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Age-dependent reduction of ghrelin- and motilin-induced contractile activity in the chicken gastrointestinal tract

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ABSTRACT

Ghrelin is an endogenous ligand for growth hormone secretagogue-receptor 1a (GHS-R1a) and stimulates gastrointestinal (GI) motility in the chicken. Since ghrelin stimulates GH release, which regulates growth, it might be interesting to compare ghrelin-induced responses in GI tract of different-aged chickens. Motilin is a ghrelin-related gut peptide that induces strong contraction in the small intestine. Aim of this study was to clarify age-dependent changes in ghrelin- and motilin-induced contractions of the chicken GI tract and expression of their receptor mRNAs. Chicken ghrelin caused contraction of the crop and proventriculus. Ghrelin-induced contraction in the proventriculus decreased gradually up to 100 days after hatching, but the responses to ghrelin in the crop were the same during the growth period. GHS-R1a mRNA expression in the crop tended to increase, but that in the proventriculus decreased depending on the age. Chicken motilin caused contraction of the chicken GI tract. Atropine decreased the responses to motilin in the proventriculus but not in the ileum. Motilin-induced contraction in the proventriculus but not that in the ileum decreased depending on post-hatching days. On the other hand, motilin receptor mRNA expression in every region of the GI tract decreased with age, but the decrease was more marked in the proventriculus than in the ileum. In conclusion, ghrelin- and motilin-induced GI contractions selectively decreased in the chicken proventriculus depending on post-hatching days, probably due to the age-related decrease in respective receptors expression. The results suggest an age-related contribution of ghrelin and motilin to the regulation of chicken GI motility.

1. Introduction

Ghrelin is a natural ligand for growth hormone secretagogue-receptor 1a (GHS-R1a) and has a unique *n*-octanoyl modification at the third serine residue (Ser^3) of the mature peptide. This modification is essential for binding to GHS-R1a and for eliciting its biological activity. Ghrelin is mainly produced in specific ghrelin-producing endocrine cells in the stomach mucosa, and GHS-R1a was shown to be distributed widely in various central and peripheral tissues [21,22]. In addition to its growth hormone (GH)-releasing activity, ghrelin regulates appetite, lipid metabolism, glucose metabolism, cardiovascular function, cellular proliferation, reproduction, and endocrine and exocrine functions [9,22]. Motilin is a 22-amino acid gastrointestinal (GI) motility stimulating peptide hormone synthesized at the mucosa of small intestine (duodenum and jejunum) and is demonstrated to be involved in the initiation of interdigestive migrating motor complex of stomach through activation of cholinergic pathway [10]. Motilin receptor (MTL-R) is G protein coupled receptor and mediates physiological actions of motilin [5]. Since ghrelin and GHS-R1a have some structural similarities with motilin and MTL-R [1,25,29], physiological roles of ghrelin in the regulation of GI motility have been investigated extensively in mammals. In the rat, mouse and guinea pig, ghrelin stimulates gastric contractility or augments phase III-like contractions *in vivo* [4,6,7,14,27]. Ghrelin is also effective in modulating neural contractions in isolated GI preparations of the mouse and rat *in vitro* [4,7,14]. Therefore, ghrelin is considered to be one of the gut hormones regulating GI motility as with motilin through activation of the vago-vagal reflex pathway and/or peripheral GHS-R1a on enteric neurons in mammals.

Ghrelin has been identified in many species of non-mammalian vertebrates [11,12]. In the chicken, ghrelin is composed of 26 amino acids, and Ser^3 has been modified by *n*-octanoic acid or *n*-decanoic acid [11,13]. Chicken ghrelin shares about 50% total sequence identity to human ghrelin and 100% identity to the N-terminal region (Gly^1-Pro^7) of human ghrelin. Chicken ghrelin mRNA and

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ghrelin immunoreactivity are mainly detected in the proventriculus [13,37]. Characteristics of chicken GHS-R have already been reported, and two types of GHS-R have been found in the chicken: GHS-R1a is considered to be a functional receptor, and GHS-R1aV (GHS-R1c) is a splice variant in which 16 amino acids (48 bp) in the transmembrane domain-6 are lacking [8,33]. GHS-R1a mRNA expression has been detected in many central tissues (pituitary, hypothalamus, telencephalon, cerebellum and brainstem) and peripheral tissues (ovary, kidney, liver and GI tract). Apparent region-dependent expression of GHS-R1a mRNA in the chicken GI tract suggests that ghrelin regulates chicken GI motility as it does in mammals [8,17,33]. In a previous study, we have already shown that chicken ghrelin caused contraction of the isolated chicken GI tract and was more effective in the upper and lower GI tract than in the middle intestine. Motilin and MTL-R have been also identified in the chicken [3,38], and motilin preferentially causes contraction of the chicken small intestine opposite to the case of ghrelin [16,17,19]. Since ghrelin itself did not cause definite contractions in isolated GI strips from the rat, mouse, guinea pig, rainbow trout and goldfish [4,14,15,27], and motilin was ineffective in the rat GI strips [4], the chicken is a unique experimental animal in which both ghrelin and motilin cause apparent GI tract contraction in a region-dependent manner [16,17].

The chicken has been used for analysis of ontogenic and developmental changes in morphology and function of the GI tract [23,24,31,35]. Since ghrelin stimulates GH release and regulates energy homeostasis, which are essential for growing animals [9,21,22], we hypothesized that the functional role of ghrelin in chickens would change depending on their growth period. In the present study, we used GI strips prepared from different-aged chickens, and we found that contractile responses to ghrelin and motilin in the proventriculus decreased depending on post-hatching days, in parallel with the decrease in expression levels of GHS-R1a mRNA and MTL-R mRNA.

2. Materials and methods

All experiments were performed in accordance with Institutional Guidelines for Animal Care at Rakuno Gakuen University, Ebetsu Hokkaido, Japan.

2.1. Animals and tissue preparations

Male white Leghorn chickens (1–100 days after hatching, Hokuren, Yuni, Japan) were used. The chickens were anesthetized with isoflurane, stunned, and bled to death. The crop, proventriculus, ileum and colon were removed after a midline incision, and their luminal contents were flushed out using ice-cold Krebs solution. The crop, proventriculus and colon were cut open longitudinally, and smooth muscle strips in the longitudinal muscle direction (1 mm in width and 10 mm in length) were prepared. In the case of the ileum, longitudinal muscle layers were peeled out mechanically using a cotton-wool swab and fine tweezers. These isolated smooth muscle preparations were used for both contraction and molecular studies.

2.2. Quantitative real-time PCR for chicken GHS-R1a and MTL-R

Quantitative real-time PCR (qPCR) for chicken GHS-R1a (acc.# AB469019), chicken MTL-R (acc.# EU122384) or β-actin (acc.# NM_205518) was performed using a LightCycler System (Roche Applied Science, Mannheim, Germany) and a QuantiFAST SYBR Green PCR Kit (QIAGEN GmbH). Total RNA was extracted separately by TRIzol reagent from muscle layers of tissues in three (days 20–100) or four (days 1–10) individuals that had been stored in RNAlater. First-strand DNA was synthesized from 1 μg total RNA using a QuantiTect Reverse Transcription Kit (QIAGEN GmbH) with an oligo-dT_{12-18} primer (Invitrogen). Primer sets used were: chGHS-R1a-Q-s: 5′-GGG CCG TCT CCT TCA TTA GTG-3′ and chGHS-R1a-Q-as: 5′-TTC CTC TTC CTC CTC CAC AGC-3′ (232-bp amplicon); chMTL-R-Q-s: 5′-AGG ATC CTG GCT GTG GTG ATC CTG-3′ and chMTL-R-Q-as: 5′-TGC CCG GTA CCT CTG TGA GAT GAG-3′ (201-bp amplicon); and chB-actin-Q-s: 5′-CCC TGA ACC CCA AAG CCA ACA-3′ and chB-actin-Q-as: 5′-GGA CTC CAT ACC CAA GAA AGA-3′ (488-bp amplicon). The amplification conditions were 95 °C for 5 min and subsequent 35 cycles at 95 °C for 10 s and 60 °C for 30 s. The reaction mixture consisted of 1× master mix and 500 nM each of primer and template (100 ng total RNA equivalent). For quantification of each mRNA copy number, the pCR II-TOPO vector containing each chicken GHS-R1a, MTL-R or β-actin fragment had been cloned was linearized by restriction with *Xba-I*, and serial dilutions of the linearized plasmid from 1×10^6 to 1×10^3 were used to generate a linear regression line.

2.3. Contraction study for GI tract of the chicken

Smooth muscle strips from different parts of the GI tracts in the chicken were suspended vertically in an organ bath (5 ml) to measure longitudinal muscle contraction. The organ bath contained warmed (37 °C) Krebs solution (mM): NaCl, 118; KCl, 4.75; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_2$, 25 and glucose, 11.5 equilibrated with 95% O_2 + 5% CO_2 (pH 7.4). Mechanical activity of the preparations was measured with an isometric force transducer (SB-11T; Nihon Kohden, Tokyo, Japan) and recorded on an ink-writing recorder. Initial load was set at 0.5 g for each preparation. The preparations were rinsed with Krebs solution every 15 min and allowed to equilibrate for 1 h. Prior to the addition of ghrelin, each strip was subjected to 3 or 4 stimulations with 50 mM KCl until a reproducible contraction was obtained. In order to examine whether ghrelin causes contraction of GI smooth muscle preparations, chicken ghrelin 26-C8 [13] at 1 μM (enough concentration to cause contraction) was applied to an organ bath and the evoked responses were observed as previously described [16], because ghrelin-induced contraction was desensitized by cumulatively applied ghrelin. The amplitude of contractions among preparations was normalized by a standard contraction of 50 mM KCl and expressed as a relative contraction (%).

Motilin-induced GI muscle contraction was also investigated using GI tracts of different-aged chickens. Chicken motilin [3] could apply cumulatively to an organ bath to construct concentration–response curves as previously described [19]. The concentration–responses curves were investigated by a sigmoid non-linear regression analysis using Origin 7.0 (Origin Lab, USA). The concentration of motilin producing 50% (EC_{50}) of its maximal effect and maximal contraction value (% to 50 mM KCl-induced contraction) were calculated at each post-hatching day and compared with each other to determine the age-related change in motilin-induced contraction.

2.4. Chemicals

The following chemicals were used in the present experiments: atropine sulfate (Wako, Osaka, Japan), carbamylcholine hydrochloride (carbachol; Sigma, St Louis, MO, USA), 5-hydroxytryptamine creatinine sulfate monohydrate (5-HT; Sigma) and tetrodotoxin (Wako, Osaka, Japan). Chicken ghrelin 26-C8 [13] was synthesized by Asubio Pharma. Co., Ltd. (Gunma, Japan). Chicken motilin (FVPFFTQSDIQKMQEKERNKGQ, [3]) was also custom-synthesized by Peptide Institute Inc. (Osaka, Japan). All agents were dissolved in distilled water and applied directly in an organ bath.