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GHRELIN REGULATES SECRETION AND PROLIFERATION OF THE BOVINE OVIDUCT EPITHELIAL CELLS *IN VITRO*

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ABSTRACT

The aim of our **in vitro** experiments was to investigate the role of ghrelin (Ghr), a newly discovered metabolic hormone in control of proliferation (PCNA, cyclin B1), apoptosis (Bax, p53) and secretory activity (IGF-I and PGF 2α release) of bovine oviductal cells *in vitro*. The cells were isolated by flushing of the oviducts from slaughtered cows. The cells were pre-cultured in a basal medium (DME/F12 medium + 20% FCS + 1% antibiotic-antimycotic solution) to form a monolayer and then were incubated in presence of Ghr at doses of 0, 1, 10 or 100 ng/ml for 72 hours. Concentrations of insulin-like growth factor I (IGF-I) and prostaglandin F2α (PGF2α) in cell-conditioned medium were measured by IRMA/RIA kits. Indices of cell apoptosis (apoptotic peptides Bax and p53) or proliferation (the proliferation associated peptides PCNA and cyclin B1) in the bovine oviduct epithelial cells were analyzed by immunocytochemistry (ICC) and Western-blotting (WB). Obtained results show that Ghr stimulated the output of PGF 2α (at all tested doses; $p< 0.05$) and IGF-I (at 1 and 100 ng/ml; $p< 0.05$) by the oviductal cells. Ghr, given at all doses, increased the proportion of PCNA-positive cells (ICC, WB). However, the proportion of Bax-, cyclin B1- and p53- positive cells (ICC) was not changed significantly after addition of Ghr. These results clearly demonstrate that Ghr is a potent regulator of the secretory activity and proliferation in the oviduct epithelial cells. Elucidation of ghrelin role in apoptosis regulation within the oviduct requires further investigations.

Key words: oviduct, apoptosis, proliferation, IGF-I, postaglandin F2α

INTRODUCTION

The oviduct (fallopian tube) and oviductal fluid play an important role in reproduction, participating in spermatozoa capacitation and providing an optimal environment for the gamete maturation, fertilization and early embryonic development. Oviductal cells produce many substances which exert the effect on oviduct contraction, cell proliferation, apoptosis and embryo development (Sayegh and Mastroianni, 1991). The production of ovarian steroids (Wollenhaupt et al., 2002), prostaglandins PGF2α and PGE2 (Harper, 1998, Zerani et al., 2005), nitric oxid (NO) (Perez Martinez et al., 1997), insulin-like growth factor I (IGF-I) (Makarevich and Sirotkin, 1997) and cytokinins (Wijayagunawardane et al., 2003) by epithelial cells of the oviduct was proved.

Oviductal functions can be regulated by growth hormone GH (Makarevich and Sirotkin, 1997), IGF-I (Adashi et al., 1986), steroid hormones (Wollenhaupt et al., 2002), osteopontin (Gabler et al., 2003) and hyaluronan (Ulbrich et al., 2004 and Berqvist et al., 2005). One of the substances, which can potentially regulate oviductal functions, is a hormone – ghrelin (Kojima et al. 1999). Ghrelin was detected in many tissues including the stomach, hypothalamus, hypophysis, human and rodent placenta, in mouse oocyte and in embryo at morula and

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later embryonal stages (Barreiro and Tena-Sempere, 2004), as well as in the ovary and the oviduct (Gaytan et al., 2005). Therefore, ghrelin can play the major role in the control of reproductive functions (Barreiro and Tena-Sempere, 2004). Importance of ghrelin in control of oviductal functions and its influence on several processes in the oviduct including proliferation and apoptosis are still unclear.

The aim of this study was to examine effects of ghrelin on the secretory activity (IGF-I and prostaglandin F2α), apoptosis (Bax, p53) and proliferation (PCNA, cyclin B1) in bovine oviductal epithelium cells *in vitro*.

MATERIAL AND METHODS

Preparation and culture of oviductal epithelial cells

Periovulatory bovine oviducts were collected from Holstein cows (n = 20, 2-6 years of age) slaughtered at a local abattoir. Epithelial cells were aseptically isolated by flushing out the ampullar-isthmic region of the oviduct using plastic syringe with a saline. The cells were washed in a saline and sedimented by 10 min centrifugation at 1200g three times. After removal of the supernatant, oviductal cells were resuspended in a sterile incubation medium DMEM/F12 (Dulbecco´s modified Eagle´s medium- HAM´S F12, 1:1, BioWhittaker™, Verviers, Belgium) supplemented with 20% of fetal calf serum (BioWhittaker™) and 1 % antibiotic-antimycotic solution (Sigma- Aldrich Chemie Gmbh, Steinheim, Germany). This suspension (at concentration of 10^6/ml, which was determinant using Bürker chamber) was placed onto 24-well culture plates (Nunclon Surface, Nunc, Roskilde, Denmark) at 1 ml per well and on chamber-slides (Nalge Nunc International, NY, USA, Nunc) at 300 µl per well and cultured at 38.5°C and 5 % CO_2 in humidified air for 3 days until the confluent monolayer was formed. Thereafter, the medium was replaced with a fresh culture medium of the same composition supplemented with ghrelin (National Hormone & Peptide Program, Torrance, California, USA) at 0, 1, 10, 100 ng/ml medium and the cells were cultured under the same conditions for 3 days. After the culture, the medium from 24- well plates was gently aspirated, collected and stored at – 20°C to await RIA. The cells were lyzed in a lysis buffer (0.06260 M Tris-base, 2% SDS, 10% Glycerol, 5% 2- mercaptoethanol, 0.003% bromphenol blue, all from Sigma- Aldrich Chemie GmbH, Steinheim, Germany) and the cell lyzates were stored at -80°C to await Western- blotting. The cells from chamber-slides were washed 3 times in an ice-cold PBS, fixed in 4 % paraformaldehyde in PBS for 20 min and then in absolute ethanol at 4°C overnight (all from Applichem, GmbH, Darstadt, Germany) and subjected to immunocytochemical analysis.

Protein gel electrophoresis and Western- immunoblotting

The frozen cell lyzates were thawed and subjected to polyacrylamide gel electrophoresis (PAGE) in presence of SDS (sodium dodecyl sulfate) according to Laemmli (1970). The samples from the gel were transfered onto Porablot PVDF membranes (Macherey–Nagel, Düren, Germany) using a Mini Trans-blott® (Bio-Rad Labs, Richmond, CA, USA) for 1 hour at 0,8 mA/cm^2 of the membrane. Endogenous peroxidase in samples was quenched by incubation in 3 % H_2O_2 for 15 min. Non-specific binding of antiserum was blocked by incubation in 5 % blot-qualified BSA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in TTBS (20 nM Tris-base, 137 nM NaCl, 0.1% Tween-20, pH 7.5). Blocked membrane was probed with a mouse monoclonal antibody against PCNA (Santa Cruz Biotechnology, Inc; Santa Cruz, USA) for 1 hour. Afterwards the membrane was incubated with a secondary horseradish peroxidase-conjugated anti-mouse IgG (Sevac, Prague, Czech Republic) for 1 hour. Positive signals on the membrane were visualized using ECL plus Western Blotting Detection System (Amersham ™, Buckinghamshire, UK) and exposure to an ECL Hyperfilm (Amersham Biosciences UK Ltd., Buckinghamshire, UK). Molecular weights of fractions were evaluated using a molecular weight calibration kit PageRuller Protein Ladder (Fermentas GMBH, Slovakia).

Immunocytochemical analysis

The cells in chamber- slides were washed 3 times in ice-cold PBS, fixed in 4% paraformaldehyde in PBS for 1 hour and in absolute ethanol (all from Applichem, GmbH, Darmstadt, Germany) at 4 °C overnight and subjected to immunocytochemical analysis. Following washing and fixation the cells were incubated in a blocking solution (1% of goat serum in phosphate- buffered saline- PBS) at room temperature for 20 minutes to block a non- specific binding of antiserum. The presence of PCNA, cyclin B1, Bax and p53 was detected in the epithelial cells plated in chamber-slides using a mouse monoclonal antibody against PCNA, BAX and cyclin-B1 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution 1:500) and rabbit monoclonal antibody against p53 (Santa Cruz) for 1 hour. For the visualization of binding sites of primary antibody, a secondary polyclonal goat anti-rabbit immunoglobulins for p53 (DakoCytomation Denmark A/S Glostrup, Denmark, dilution 1:1000) and a secondary horseradish peroxidase-conjugated anti-mouse IgG (Sevac, Prague, Czech republic) for PCNA, Bax and cyclin B1 were used. The proportion of positive cells was determined by a light microscopy.

Radioimmunoassay

IGF-I and PGF 2α concentrations in the incubation medium were determined by RIA/IRMA, using commercial IRMA kit for IGF-I (DRG Instruments GmbH, Marburg, Germany) and RIA kit for PGF 2α (Institute of Isotopes Co., Ltd., Budapest, Hungary) according to instructions of the manufacturer. A radioactivity of samples was measured using Cobra II gamma-counter (Canberra-Packard, Meriden, USA).

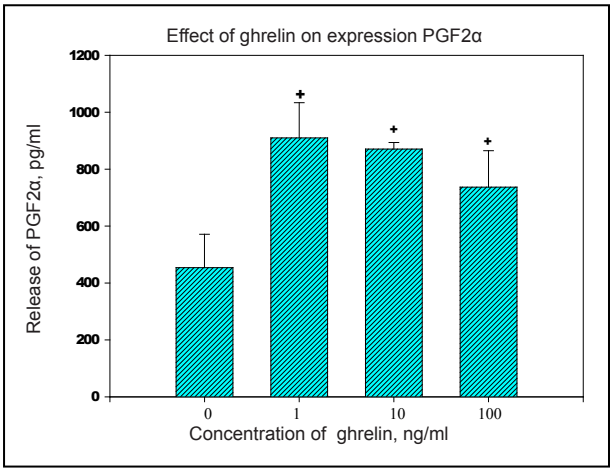
Statistics

Significant differences between the experiments were evaluated by paired- t test using Sigma Plot 9.0. statistic sotware (Systat Software, Inc., CA, USA). Differences from control at $p<0.05$ were considered as significant.

RESULTS

Isolated epithelial cells of the oviduct were able to survive in culture and to produce PCNA, cyclin B1, Bax and p53 and to secrete prostaglandin (PGF 2α) and IGF-I. Epithelial cells were attached to the bottom of culture wells and a confluent monolayer was formed.

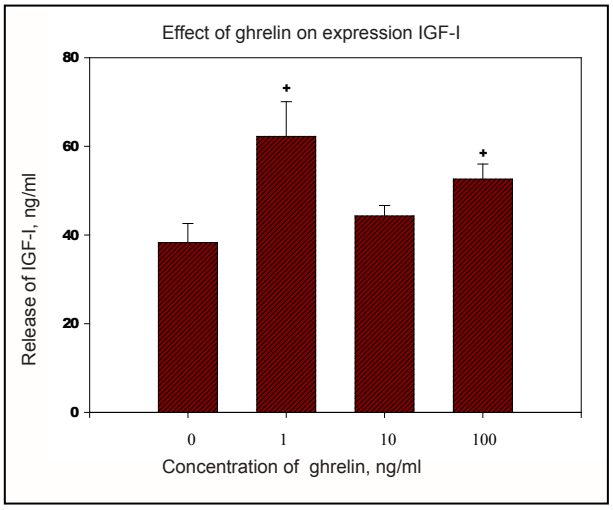
RIA analysis showed, that the addition of ghrelin into culture medium increased the release of prostaglandin F2α at all tested doses (1, 10, 100 ng/ml; Fig. 1).



+ significant (P<0.05) difference compared to control (serum-supplemented medium without additions)

Fig.1: Effect of ghrelin (0, 1, 10, 100 ng/ml) on the secretion of PGF 2a by cultured epithelial oviduct cells. Values are means ± S.E.M.

The oviductal cells produced IGF-I in detectable amounts. Ghrelin considerably stimulated secretion of IGF-I, particularly at concentrations of 1 ng/ml, but also at 100 ng/ml of ghrelin, though in a lesser extent (Fig. 2).



+ significant (P<0.05) difference compared to control (serum- supplemented medium without additions)

Fig. 2: Effect of ghrelin (0, 1, 10, 100 ng/ml) on the secretion of IGF-I by cultured epithelial oviduct cells. Values are means ± S.E.M.

Table 1: Results of imunocytochemical analysis of bovine epithelial cells of the oviduct

Ghrelin (ng/ml)	proliferative peptides		pro-apoptotic peptides	
	PCNA	Cyclin B1	BAX	P53
0	5.256±0.980 (2114)	7.991 ± 1.735 (2205)	3.929 ± 0.9482 (2065)	6.181 ± 2.233 (1959)
1	9.995±0.570* (2059)	8.124 ± 1.700 (2123)	5.185 ± 1.143 (1871)	5.086 ± 1.721 (1980)
10	11.085± 1.178* (2195)	8.124 ± 1.700 (2123)	3.487 ± 1.049 (1813)	8.,682 ± 1.604 (1931)
100	9.625 ± 0.657* (2038)	6.543 ± 0.930 (1074)	2.153 ± 1.076 (1839)	4.010 ± 1.477 (1936)

Values are mean (% of cells) ± S.E.M
Values in brackets: number of all analysed cells
* significant difference (P<0.05) compared to control (medium without ghrelin)

Percentages of PCNA-, cyclin B1-, BAX- and p53- positive cells after the addition of ghrelin are presented in table 1. Results of imunocytochemical analysis demonstrated that ghrelin had no effect on cyclin B1, BAX and p53 expression.

Addition of ghrelin to the incubation medium at concentrations of 1, 10 and 100 ng/ml induced a significant increase in proportion of cells containing PCNA immunoreactivity in bovine oviductal cells (Tab. 1). The highest stimulation of PCNA was noted at the concentration of 10 ng/ml. These results were confirmed by Western-blotting, where the intensity of 36 kDa fraction of PCNA was higher at concentration of 1, 10 and 100 ng/ml compared to control (Fig. 3).

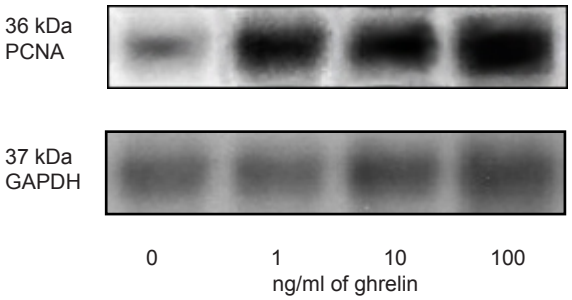


Fig. 3: PCNA identified in lyzates of epithelial cells of the oviduct after 3 days of culture in a serum-supplemented medium with ghrelin (0, 1, 10, 100 ng/ml) using SDS PAGE-Western-blotting. GAPDH - housekeeping protein as a control for protein content in samples. Molecular weights (kDa) of protein fractions are marked to the left of the gel lanes

DISCUSSION

The basic function of the oviduct is a transport of an ovulated oocyte to the uterus and intensive metabolism - secretion and resorption. The luminal epithelium of the oviduct is composed of ciliated cells and secretory cells, but it is assumed for several species that under the control of steroid hormones secretory cells are able to transform into ciliated cells. In the oviduct the cell diferentiation is dependent on the amount of steroid hormones and stages of the oestrous cycle (Steffl et al., 2004). These processes are coupled with proliferation and apoptosis. Relations between proliferation and apoptosis can be regulated by hormones, growth factors, cytokines and other biological active substancies. A study of proliferation and apoptosis in oviductal tissues can help to understand mechanism of possible effect of these substances on important functions of the oviduct.

In our study we investigated a regulatory effect of ghrelin on apoptosis, proliferation and secretory activity of bovine oviduct epithelium cells. We have observed the stimulatory effect of ghrelin on the secretion of IGF-I and prostaglandin F2α. Prostaglandins directly regulate contractions of the oviduct. In particular, PGF2α regulates the contraction of smooth muscles, while PGE_2 can relax this muscles in the oviduct (Wijayagunawardane et al., 1999). Prostaglandins play very important role in transport of oocyte and embryo throughout the oviduct. Concentration of prostaglandins and their receptors in the bovine oviduct is highest before ovulation. Production of prostaglandins was stimulated by some substances, including luteal hormone (Wijayagunawardane and Miyamoto, 2004).

The obtained data from RIA/IRMA in our experiments show, that dose-dependent elevated concentrations of ghrelin stimulated secretion of prostaglandin F2α in epithelial cells of the oviduct. This data may indirectly suggest on the role of ghrelin in oviductal contractions.

Insulin-like growth factors (IGF) have very important position among growth factors, because it can affect hormone level via systemic action and also locally by an autocrine or paracrine manner. IGF-I is known to stimulate the cell secretion, proliferation, maturation of oocytes and embryo development, the processes, which under physiological conditions take place in the oviduct (Buhi, 1996). We confirm a stimulatory effect of ghrelin on the expression of IGF-I. It is assumed that some effects of ghrelin can be mediated via IGF-I.

Proliferation of epithelial cells is accompanied by higher production of proliferation peptides, in particular PCNA, which is a marker of S- phase of the cell cycle (Mathews et al, 1984). PCNA is a proliferative antigen in the cell nucleus, which plays very important role in DNA repair and replication. In ovarian granulosa cells of rabbits ghrelin elevated the expression of PCNA (Sirotkin et al., 2009). Similarly in our experiments ghrelin also increased percentage of PCNA- positive cells in the bovine oviduct at all concentrations of ghrelin (1, 10, 100 ng/ml). Our results may suggest the participation of ghrelin in the regulation of the cell cycle. Since ghrelin at rising concentrations significantly elevated a percentage of PCNA-positive cells, we can assume, that ghrelin affects proliferation of the oviduct epithelium cells and can influence a microenvironment of the oviduct thus affecting processes running in the oocyte, spermatozoa and embryo.

CONCLUSION

In our experiments ghrelin affected proliferation and secretory activity of oviductal cells. Since pro-apoptotic markers were not affected by ghrelin in our study, elucidation of its role in apoptosis regulation within the oviduct requires further investigations. Studies involving effects of biological active substancies on elements of reproduction system can help to understand the mechanism of hormone action and provide a basis for the application of these findings in assisted reproduction.

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