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Expression of ghrelin receptor, GHSR-1a, and its functional role in the porcine ovarian follicles

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Abstract

Recently, we reported stimulatory effect of ghrelin alone and in combination with growth hormone (GH) on estradiol secretion, aromatase activity in parallel with inhibitory effect on cell apoptosis. The aim of this study was to analyze the expression of the functional ghrelin receptor (GHS-R type 1a) and the effect of GH on GHSR-1a expression in cultured whole porcine follicles. Using RT-PCR and Western Blots, we demonstrated the presence of GHSR-1a in prepubertal pig ovary and found no influence of GH on either GHSR-1a protein levels or mRNA expression. Additionally, to show if, noted previously by us action of ghrelin on ovarian follicular function is dependent of its binding to GHSR-1a, we used an antagonist of the ghrelin receptor, (D-Lys-3)-GHRP-6. In cultures treated together ghrelin and (D-Lys-3)-GHRP-6, estradiol secretion, aromatase activity and cell proliferation returned to control levels. Inhibitory action on caspase-3 activity was not reversed by a selective antagonist of GHSR-1a. In conclusion, results of the present data clearly showed: (1) the presence of GHSR-1a in prepubertal pig ovary and found no influence of GH on GHSR-1a protein levels and mRNA expression, and (2) ghrelin effect on estradiol secretion, aromatase activity and cell proliferation dependent of its binding to GHSR-1a, while the effect on cellular apoptosis was independent of its binding to GHSR-1a.

Keywords: GHS-R1a; GH; Ovarian cells; Estradiol; Aromatase activity; Apoptosis; Proliferation

1. Introduction

Ghrelin, a 28-amino acid octanoylated peptide, was recently isolated from the stomach of rat and identified as the endogenous ligand for the growth hormone secretagogue receptor, GHSR [1]. GHSs are a family of small synthetic peptide or non-peptide molecules that stimulate the release of growth hormone from the anterior pituitary via a specific seven-transmembrane G protein-coupled receptor [2]. Two GHSR subtypes generated by alternative splicing of a single gene have been identified: the full-length type 1a receptor and the truncated type 1b [3]. GHS-R1a is the functionally active, signal transduction form of the receptor. In contrast, GHS-R1b lacks transmembrane domains 6 and 7 and is unable to bind a ligand or transduce a signal.

Like ghrelin, the ghrelin receptor also has a widespread distribution in various tissues, suggesting multiple paracrine, autocrine and endocrine roles for ghrelin. Expression of the ghrelin receptor, GHSR-1a, has been detected in the pituitary, thyroid, pancreas, stomach, heart, lung, adrenal cortex, immune system, adipose tissue and human breast carcinoma [4–6]. Gaytan et al. [7] reported that GHS-R1a peptide expression paralleled follicular development, with stronger immunostaining in the granulosa and theca cell layers of

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healthy human antral follicles. Moreover, expression of ghrelin in the ovary has been demonstrated in steroidogenically active luteal cells and interstitial hilus cells. This suggests a potential relationship between GHSR-1a expression, follicular growth and regulation of ovarian production. In addition, ghrelin and GHSR-1a expression were detected in reproductive tissues including placenta [8], ovary [9] and testis [10]. To date, we have demonstrated that ghrelin is secreted from ovarian follicles, that it increases estradiol secretion by modifying aromatase activity and that it acts as an anti-apoptotic and proliferation stimulate factor in prepubertal pig ovaries [11]. Moreover, we have shown that ghrelin stimulates local, ovarian growth hormone (GH) secretion and cooperates with GH in ovarian function [12].

To better define, characterize and understand the receptor dependent mechanism of ghrelin action in the pig ovary, we examined: (1) expression of GHSR-1a in ovarian follicles and the influence of GH on GHSR-1a expression, and (2) whether ghrelin through the GHSR-1a control ovarian function such as estradiol secretion, aromatase activity, follicular cell apoptosis and proliferation.

2. Materials and methods

2.1. Reagents

M199, trypsin, fetal bovine serum (FBS, heat-inactivated), antibiotic/antimycotic solution (100×), Trypan blue, Tris, Na-deoxycholate, Nonidet NP-40, sodium dodecyl sulfate (SDS), protease inhibitor (EDTA-free), DTT, Tween 20, bromophenol blue, dibenzylfluorescein (DBF), NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, selective antagonist of ghrelin receptor, GHSR-1a-(D-Lys-3)-GHRP-6, porcine GH and human ghrelin were obtained from Sigma (Chemical Co. St. Louis, MO, USA). Human ghrelin was utilized in this experiment because porcine ghrelin was not readily available at the onset of this experiment. Human ghrelin differs from porcine ghrelin by three amino acids [1].

2.2. Experimental procedures

Prepubertal porcine ovaries obtained from a local abattoir were collected in a bottle filled with sterilized ice-cold saline and transported to the laboratory. Approximately, 15 min elapsed from slaughter to ovary collection.

2.2.1. Experiment 1 was conducted to examine mRNA and protein expression of GHSR-1a and the influence of GH on GHSR-1a in ovarian follicles

To look at expression of the ghrelin receptor, GHSR-1a, whole follicles (~2–3 mm in diameter) were cut with small scissors and placed individually in 24-well plates (Nunc) with 2 ml of M199 medium supplemented with 5% of fetal bovine serum (FBS). Ovarian follicles were incubated for 24 h at 37 °C in humidified atmosphere containing 5% CO_2, either alone or with GH (100 ng/ml). The dose of GH was chosen based on Kolodziejczyk et al. [13].

2.2.2. Experiment 2 was conducted to evaluate participation of GHSR-1a in estradiol secretion, aromatase activity, follicular cell proliferation and apoptosis in the prepubertal pig ovary

Granulosa cells (Gc) and theca interna cells (Tc) were isolated from prepubertal follicles (2–3 mm) according to the technique described by Stokłosowa [14]. Briefly, Gc were scrubbed from the follicular wall with round-tipped ophthalmologic tweezers and rinsed several times with PBS. After isolation, Gc were exposed to DNAse I (500 U for 1 min), washed three times in M199, collected and resuspended in M199 supplemented with 5% FBS. The Tc were prepared from the same follicles by placing the theca layers in a drop of saline under the dissecting microscope. Isolated theca interna tissue was then washed with PBS, cleaned, cut with scissors and exposed to 0.25% trypsin in PBS for 10 min at 37 °C. Isolated cells were separated by decantation, and the procedure was repeated three times. Finally, the cells were centrifuged and resuspended in M199/FBS. The viability of the cells was determined before seeding by the Trypan blue exclusion test, and viability was found to be 93% for granulosa cells and 85% for theca cells. For co-culture experiments, granulosa and theca cells were inoculated at concentrations of 4×10^4 and 1×10^4 viable cells/well, respectively, in 96 well tissue culture plates. This ratio was similar to that observed *in vivo* (Gc:Tc = 4:1) according to Stokłosowa et al. [15]. After 24 h in culture, the medium was changed, and (D-Lys-3)-GHRP-6 (50 ng/ml) was added 1 h before ghrelin (500 pg/ml) or GH (100 ng/ml). The doses of ghrelin were chosen based on our previous research [11], and (D-Lys-3)-GHRP-6 dosage (50 ng/ml) was based on preliminary dose-dependent experiments. After 48 h the cultures were terminated and Alamar Blue was added into the medium. The remaining amount of the media was frozen for estradiol determination. Cells from culture were stored at −20 °C for estimation of caspase-3 activity, or at −70 °C for estimation of aromatase activity. Every treatment was conducted in four wells each, and each experiment was repeated three times.

2.3. Western immunoblotting

For immunoblotting, whole follicles were homogenized twice in 50 μl of ice-cold lysis buffer, containing 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1 mM