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Tissue distribution and developmental changes of ghrelin and GOAT expression in broiler chickens during embryogenesis

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ABSTRACT

Ghrelin plays important roles, such as regulating growth hormone release and energy metabolism, but little is known about its developmental changes in the proventriculi of chicken embryos. This study was designed to elucidate the distributions and developmental changes of ghrelin and ghrelin-O-acyl-transferase (GOAT) expression in broiler embryos using qRT-PCR and immunohistochemistry. Our results demonstrated the following: (1) on E18, ghrelin and GOAT are ubiquitously expressed in every tissue examined. The expression level of ghrelin mRNA was the highest in the proventriculus, reaching a level that was 50-fold higher than that in the hypothalamus, while GOAT mRNA expression was low in the proventriculus and it was only 67.6% as high as that of hypothalamus; (2) ghrelin and GOAT mRNA expression were detected in the proventriculus on E9, but only at 1.9% and 1.7% of the level expressed on E18, respectively, and their expression levels increased rapidly from E18 to E21. There was similar developmental pattern in the ghrelin and GOAT mRNA expression; and (3) ghrelin-immunopositive cells were first detected in the proventriculus on E15, were located only in the compound tubular glands of the proventriculus, and were of the closed-cell type. The density of ghrelin-immunopositive cells increased significantly from E15 to E21. These results suggest that ghrelin may be an important regulating factor that plays a vital role during the development of chicken embryos.

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1. Introduction

Ghrelin is a 28-amino acid peptide that was originally isolated from the rat stomach, and it acts as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999). According to its acylation status, the peptide can be divided into unacylated ghrelin (UAG) or acylated ghrelin (AG). AG was the active peptide able to bind its receptor GHSR1a (Gahete et al., 2014). The enzyme GOAT (ghrelin-O-acyltransferase) that belonged to the superfamily of membrane bound O-acyltransferases, which is named MBOAT4 (Lim et al., 2011), was known as to be responsible for ghrelin acylation (Gahete et al., 2014; Yang et al., 2008). In mammals, ghrelin is distributed in various tissues (Kaiya et al., 2007; Kojima and Kangawa, 2005; Lee et al., 2002) and has extensive biological functions, including the regulation of growth hormone (GH) release, food intake, gastrointestinal function, reproduction and cardiovascular function (Date et al., 2000; Fernández-Fernández et al., 2005; Masuda et al., 2000; Nagaya and Kangawa, 2003; Nakazato et al., 2001; Wren et al., 2000; Yamazaki et al., 2002). Ghrelin has also been identified in many non-mammalian species, such as bullfrogs, chickens, turtles, and rainbow trout (Kaiya et al., 2011). Chicken ghrelin consists of 26 amino acids. This 26-amino acid peptide also plays important roles including acting as a GH-releasing peptide in young and adult chickens and an inhibitor for food intake (central ghrelin), promoting corticosterone release from the adrenal gland, and regulating body temperature, reproduction, behaviour, and energy metabolism (Geelissen et al., 2006; Kaiya et al., 2002, 2007, 2009, 2013; Saito et al., 2005; Shousha et al., 2005; Song et al., 2013; Yoshimura et al., 2005). Recently, studies showed that ghrelin had an anti-lipogentic action (Buyse et al., 2009), and had contractile activity on gastrointestinal tract and stimulates motility of the middle intestine in birds (Kitazawa et al., 2007, 2009).

RT-PCR analyses have revealed that ghrelin mRNA is distributed primarily in the proventriculus, followed by the brain, lungs, pancreas, spleen, and intestines, in 8-day-old layer chickens and in 3-week-old broiler chickens (Kaiya et al., 2002; Richards et al., 2006). Wada et al. (2003) detected ghrelin mRNA expression only in the proventriculus of newly hatched Leghorn chicks and detected ghrelin mRNA in the pylorus, duodenum, and proventriculus of

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adult Leghorn chickens. Ghrelin-immunopositive (ghrelin-ip) and ghrelin-expressing (ghrelin-ex) cells are found in the mucosal layer of the proventriculus (Neglia et al., 2005; Wada et al., 2003; Yamato et al., 2005) and the gastrointestinal tissue (Neglia et al., 2005) of chickens. These data indicate that the proventriculus is the main ghrelin-producing site and that ghrelin mRNA and ghrelin-ip cells exhibit developmental and tissue-specific changes during the hatching and growth processes of chickens. However, these studies were almost restricted to layer chickens, which have large difference from broilers in the growth rate resulting from different basal metabolism (Muramatsu et al., 1987) and muscle protein degradation (Nakashima et al., 2009), fat deposition (Griffin et al., 1987), heat production and lipid metabolism (Sato et al., 2006), and so on. So it is necessary to investigate both the distribution and the development of ghrelin in broilers. In addition, ghrelin has also been identified in both the yolk and the albumen of fresh fertilised eggs and embryos that have been incubated for five days (Yoshimura et al., 2009). Recent studies found that *in ovo* administration of exogenous ghrelin can improve gizzard and intestine weight of newly-hatched chicks (Lotfi and Shahryar, 2012) and hatching weight and post-hatch performance of chick (Lotfi et al., 2013). These results suggest that ghrelin mRNA is present in chicken embryos and may play regulatory roles in organ development during chicken embryogenesis. Therefore, the present experiment was designed to determine the distribution of ghrelin and the enzyme GOAT involved in ghrelin acylation in the tissues of chicken embryos on E18, and the developmental changes in ghrelin and GOAT mRNA and ghrelin-ip cells in the proventriculus during chicken embryogenesis using quantitative RT-PCR (qRT-PCR) and immunohistochemistry. This study will provide basic materials for further studies of the effects of ghrelin on the development of chicken embryos and the associated regulatory mechanisms.

2. Material and methods

2.1. Animal treatment and tissue harvesting

A total of 70 fertile Ross broiler eggs were obtained from a commercial hatchery (Dayong Chicken Breeding Farm, Henan Province, China) and incubated in an intelligent automatic incubator (Dezhou Ruike Incubation Equipment Factory, Shandong Province, China) at 37.5 ± 0.2 °C and 55 ± 2% relative humidity. Ten embryos were randomly sampled on days 9, 12, 15, 18, and 21 (piped but not hatched) of incubation (termed E9, E12, E15, E18, and E21, respectively). Samples of six chicken embryos on E18 that included the spleen, duodenum, jejunum, ileum, yolk sac, kidney, heart, proventriculus, lung, hypothalamus, subcutaneous fat, breast muscle, leg muscle, liver, bursa of Fabricius, and proventriculi of six chicken embryos on E9, E12, E15 and E21 were removed, frozen in liquid nitrogen and stored at −80 °C for RNA expression analysis. The proventriculi of the other four embryos on E9, E12, E15, E18 and E21 were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 4 °C) for 48 h, and paraffin cross-sections (5-μm-thick) were prepared for immunohistochemical staining. All of the embryos were euthanized with cervical dislocation under deep Nembutal anaesthesia (45 μg/g of BW, intraperitoneal injection; Shanghai Chemical Factory, Shanghai, China). All of the procedures were approved by the Animal Care and Use Committee of China.

2.2. Quantitative RT-PCR analysis of the expression of ghrelin and GOAT mRNA

The total RNAs from the proventriculus and other tissues were isolated with the TRIzol reagent (Life Technologies, 15596018, Carlsbad, USA). DNA degradation of the total RNAs and first-strand cDNA synthesis were performed using PrimeScript™ RT reagent kits with gDNA eraser (Taraka, RR047A, Dalian, China) according to the manufacturer's instructions. The primers (ghrelin: ggrnF, GAAACTGCTCTGGCTGGCTCTAG, ggrnR, CTGTGCCTCGGCGATG-TAATC; GOAT: GgoatF, GCTCTTCAGACTGGCGTACTACTCG, GgoatR, GAAGACAGACAGGCGATGTGTGG; Actin: gacnF, TGTGCGTGACAT-CAAGGAGAAG, gacnR, TACCACAGGACTCCATACCCAAG) were designed against the complete nucleotide sequences of *Gallus gallus ghrelin* (GenBank NM_001001131), *GOAT* (GenBank NM_001199289) and *actin* (NM_205518). The actin gene was used for internal standardisation. To analyse the data of qRT-PCR with the $2^{-\Delta\Delta CT}$ method, the efficiency of ghrelin, GOAT, and actin were tested by the standard curve. Real-time quantitative PCR was performed with SYBR® Premix Ex Taq™ II (Takara, RR820A, Dalian, China) according to the manufacturer's instructions. Dissociation curve analyses were performed for each sample after PCR to detect the specificities of the PCR products. Each sample was repeated triplicate. After PCR, the data were analysed using the ABI 7300 instrument (Applied Biosystems, Marsiling, Singapore) with the $2^{-\Delta\Delta CT}$ method.

2.3. Immunohistochemistry staining

For immunohistochemistry, the sections were incubated overnight at 4 °C with rabbit anti-human ghrelin antibody (1:200, Boster, BA1619, Wuhan, China). Next, the sections were rinsed in 0.01 M phosphate buffer saline (PBS, pH 7.4), and incubated with biotinylated goat anti-rabbit secondary antibody (1:400, CWBIO, CW0107, Beijing, China) for 2 h at room temperature. After washing, the tissues were incubated with streptavidin-horseradish peroxidase (1:400, Vector Labs, SA5004, Burlingame, CA, USA) for 1 h at 25 °C. The immunoreactivity was visualised by incubation in 0.01 M PBS containing 0.05% 3′,3-diaminobenzidine tetrahydrochloride (DAB, D5637, Sigma–Aldrich, St. Louis, USA) and 0.003% hydrogen peroxide for 10 min in the dark. After the final rinse, the sections were mounted. The specificities of the immunostainings were verified by omitting the primary antibodies from the first incubation. The stomach of mice was used for positive control. To test the specificity of the primary antibody of rabbit anti-human ghrelin, the absorption test was conducted. The diluted rabbit anti-human ghrelin antiserum (1:200) was incubated with a synthetic human ghrelin (gssflspehqrvqqrkeskkppaklqpr) (5 μg/mL) at room temperature for 8 h, and mixtures were centrifuged at 13,000*g* for 20 min at 4 °C to obtain the supernatant. The primary antiserum was replaced by the supernatant for absorption tests.

2.4. Morphological measurements

The number of ghrelin-ip cells in the compound tubular gland areas of twenty cross-sections of each proventriculus was counted. The densities of the ghrelin-ip cells were calculated as the number of cells per unit of compound tubular gland area in the proventriculus (cells/mm^2).

2.5. Statistical analysis

One-way ANOVAs were performed with SAS software (version 8.01; SAS Institute, Cary, NC, USA) to examine the differences. Differences with *P* values below 0.05 were considered statistically significant.

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