The chicken is an interesting animal for study of the functional role of ghrelin in the gastrointestinal tract

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Abstract. Ghrelin has been identified in vertebrates from fish to mammals, and it has multiple biological activities including gastrointestinal (GI) motor-stimulating action. In some non-mammalian vertebrates, we examined the effects of ghrelin on contractility of the isolated GI tract as well as the mRNA expression of growth hormone secretagogue-receptor 1a (GHS-R1a) to determine whether the motor-stimulating action of ghrelin is common in vertebrates. The expression level of GHS-R1a mRNA differed depending on the species and on the GI region (stomach, small intestine, and colon). GI region-dependent expression of GHS-R1a mRNA was remarkable in chickens, and the expression levels changed depending on age. In a functional study, ghrelin did not cause contraction of unstimulated GI strips in fish (goldfish and rainbow trout) or amphibians (bullfrog and Japanese fire belly newt) even using their homologous ghrelin. In avian species, ghrelin caused contraction of the unstimulated GI tract of the chicken but not of the Japanese quail, and the responses to ghrelin in the chicken GI tract decreased with aging. Our in vitro studies show that the motor-stimulating action of ghrelin is not conserved across vertebrates and that the chicken is a unique animal species for evaluation of the GI-stimulating action of ghrelin of different age.

Key words: Ghrelin, Gastrointestinal contraction, GHS-R1a, Non-mammals, Chicken

GHRELIN is a natural ligand for growth hormone secretagogue-receptor 1a (GHS-R1a), which has a unique n-octanoyl modification at the third serine residue of the mature peptide [1]. Ghrelin is mainly produced in X/A-like endocrine cells in the mucosa of the stomach, and GHS-R1a is distributed in various central and peripheral organs [1, 2]. Since ghrelin and GHS-R1a have structural similarities with motilin and motilin receptor [3], physiological roles of ghrelin in the regulation of gastrointestinal (GI) motility have been extensively examined in mammals. In rodents, ghrelin stimulates GI contraction in vivo and in vitro [4-6], indicating that ghrelin is one of the gut peptides regulating GI motility of mammals.

Ghrelin and GHS-R1a have also been identified in non-mammalian vertebrates from fish to birds [7, 8]. Ghrelin is produced in the stomach and/or intestine in all animals examined. The fundamental structure of ghrelin has been conserved during vertebrate evolution.

Functional roles of ghrelin have been investigated in detail in birds and fish. Ghrelin stimulates GH release in both species, whereas the effects of ghrelin on food intake are different depending on the species, e.g., intracerebroventricular (ICV) or peripheral injection of ghrelin increases food intake in rodents, tilapia and goldfish, whereas ICV injection of ghrelin decreases food intake in chickens and rainbow trout [2, 7-9]. Species-dependent actions of ghrelin among vertebrates prompted us to investigate the effect of ghrelin on GI contractility in several vertebrates. Until now, the effects of ghrelin on GI motility have been investigated in vivo (conscious or anesthetized animals) and in vitro (stimulated and unstimulated muscles). In this study, we used isolated GI strips of fish (rainbow trout and goldfish), amphibians (bullfrog and Japanese fire belly newt) and birds (chicken and Japanese quail) to compare the contractile actions of ghrelin in the GI tract. In addition, expression of GHS-R1a mRNA was determined using quantitative RT-PCR. In chickens, changes in contractile response to ghrelin at different ages were examined to determine the developmental changes in the response of ghrelin.
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The goldfish intestinal bulb, mRNAs for two types of GHS-R1a, GHS-R1a-1 and GHS-R1a-2, were detected in the goldfish intestine, and the expression level of GHS-R1a-2 was 4-times higher than that of GHS-R1a-1. The expression levels of GHS-R1a-1 and GHS-R1a-2 in four different regions of the goldfish intestine were almost the same.

Amphibians
Isolated GI strips from the bullfrog exhibited spontaneous contraction, and substance P (1 µM) and carbachol (10 µM) caused marked contraction. However, bullfrog ghrelin (1 µM) did not affect the contractility of GI strips. GHS-R1a mRNA was detected in both the mucosa and muscle layers, and the expression level in the gastric mucosa was lower than that in the intestinal mucosa. Similar results were obtained in another amphibian, Japanese fire belly newt. Newt ghrelin (1 µM) did not cause any contraction of GI longitudinal muscle despite detectable expression of GHS-R1a mRNA.

Birds
Chicken ghrelin (10 nM-1 µM), but not rat ghrelin (1 µM), caused the contraction of chicken crop, proventriculus (stomach) and colon. However, the small intestine was insensitive to ghrelin. The ghrelin-induced contraction in the crop was resistant to tetrodotoxin but was partially decreased in the proventriculus by atropine and tetrodotoxin. Ghrelin potentiated the EFS-induced contraction of the proventriculus. These results indicated that the mechanisms of contraction induced by ghrelin are different in the crop (myogenic) and proventriculus (neurogenic and myogenic).

The expression level of GHS-R1a mRNA differed depending on the GI regions, with high levels in the esophagus and colon, medium levels in the crop and proventriculus, and low levels in the gizzard and small intestine. A significant correlation was found between expression level of GHS-R1a mRNA and amplitude of ghrelin-induced contraction.

Since ghrelin stimulates GH release and regulates energy homeostasis, we hypothesized that the action of ghrelin in chickens would change depending on their age. The contractile response to ghrelin in the proventriculus decreased with increase in number of post hatching days, but the response did not change in the crop (Fig. 1A and 1B). Consistent with the decrease

Materials and Methods

Contraction study
Several vertebrates (rainbow trout, goldfish, bullfrog, Japanese fire belly newt, chicken and Japanese quail) were used in this study. After anesthesia (fish and birds) or immobilization by spinal destruction (bullfrog and fire belly newt), the stomach and intestine were isolated. The isolated GI strips (3×10 mm) were suspended vertically in an organ bath containing physiological salt solutions [10-14]. Ghrelin and muscle stimulants were applied to the bath, and the induced mechanical changes were analyzed. In some experiments, enteric nerve-mediated responses were elicited by electrical filed stimulation (EFS), and the effect of ghrelin was examined. GI strips from chickens of different ages (from 1 day to 100 days after hatching) were used to compare the age-related changes in ghrelin responses and GHS-R1a mRNA expression.

Quantitative RT-PCR
The GI tract of each vertebrate was stored in an RNA stabilizing solution for analysis of GHS-R1a mRNA expression. The primer sets and protocol used in the following species (goldfish, bullfrog, Japanese fire belly newt, chicken and Japanese quail) have been published in respective papers [10-14].

Measurement of chicken ghrelin
In growing chickens, ghrelin concentrations in plasma and the stomach were measured by the specific radioimmunoassay for acylated ghrelin [13].

Results

Fish
Neither rainbow trout ghrelin (1 µM) nor rat ghrelin (1 µM) affected the contractility of GI strips of rainbow trout. Similarly, goldfish ghrelin-17 (1 µM) and rat ghrelin did not cause any mechanical changes in the goldfish intestinal bulb. On the other hand, substance P, goldfish neuromedine U and carbachol showed apparent contraction in the same preparation. Frequency-dependent contractions induced by EFS (1–20 Hz) were abolished by tetrodotoxin. Goldfish ghrelin-17 and rat ghrelin did not modify the EFS-induced contractions.

Although no response to ghrelin was seen in the goldfish intestinal bulb, mRNAs for two types of GHS-R1a, GHS-R1a-1 and GHS-R1a-2, were detected in the goldfish intestine, and the expression level of GHS-R1a-2 was 4-times higher than that of GHS-R1a-1. The expression levels of GHS-R1a-1 and GHS-R1a-2 in four different regions of the goldfish intestine were almost the same.

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Since ghrelin stimulates GH release and regulates energy homeostasis, we hypothesized that the action of ghrelin in chickens would change depending on their age. The contractile response to ghrelin in the proventriculus decreased with increase in number of post hatching days, but the response did not change in the crop (Fig. 1A and 1B). Consistent with the decrease
of ghrelin-induced contraction, the expression level of GHS-R1a mRNA in the proventriculus decreased and a significant correlation was seen between the receptor mRNA expression level and the response (Fig. 1C). With an increase in the number of post hatching days, plasma ghrelin level and ghrelin content in the proventriculus increased. There was a significant negative correlation between plasma ghrelin and expression of GHS-R1a mRNA (Fig. 1D). Therefore, it is likely that the expression level of GHS-R1a mRNA is regulated by endogenous ghrelin.

The effects of ghrelin were also examined in the Japanese quail. Different from the results obtained in the chicken, quail ghrelin and chicken ghrelin did not cause any marked contraction of quail GI strips, although the expression level of GHS-R1a mRNA was comparable to that of the chicken.

**Discussion**

When ghrelin was discovered, it was focused on a growth hormone-releasing peptide, but accumulating evidence has indicated that ghrelin is a multifunctional peptide that regulates glucose metabolism, feeding, cardiovascular function, gastrointestinal function, and endocrine and exocrine functions [1-3]. The ghrelin system has been confirmed in all vertebrates from fish to mammals [2, 7, 8]. Conservation of the ghrelin system suggests that ghrelin plays indispensable physiological roles in vertebrates. In this study, to know what the general action of ghrelin is in vertebrates, we compared the actions of ghrelin on contractility of isolated GI strips from fish to birds (Table 1). *In vitro* study using isolated GI strips is useful to examine the actions of ghrelin on smooth muscle and enteric neurons.
Ghrelin did not cause any mechanical responses in GI strips of fish and amphibians even at 1 µM. The enteric neuron-mediated response was also not affected by ghrelin in the case of goldfish despite detectable expression of GHS-R1a mRNA. The results suggest that ghrelin does not affect the GI motility of fish and amphibians [10, 11], although we should examine the in vivo effects. It is necessary to examine the involvement of ghrelin in other physiological events in the GI tract of these species. In contrast, ghrelin caused contraction of unstimulated GI strips and potentiation of EFS-induced contraction in chickens [15]. The responsiveness of ghrelin was GI region-dependent, and expression of receptor mRNA is correlated with ghrelin-induced contraction [13-15]. However, the excitatory response is not general in avian species because ghrelin had no effect on GI motility in the Japanese quail despite the expression GHS-R1a mRNA [14]. In rodents, ghrelin caused contraction of electrically stimulated GI strips but did not cause contraction of unstimulated strips, probably due to the lack of myogenic GHS-R1a [4, 5]. Therefore, the chicken is a specific species because ghrelin causes contraction of unstimulated GI strips through activation of myogenic GHS-R1a. A myogenic contractile mechanism of ghrelin has not been reported in mammals (only neurogenic contraction) (Table 1).

In the chicken GI tract, ghrelin-induced contraction decreased with advance of age, and the decrease was seen in a GI region-dependent manner. A significant correlation was found between contractile responses and expression of GHS-R1a mRNA in growing chickens [12, 13]. The decrease in GHS-R1a mRNA expression is negatively correlated with plasma ghrelin level. Therefore, the decrease in GHS-R1a mRNA expression is probably due to the down-regulation of receptor mRNA by endogenous ghrelin [13]. Changes in ghrelin-induced contraction and receptor expression during growth process of vertebrates have so far only been found in chickens.

In conclusion, ghrelin, a GI motility-stimulating peptide in mammals, does not affect contractility of GI smooth muscles in fish and amphibians, but ghrelin induces the GI contraction through myogenic and neurogenic receptors in chickens. These results suggest that there is a diversity of ghrelin actions on the GI tract across vertebrates and that motor-stimulating action is not a general action of ghrelin. Among the species examined, chickens have a unique characteristic for evaluating the GI-stimulating action of ghrelin and the age-dependent changes of ghrelin functions.

Table 1  Comparison of ghrelin-induced mechanical responses in the gastrointestinal tract and expression of GHS-R1a mRNA in vertebrates

| Species      | In vitro (unstimulated) | In vitro (EFS-response) | In vivo | GHS-R1a mRNA | Mechanisms of contraction | Reference |
|--------------|-------------------------|-------------------------|---------|--------------|---------------------------|-----------|
| Fish         |                         |                         |         |              |                           |           |
| Rainbow trout| No effect               |                         |         | Detectable   |                           | 10        |
| Goldfish     | No effect               | No effect               |         | Detectable   |                           | 10        |
| Amphibians   |                         |                         |         |              |                           |           |
| Bullfrog     | No effect               |                         |         | Detectable   |                           | 11        |
| Japanese fire belly newt | No effect               |                         |         | Detectable   |                           | 11        |
| Birds        |                         |                         |         |              |                           |           |
| Chicken      | Contraction             | Potentiation            |         | Detectable   | Enteric neurons/ Smooth muscle | 12, 13, 14, 15 |
| Japanese quail | No effect              |                         |         | Detectable   |                           | 14        |
| Mammals      |                         |                         |         |              |                           |           |
| Mouse        | No effect               | Potentiation            | Contraction | Detectable   | Enteric neurons         | 5         |
| Rat          | No effect               | Potentiation            | Contraction | Detectable   | Enteric neurons         | 4, 6      |

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