JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2012, 63, 2, 195-199
www.jpp.krakow.pl

Short communication

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EXPRESSION OF GHRELIN AND ITS RECEPTOR IN PORCINE OVARIAN FOLLICLES COLLECTED FROM PREPUBERTAL AND ESTROUS CYCLE ANIMALS

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Ghrelin, a hormone predominantly found in the stomach, was described as a factor that controls female reproductive function. Using real time PCR and western blot, we measured gene and protein expression of ghrelin and its receptor. Enzyme-like immunoassay (ELISA) was used to measure the concentration of acylated (Ac) and unacylated (UnAc) forms of ghrelin as well as levels of estradiol (E2) in follicular fluid. For all analyses, we compared small, medium and large ovarian follicles collected from ovaries of prepubertal and estrous cycling pigs. We demonstrated that the gene expression levels of ghrelin significantly increased in ovarian follicles from cycling animals, with the maximum expression in large follicles, without any change in prepubertal. However, the protein expression of ghrelin and its concentration was increased with increasing follicle size both in prepubertal and cycling animals and it was positively correlated with E2 levels in follicular fluid. In addition, both receptor growth hormone secretagogue receptor (GHSR) and GHSR type GHSR-1a expression were significantly higher in ovarian follicles from cycling animals than prepubertal. Results of this study suggest the possibility of local synthesis of ghrelin in the ovarian follicles and point to important modulatory functions for ghrelin before puberty and during the estrous cycle.

Key words: *estradiol, estrous cycle, ghrelin, growth hormone secretagogue receptor-1a, ovary, prepubertal, protein expression*

INTRODUCTION

Ghrelin, an endogenous ligand of GHSR, is a 28-amino acid peptide recognized to have a wide range of physiological roles in many species including regulation of food intake and stimulation of growth hormone secretion (1). It is synthesized predominantly in the stomach but has also been identified in a variety of other organs such as the bowels, kidney, thyroid, lung, lymphatic tissue, placenta, hypothalamus, and pituitary (2).

The expression of ghrelin and its receptor have been detected in reproductive tissues in the human, rat, pig, sheep and chicken (3-7). Ghrelin mRNA expression was consistently detected in the rat ovary throughout the estrous cycle and pregnancy, with higher levels present in the corpus luteum and during the first days of gestation (5). Zhang *et al*. (6) showed that the highest ghrelin mRNA expression levels in the pig ovary occur in the diestrous phase and the lowest in the proestrous phase. Our previously published data demonstrated that ghrelin was secreted by porcine ovarian follicles collected from ovaries of prepubertal animals and by modification of aromatase activity stimulated estradiol secretion (8, 9). Moreover, the ERK 1/2 and PI-3 kinase pathways may be potential signals of ghrelin mediate the cell proliferation and apoptosis of porcine ovary cells (10).

There are no data showing changes of ghrelin and its receptor in porcine ovarian follicles collected from animals during prepubertal and regular estrous cycle. In this study, we examined the gene and protein expression of ghrelin, and its receptor, and the concentration of both forms of ghrelin, Ac and UnAc, in different size of ovarian follicles collected from ovaries of prepubertal and cycling animals.

MATERIALS AND METHODS

Sample collection

Crossbed gilts (Large White×Polish Landrace) of prepubertal (5-6 months age) and cycling (7-8 months age) animals were used in the experiment. Small (3-4 mm; SF, n=5), medium (4-5 mm; MF, n=6) and large (6-7 mm; LF, n=6) follicles were obtained from porcine prepubertal ovaries. From cycling animals, follicles were obtained at days 4-6 (2-4 mm; SF, n=6), 10-12 (4-6 mm; MF, n=6) and 16-18 (8-12 mm; LF, n=5) of the estrous cycle. Follicular fluids were aspirated from the ovarian follicles.

Experimental procedure

To assay ghrelin and GHSR gene expression, ovarian follicles were collected, immediately frozen in liquid nitrogen and stored at -70°C until RNA extraction. To determinate concentrations of Ac and UnAc forms of ghrelin and protein expression of ghrelin and GHSR-1a, whole ovarian follicles were homogenized in lysis buffer, centrifugation and the whole protein

content in lysates was measured with Bradford method. After homogenization, supernatants and follicular fluid were collected and stored at -70°C.

Real-time PCR analysis

We examined ghrelin and GHSR gene expression by reverse transcription real-time PCR method as describe in our previous study (11). Briefly, total RNA was isolated using the High Pure RNA Tissue Kit (Roche, Germany) and 1 µg of total RNA was treated with deoxyribonuclease I and reverse transcribed using the transcriptor first strand cDNA Synthesis Kit (Roche, Germany). Real-time PCR analyses were performed using the StepOne real-time PCR system (Applied Biosystems). The mRNA expression of the ghrelin and GHSR was quantified using TaqMan Gene Expression Assays (Ss03392360_m1, RefSeq: NM_213807.1; and Ss03383123_s1, RefSeq: NM_214180.1 respectively, Applied Biosystems). 18S rRNA levels were determined as an endogenous control assay (Hs99999901_s1, RefSeq: X03205.1, Applied Biosystems). Quantitative PCR was performed with 100 ng of cDNA, 1 µL gene expression assay, and 10 µL TaqMan PCR master mix (Applied Biosystems). Expression of ghrelin and GHSR mRNA were normalized to the levels of 18S rRNA.

Western blot method

Samples were subjected to SDS-page and immunoblotting using specific antibodies against ghrelin and GHSR-1a (both 1:200, Santa Cruz Biotechnology) followed by peroxidase-conjugated secondary antibodies and chemiluminescence detection. The blots were visualised using the ChemiDocTM and quantified using the Image Lab™ 2.0 Software (BioRad Laboratories).

Hormone determination

The levels of Ac, UnAc isoforms of ghrelin and E2 were determined using commercially available ELISA kits (Biovendor). The inter- and intra-run precision of Ac ghrelin: of 8.3% and 8.1%, and of UnAc ghrelin: 3.80% and 3.20%, and of E2: 6.72% and 2.71%, respectively.

Statistical analyses

Statistical analysis was performed using Statistical 6.0 (StatSoft, Poland). Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey honestly significant difference (HSD) multiple range test. All data are expressed as the mean ±S.E.M.

RESULTS

Expression of ghrelin receptor and ghrelin in porcine ovarian follicles from prepubertal and estrous cycle animals

Both mRNA and protein of ghrelin receptor were significantly increased in ovarian porcine follicles from cycling animals compared to prepubertal ($p<0.05$, *Fig. 1*).

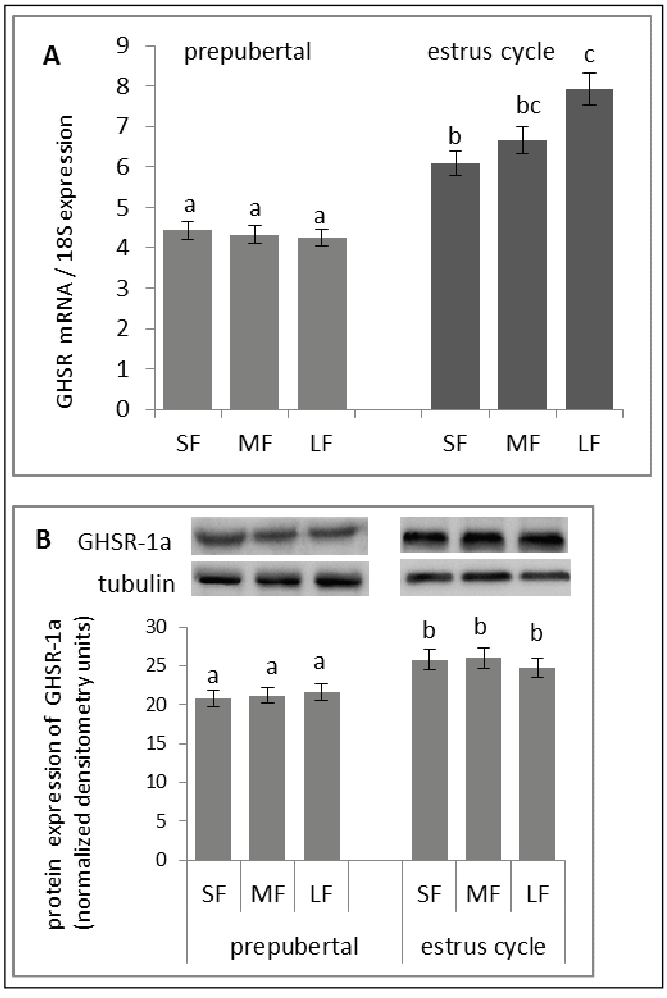

Fig. 1. Expression of ghrelin receptor in small (SF), medium (MF) and large (LF) ovarian follicles collected from ovaries of prepubertal and cycling animals; (*A*) mRNA of GHSR, (*B*) protein and semi-quantitative densitometry of Western blot analysis of GHSR-1a. All data are expressed as the mean ±S.E.M. Different letters indicate statistically significant differences among groups ($p<0.05$).

Ghrelin mRNA are expressed in the ovarian follicles collected from prepubertal and cycling animals (*Fig. 2*). The expression levels were consistent across all sizes of follicles in prepubertal, but significantly increase ghrelin mRNA expression was observed in cycling animals, with the maximum expression in large follicles ($p<0.05$, *Fig. 2A*). In both prepubertal and cycling ovarian follicles, the protein expression of ghrelin was significantly increased with increasing follicle size ($p<0.05$, *Fig. 2B*).

Concentrations of ghrelin in ovarian follicles from prepubertal and estrous cycling animals

In prepubertal animals the total concentration of ghrelin was higher in ovarian follicles (68.77, 71.01 and 95.19 pg/ml for SF, MF and LF, respectively) than it was in the follicular fluid (35.48, 42.14 and 46.02 pg/ml, *Fig. 3A*). The concentration of ghrelin in prepubertal ovarian follicles was correlated with increasing levels of estradiol (3.87, 7.22 and 9.58 pg/ml in SF, MF and LF, respectively; $p<0.05$; *Fig. 3B*).

The total concentration of ghrelin (both forms) in ovaries of cycling animals was higher in ovarian follicles than follicular fluid (ovarian follicles: 109.19, 130.8 and 148.36 pg/ml and follicular fluid: 46.47, 52.34 and 53.32 pg/ml in SF, MF, and LF, respectively; *Fig. 3C*). As was the case for prepubertal animals, ghrelin was correlated with levels of estradiol (4.73, 7.35 and 14.49 pg/ml, respectively in SF, MF and LF; *Fig. 3D*, $p<0.05$) in follicular fluid.

DISCUSSION

In the present study, we demonstrated that ovarian ghrelin mRNA expression significantly increased in cycling animals, without any change in prepubertal. However, protein expression of ghrelin and its concentration was increased with increasing follicle size both in ovaries of prepubertal and cycling animals. We demonstrated that both GHSR and GHSR-1a expression was significant higher in cycling animals than prepubertal. The presented study was agreement with observation by Barreiro *et al.* (12) who showed that the expression of the GHSR-1a receptor increases after puberty and reaches peak values in adulthood; this suggests that during pubertal development a shift in the pattern of splicing of the GHSR gene takes place in rat testis, thereby favoring the expression of the biologically active GHSR-1a, which might imply considerable changes in net ghrelin sensitivity with sexual maturation.

Puberty is characterized by increasing concentrations of estradiol, which are driven by increasing concentrations of pituitary gonadotrophins. Ghrelin by inhibiting GnRH and LH secretion in the prepubertal period (13, 14), could be involved in puberty. Our previously published data demonstrated a stimulatory action of ghrelin on estradiol secretion and aromatase protein expression with parallel antiapoptotic actions in prepubertal ovarian follicles, supported a possible local action of ghrelin just before puberty (9). Results of the present study show that levels of ghrelin in prepubertal ovarian follicles are correlated with estradiol concentration. Lebenthal *et al*. (15) demonstrated that increased

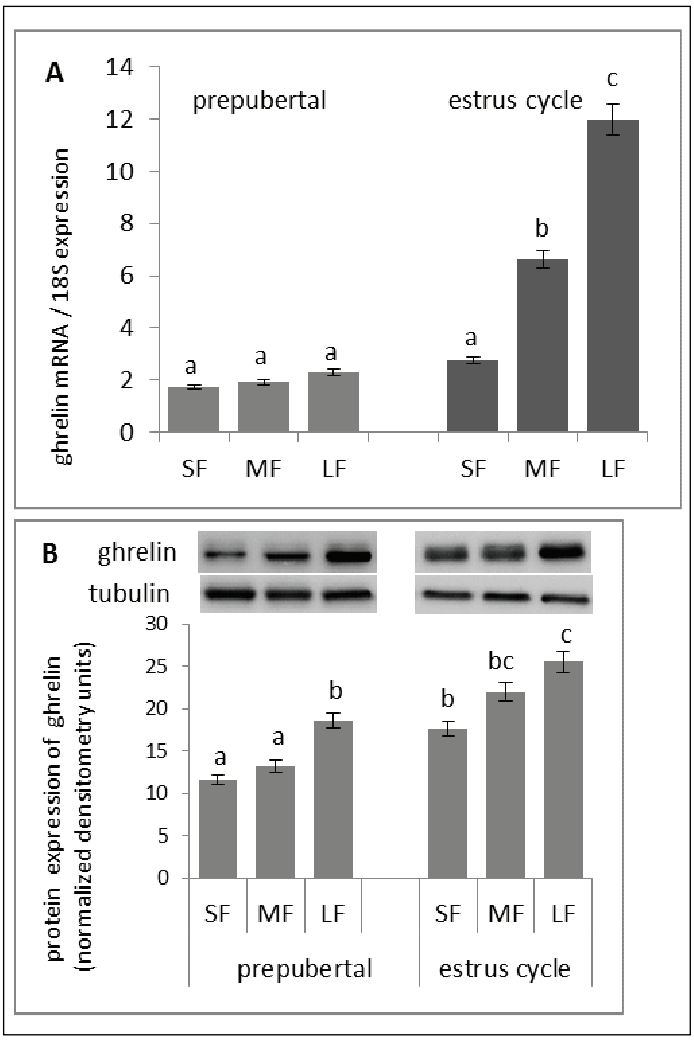

Fig. 2. Expression of ghrelin in small (SF), medium (MF) and large (LF) ovarian follicles collected from ovaries of prepubertal and cycling animals; (*A*) mRNA, (*B*) protein and semi-quantitative densitometry of Western blot analysis. All data are expressed as the mean ±S.E.M. Different letters indicate statistically significant differences among groups ($p<0.05$).

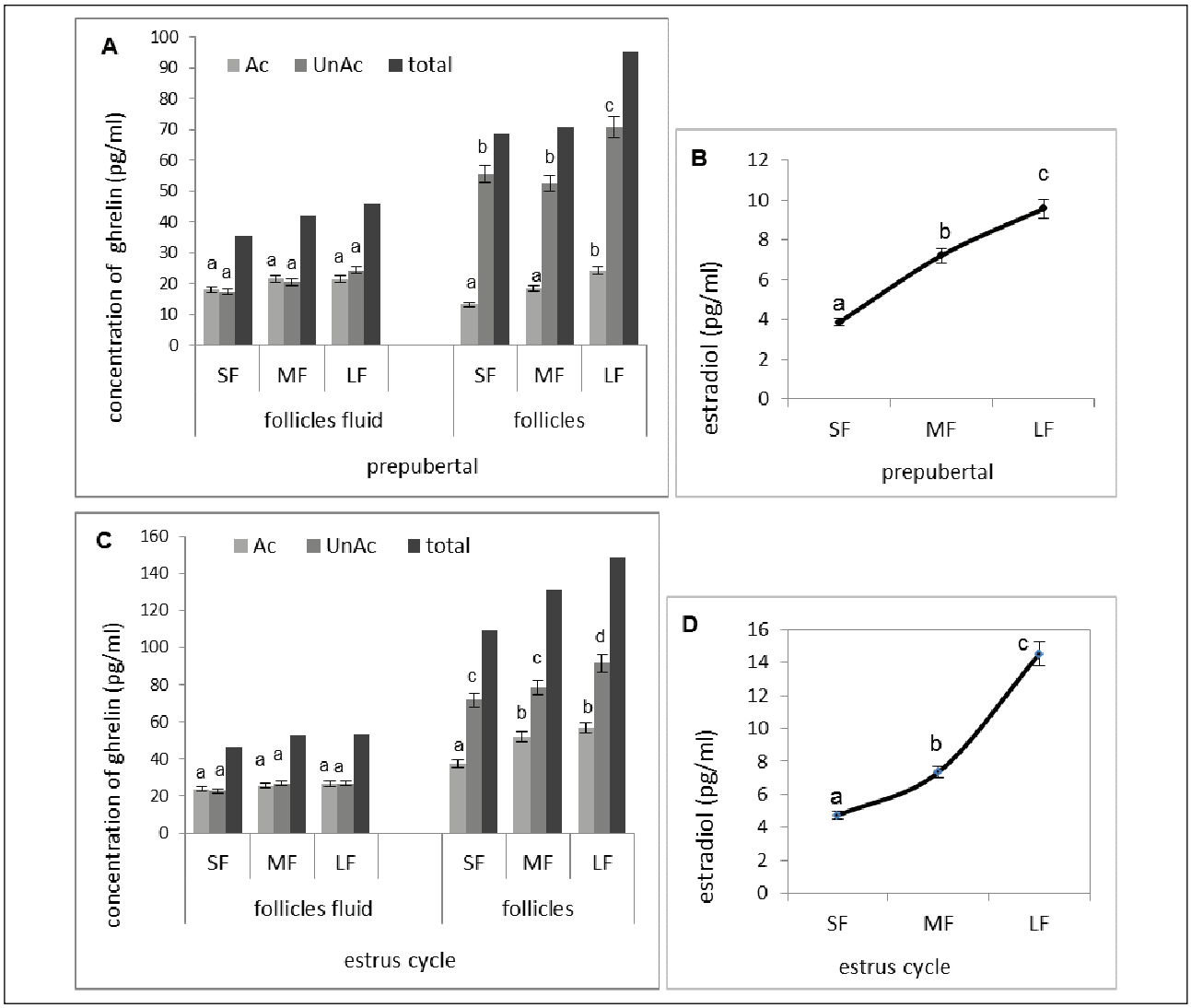

Fig. 3. Concentrations of (*A*) both Ac and UnAc of ghrelin in follicular fluid and in small (SF), medium (MF) and large (LF) ovarian follicles collected from prepubertal animals; (*B*) estradiol in follicular fluid from prepubertal animals; (*C*) both Ac and UnAc of ghrelin in follicular fluid and ovarian follicles collected from cycling animals; (*D*) estradiol in follicular fluid from cycling animals. All data are expressed as the mean ±S.E.M. Different letters indicate statistically significant differences among groups ($p<0.05$).

steroid hormone levels in prepubertal children led to a marked decline in circulating plasma levels of ghrelin in boys, but not girls. They suggested that estradiol can be positively correlated with ghrelin levels, which is in accordance with our suggestion.

In cycling animals, expression of ghrelin and concentrations of both, Ac and UnAc, isoforms of ghrelin increased with the size of ovarian follicles. UnAc ghrelin is the major circulating form and constitutes 80-90% of circulating total ghrelin. We suggest, as we did for the large follicles of prepubertal animals, that the increasing levels of ghrelin are a result of local production of ghrelin in the follicles. However, high concentrations of UnAc ghrelin could be also the result of the filtrate from plasma not only local synthesis. To our knowledge there are no data showing effect of UnAc ghrelin in the ovarian follicles function. The last paper by Romani *et al*. (16) showed that UnAc ghrelin significantly reduced progesterone and vascular endothelial growth factor (VEGF) release in cultured human luteal cells. Zhang *et al*. (6) showed that ghrelin mRNA expression depended on the stage of the porcine estrous cycle, with lowest expression in proestrous and maximum expression in estrous and diestrous. Similarly, ghrelin mRNA expression in the mouse ovary was dependent on the estrous cycle, with the lowest expression in proestrous and the maximum value in the diestrous phase (5). Expression of ghrelin and its receptor also increased in hypothalamus and pituitary during pregnancy (17). Moreover, ghrelin mediated circadian timing in the gastrointestinal tract (18).

In summary, this study provides evidence that expression and concentration of ghrelin increases in ovarian follicles from prepubertal pig and follicular development in cycling animals. Results of this study suggest of local synthesis of ghrelin in the ovarian follicles and points to important auto/paracrine modulation of ghrelin in puberty and during estrous cycle.

Acknowledgements: This work was supported by the DS/MND/WBiNoZ/IZ/6/2011.

Conflict of interests: None declared.

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Received: February 20, 2012
Accepted: April 6, 2012

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