP-2	32.0 ± 6.6	30.2 ± 6.6	33.9 ± 8.9
0.75	Chicken ghrelin	40.7 ± 6.3	44.6 ± 4.1[c]	42.4 ± 5.3[a]
	Bullfrog ghrelin	54.0 ± 8.6	58.2 ± 7.8	67.0 ± 13.8[b,c]
	GHRP-2	38.9 ± 8.2	34.5 ± 8.5	17.1 ± 7.7
1.5	Chicken ghrelin	46.8 ± 8.3	46.3 ± 9.6	49.9 ± 9.4
	Bullfrog ghrelin	64.5 ± 5.4[a]	66.9 ± 4.8[a]	69.8 ± 4.0
	GHRP-2	44.4 ± 6.0	41.2 ± 8.1	46.9 ± 10.4

Values are means ± S.E.M.
[a] $P<0.05$ compared to corresponding GHRP-2 group.
[b] $P<0.05$ compared to corresponding chicken ghrelin group. Comparison between groups were examined by Mann–Whitney U-test.
[c] $P<0.01$ compared to corresponding GHRP-2 group.

shows higher homology to mammalian ghrelins than bullfrog ghrelin.

Some avian species do not eat food at certain times such as during migration or brooding. Ghrelin may be involved in suppression of food intake during these fasting conditions (Toshinai et al., 2001). Enhanced ghrelin may suppress appetite, stimulate GH release and enhance metabolism during migration and brooding. The relationship among ghrelin levels and feeding, flying or nesting should be studied further.

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