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Characterization of turkey and chicken ghrelin genes, and regulation of ghrelin and ghrelin receptor mRNA levels in broiler chickens

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Abstract

Ghrelin, a peptide hormone produced by the stomach in mammals, stimulates growth hormone release and food intake. Recently, ghrelin was identified and characterized in chicken proventriculus and shown to stimulate growth hormone release but inhibit feed intake. The purpose of this work was to identify and further characterize the ghrelin gene in chickens and in turkeys. Using molecular cloning techniques we have sequenced cDNAs corresponding to chicken (White Leghorn) and turkey ghrelin mRNAs. A total of 844 (chicken) or 869 (turkey) bases including the complete coding regions (CDS), and the 5′- and 3′-untranslated regions (UTRs) were determined. Nucleotide sequence (CDS) predicted a 116 amino acid precursor protein (preproghrelin) for both the chicken and the turkey that demonstrated complete conservation of an N-terminal 'active core' (GSSF) including a serine (position 3 of the mature hormone) known to be a modification (acylation) site important for ghrelin bioactivity. Additional nucleotide sequence was found in the 5′-UTRs of both Leghorn and turkey cDNAs that was not present in broilers or the red jungle fowl. The turkey ghrelin gene, sequenced from genomic DNA templates, contained five exons and four introns, a structure similar to mammalian and chicken ghrelin genes. Ghrelin was highly expressed in proventriculus with much lower levels of expression in other tissues such as pancreas, brain, and intestine. RT-PCR was used to quantify ghrelin mRNA levels relative to 18S rRNA in 3-week-old male broiler chickens. The level of ghrelin mRNA increased in proventriculus in response to fasting but did not decline with subsequent refeeding. Plasma ghrelin levels did not change significantly in response to fasting or refeeding and did not appear to reflect changes in proventriculus ghrelin mRNA levels. Ghrelin mRNA levels declined in broiler pancreas after a 48 h fast and increased upon refeeding. Expression of the gene encoding the receptor for ghrelin (growth hormone secretagogue receptor, GHS-R) and a variant form was detected in a variety of tissues collected from 3-week-old male broiler chickens possibly suggesting autocrine/paracrine effects. These results offer new information about the avian ghrelin and ghrelin receptor genes and the potential role that this system might play in regulating feed intake and energy balance in poultry.
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1. Introduction

The ghrelin system consists of a small acylated peptide hormone and its cognate receptor, the growth hormone secretagogue receptor (GHS-R). As a member of the G-protein-coupled receptor superfamily, GHS-R signals via activation of the protein kinase C system and hydrolysis of phosphatidylinositol (phospholipase–IP3 pathway) resulting in the elevation of intracellular calcium levels (Korbonits et al., 2004; Ueno et al., 2005). Ghrelin was originally isolated from mammalian stomach tissue and subsequently shown to be a naturally occurring ligand for GHS-R (Kojima et al., 1999). This unique acylated peptide hormone has now been identified in several non-mammalian vertebrate species such as bullfrog

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(Kaiya et al., 2001), chicken (Kaiya et al., 2002), and fish (Kaiya et al., 2003; Parhar et al., 2003; Unniappan et al., 2002; Unniappan and Peter, 2005). Although ghrelin is predominantly a product of gastric tissue, expression has been detected in a variety of tissue sites including intestine, pancreas, placenta, brain, pituitary, hypothalamus, lung, cells of the immune system, kidney, and thyroid gland (Korbonits et al., 2004; Ueno et al., 2005). Similarly, GHS-R expression occurs in a variety of sites including the central nervous system and peripheral tissues indicating the potential for systemic (endocrine) as well as localized (autocrine/paracrine) functioning of this important hormone system (Korbonits et al., 2004; Ueno et al., 2005).

In mammals, ghrelin plays important roles in regulating growth hormone (GH) release from the pituitary gland as well as in regulating appetite, energy balance, adiposity, gastrointestinal function, immune function, reproductive function, and cardiovascular function (Korbonits et al., 2004; Tena-Sempere, 2005; Ueno et al., 2005; Van der Lely et al., 2004). Ghrelin and its receptor have been identified in the pancreas (Kageyama et al., 2005) where the ghrelin system has been suggested to function as part of a physiological control mechanism (calcium signaling via GHS-R) regulating β cell development and insulin secretion, as well as exocrine function in the pancreas (Date et al., 2002; Dezaki et al., 2004; Lai et al., 2005) and in the central regulation of pancreatic exocrine secretion mediated via vagal nerve efferent pathways (Sato et al., 2003). Moreover, a new ghrelin-secreting cell type (epsilon cell) has been described in pancreatic islet tissue (Prado et al., 2004). In general, as a regulatory component of the complex brain–gut anabolic neuroendocrine network controlling food intake, energy balance, and body weight, ghrelin may play a fundamental role in coordinating energy needs with the processes involved in growth.

Considerably less is known concerning the ghrelin system in avian species as compared to mammals. Chicken ghrelin has been purified from proventriculus and found to contain 26 amino acids (as opposed to 28 in mammals) with the third serine residue from the N-terminus of the mature peptide being acylated with *n*-octonoic or *n*-decanoic acid (Kaiya et al., 2002). Fatty acylation has been reported to confer bioactivity to the modified peptide hormone by enabling it to bind to, and signal through, the GHS-R (Kaiya et al., 2002). A gene homologue that codes for the chicken GHS-R (GHS-R1a) and a variant form (GHS-R1aV also designated GHS-R1c, truncated in the sixth transmembrane domain by alternative splicing of the gene transcript) have been cloned and their expression pattern in various tissues studied (Geelissen et al., 2003; Tanaka et al., 2003). Ghrelin immuno-positive cells have been detected in the hypothalamus, proventriculus, and gastrointestinal tract of birds (Ahmed and Harvey, 2002; Neglia et al., 2004, 2005; Wada et al., 2003; Yamato et al., 2005). There have been several reports that injections of acylated ghrelin peptide increase pituitary GH release (Ahmed and Harvey, 2002; Baudet and Harvey, 2003; Kaiya et al., 2002), increase plasma corticosterone levels (Kaiya et al., 2002; Saito et al., 2005), and inhibit feeding behavior (Furuse et al., 2001; Geelissen et al., 2005; Kaiya et al., 2002; Saito et al., 2002; Saito et al., 2005) in chickens. Recently, conflicting (stimulatory or inhibitory) effects of peripheral versus central ghrelin administration were reported on food intake in the Japanese quail (Shousha et al., 2005). However, the exact role that the ghrelin system plays in birds still remains largely unknown. The purpose of this work was to further characterize the ghrelin gene in chickens and turkeys, and to follow its expression as well as the expression of GHS-R in 3-week-old broiler chickens under different energy states with the goal of better understanding the function of the ghrelin system in different avian species.

2. Materials and methods

2.1. Animals and materials

Chicks (*Gallus gallus*, broiler, and White Leghorn) and turkey (*Meleagris gallapavo*) poults were reared from hatching to 3 weeks of age in heated battery/brooder units. All birds received a standard starter poultry ration and water ad libitum. At 3 weeks, tissue samples were collected and snap-frozen in liquid nitrogen prior to RNA isolation. In a separate experiment, 30 broiler chickens (3-week-old males) were divided into five groups of six birds each and subjected to the following treatments: (1) fed ad libitum, (control), (2) fasted for 24 h (S24), (3) fasted for 48 h (S48), (4) fasted for 24 h and refed for 24 h (S24-RF24), and (5) fasted for 48 h and refed for 24 h (S48-RF24). All birds had free access to water throughout the experimental period. Upon termination of the experiment, samples of pancreas, whole brain, and proventriculus were collected and snap-frozen in liquid nitrogen prior to RNA isolation. Total RNA was isolated from tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA). Blood was collected by heart puncture. Following centrifugation (8000*g* 10 min at 4 °C) plasma was collected and stored at −80 °C prior to analysis. Genomic DNA was extracted from 50 μl of whole blood using the Easy DNA extraction kit (Invitrogen). All protocols involving the use of animals received prior approval from the Beltsville Animal Care and Use Committee.

2.2. Nucleotide sequencing

Total RNA from proventriculus and primer-directed RT-PCR were used to generate turkey and chicken ghrelin cDNAs that were sequenced. Primer sets based on sequence reported for the broiler chicken (GenBank Accession No. AB075215) were initially used to generate a series of overlapping PCR products. The sequence of the turkey ghrelin gene was derived from genomic DNA templates using a primer-directed PCR strategy. PCR products were evaluated by agarose gel (1.5%) electrophoresis, and bands of the appropriate size were excised from the gel and purified using a GenElute gel extraction kit (Sigma Chemical, St. Louis, MO) or purified directly from the PCR sample by a