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Expression of the orexigenic peptide ghrelin in the sheep ovary

Chenguang Du [a,b], Xilingaowa [a], Guifang Cao [a,*], Caiyun Wang [a], Haijun Li [a], Yanhong Zhao [a], Siqingaowa [b], Jinshan Cao [a]

[a] *College of Animal Science and Animal Medicine, Inner Mongolia Agricultural University, Hohhot 010018, China*
[b] *Vocational and Technical College, Inner Mongolia Agricultural University, Baotou 014109, China*

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Abstract

Ghrelin has been implicated in the control of cell proliferation in reproductive tissue. Here, we provide evidence that both ghrelin mRNA and protein are present in ovarian follicles. Persistent expression of ghrelin was also demonstrated in sheep ovary throughout the estrous cycle and pregnancy. In fact, the relative mRNA levels of ghrelin varied depending on the stage of the cycle, with the highest expression during the development of the corpora lutea (CL) and minimal expression in the regressing CL. A similar pattern was seen during pregnancy. Dynamic changes in the profile of ghrelin expression during the estrous cycle and throughout pregnancy suggest a precise regulation of ovarian expression of ghrelin, which could represent a potential role for ghrelin in the regulation of luteal development. In conclusion, the presence of the ghrelin signaling system within the sheep ovary especially in the oocytes opens up the possibility of a potential regulatory role of this novel molecule in reproductive function.
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Keywords: Ghrelin; Sheep ovary; Follicle; Oocyte; Corpora lutea

1. Introduction

Ghrelin was identified in 1999 as the natural ligand of the GH secretagogue receptor (GHS-R1a), a 7-transmembrane G protein-coupled receptor [1]. A striking feature of ghrelin is its widespread pattern of expression [2,3]. Expression of ghrelin has been demonstrated in an array of tissues and cell types including the stomach, small intestine, pancreas, lymphocytes, placenta, kidney, lung, pituitary and brain [3].

This ubiquitous pattern of expression strongly suggests that in addition to systemic actions of the gut-derived peptide, locally produced ghrelin might have paracrine/autocrine regulatory effects in different tissues [4–6]. This may be the case in various reproductive organs such as endometrium, placenta, testis and ovary [7,8]. Interestingly, a wide range of endocrine and non-endocrine tissues, including the gonads [9], possess GHS-binding sites, and many of these tissues also have significant levels of ghrelin mRNA. Expression of ghrelin mRNA and its cognate receptor have been demonstrated in rat, pig, sheep and human gonads [10–13]. Persistent expression of the ghrelin gene was also demonstrated in rat and human ovary throughout the estrous cycle, and its relative mRNA levels varied depending on the stage of cycle [10,13]. In sheep, ghrelin immunostaining also was detected in ovarian follicles at all developmental stages, mainly in the granulosa cells [12]. At the same time, strong ghrelin immunostaining was evident in the CL of the sheep ovary, similar to the findings in the rat and human CL. These data provide further evidence for a reproductive role for this relatively new hormone.

* Corresponding author. Tel.: +86 471 4303140; fax: +86 471 4303140.
E-mail address: guifangcao@126.com (G. Cao).

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It is unknown whether the ghrelin system is regulated in sheep ovary and it remains equivocal as to whether ghrelin is expressed in the different stages of the oocyte. The aim of this study was to characterize in detail the pattern of expression of ghrelin in the sheep ovary, especially in the oocytes, with special attention to the cellular distribution of ghrelin peptide within the ovarian tissue, as well as the influence of the estrous cycle and pregnancy on ovarian ghrelin mRNA expression levels.

2. Materials and methods

2.1. Collection of ovine ovarian tissue and isolation of oocytes, cumulus cells, complex of granulosa cells and theca cells

All experiments involving animals were approved by the animal care and use committee at the institution where the experiment was conducted.

A total of 40 Mongolia sheep (12–24 months of age) were used in this study from September to January. In half of the animals ($n = 20$), estrus was synchronized by treatment for 8 days with a CIDR device (Hamilton, New Zealand). Appearance of estrus behaviour was detected with adult rams at 24 h after CIDR withdrawal.

For RNA extraction, ovarian CL were immediately snap frozen in liquid nitrogen, while the remainder of ovaries used for cell extraction were stored in ice cold saline for transport to the laboratory. Ovarian tissues representing different stages of the estrous cycle were used from: young CL (1–3 days after ovulation, $n = 6$); mature CL (9–11 days after ovulation, $n = 7$); and regressing CL (14–16 days after ovulation, $n = 7$). CL from very early (<6 weeks, $n = 5$), early (6–8 weeks, $n = 5$), middle (9–14 weeks, $n = 5$) or late (15–18 weeks, $n = 5$) stages of pregnancy were also used. Ovarian CLs were sampled from 20 pregnant sheep immediately after slaughter at a local abattoir. The stage of pregnancy was estimated by measuring foetal size as previously described [14].

COCs (cumulus-oocyte complexes) were collected with a surgical blade from the surface of intact healthy antral follicles 3–5 mm in diameter. COCs having an evenly granulated cytoplasm with at least four layers of unexpanded cumulus cells (CCs) were selected. Complexes of granulosa cells (GCs) and theca cells were collected using a small scoop at the same time. Denuded oocytes were generated by vortexing for 2–3 min. Any remaining CCs were mechanically removed by repeated passage through a fine bore polished glass pipette and collected separately.

2.2. Immunohistochemistry

Sheep ovaries were bisected and fixed in 4% paraformaldehyde (PFA) for 6 h. Fixed tissues were embedded in paraffin and sliced into 5 μm sections, which were placed on poly-L-lysine (Sigma, USA) coated slides. Sections were deparaffinized by two 30 min exposures to xylene, then hydrated by successive 5 min washes in 95% then 85% ethanol. The remaining formaldehyde was removed from each section by incubation in 10% ammonium hydroxide in ethanol for 10 min, followed by a brief wash in distilled water. Antigen retrieval was achieved by incubating in 10 mM EDTA, pH 8.0 at 96 °C for 25 min. Endogenous peroxidase was quenched by incubation in hydrogen peroxide for 20 min, then the slides were rinsed twice for 5 min in distilled water and once in 0.5 M Tris buffer, pH 7.4. Tissue sections were placed in a blocking solution for 1 h at room temperature in a humidified chamber. Blocking solution consisted of 0.1 M glycine, 1% goat serum, 0.01% Triton X-100, 1% powdered non-fat dry milk, 0.5% BSA, and 0.02% sodium azide in PBS. After blocking, tissue sections were incubated with anti-human ghrelin (Phoenix Pharmaceuticals, CA) at a 1:600 dilution overnight at 4 °C. Ghrelin peptide sequences are highly conserved between species [15] and specificity of the antibody used in this study for sheep ghrelin has been previously demonstrated [12]. Slides were washed in Tris buffer twice for 5 min, then incubated with horseradish peroxidase-conjugated secondary antibody (EnVision Dual Link System; DakoCytomation). Immunostaining was revealed with liquid DAB (DakoCytomation). Negative controls were performed by omitting primary antibody from the procedure.

2.3. Immunofluorescence staining

A similar immunohistochemical procedure was used for assessment of the presence and pattern of sheep antral follicles by using the same antibody. Briefly, COCs (denoted denuded CCs), oocytes and GCs were fixed for 2 h in 4% PFA at 4 °C and permeabilized with 0.2% Triton X-100 for 10 min. COCs, oocytes and GCs were then incubated first with anti-human ghrelin antibody for 1 h and then with goat anti-rabbit FITC-labeled secondary antibodies (Santa Cruz Biotech) for 1 h at a 1:500 dilution. After washing for 15 min in four changes of PBS, COCs, oocytes and GCs were stained with propidium iodide (0.1 mg/mL final concentration) for 1 h, rinsed in PBS-T for 30 min, mounted on a drop of PBS containing 0.5% FBS and analyzed with a laser scanner confocal microscope (Bio-Rad MRC 1024ES) equipped

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