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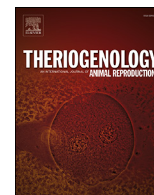

Bovine ovarian follicular growth and development correlate with lysophosphatidic acid expression

Emilia Sinderewicz, Katarzyna Grycmacher, Dorota Boruszewska, Ilona Kowalczyk-Zięba, Joanna Staszkiewicz, Tomasz Ślężak, Izabela Woclawek-Potocka *

Department of Gamete and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, 10-747 Olsztyn, Poland

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A B S T R A C T

The basis of successful reproduction is proper ovarian follicular growth and development. In addition to prostaglandins and vascular endothelial growth factor, a number of novel factors are suggested as important regulators of follicular growth and development: PGES, TFG, CD36, RABGAP1, DBI and BTC. This study focuses on examining the expression of these factors in granulosa and thecal cells that originate from different ovarian follicle types and their link with the expression of lysophosphatidic acid (LPA), known local regulator of reproductive functions in the cow.

Ovarian follicles were divided into healthy, transitional, and atretic categories. The mRNA expression levels for PGES, TFG, CD36, RABGAP1, DBI and BTC in granulosa and thecal cells in different follicle types were measured by real-time PCR. The correlations among expression of enzymes synthesizing LPA (autotaxin, phospholipase A2), receptors for LPA and examined factors were measured. Immunolocalization of PGES, TFG, CD36, RABGAP1, DBI and BTC was examined by immunohistochemistry.

We investigated follicle-type dependent mRNA expression of factors potentially involved in ovarian follicular growth and development, both in granulosa and thecal cells of bovine ovarian follicles. Strong correlations among receptors for LPA, enzymes synthesizing LPA, and the examined factors in healthy and transitional follicles were observed, with its strongest interconnection with TFG, DBI and RABGAP1 in granulosa cells, and TFG in thecal cells; whereas no correlations in atretic follicles were detected. A greater number of correlations were found in thecal cells than in granulosa cells as well as in healthy follicles than in transitional follicles. These data indicate the role of LPA in the growth, development and physiology of the bovine ovarian follicle.

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1. Introduction

The preovulatory follicle is considered to be the limiting ovarian unit in successful reproduction [1]. The main role of the preovulatory follicle is to provide appropriate conditions for oocyte growth and development. Based on the estradiol: progesterone (E2:P4) ratio, ovarian follicles can be divided into three groups: healthy, transitional and atretic [2]. The ovarian follicle includes two main types of cells, granulosa and thecal, which are responsible for nurturing growing oocytes until ovulation and releasing mature oocytes capable of producing an embryo. The proper differentiation of the thecal and granulosa cells is required for appropriate follicle development and the ovulation process. Among many factors regulating these processes in various species are prostaglandins (PGs) [3–5], vascular endothelial growth factor (VEGF) [6,7], and a number of factors with known functions that are important for follicular development and (or) ovulation: prostaglandin E synthase (PGES), TRK-fused gene (TFG), thrombospondin receptor (CD36), Rab GTPase activating protein 1 (RABGAP1), diazepam-binding inhibitor (DBI) and betacellulin (BTC) [8]. Prostaglandin E synthase is one of the enzymes that metabolize arachidonic acid and is crucial in the ovulation process in cattle [5]. TRK-fused gene was originally identified as part of an oncogenic fusion gene involved in promoting cumulus expansion in mice [9]. Thrombospondin receptor is a multifunctional receptor-binding autocrine

* Corresponding author.
E-mail addresses: e.sinderewicz@pan.olsztyn.pl (E. Sinderewicz), k.grycmacher@pan.olsztyn.pl (K. Grycmacher), d.boruszewska@pan.olsztyn.pl (D. Boruszewska), i.kowalczyk@pan.olsztyn.pl (I. Kowalczyk-Zięba), j.staszkiewicz@pan.olsztyn.pl (J. Staszkiewicz), t.slezak@pan.olsztyn.pl (T. Ślężak), i.woclawek-potocka@pan.olsztyn.pl (I. Woclawek-Potocka).

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growth factor that can regulate cell growth, adhesion, and angiogenesis [10]. Rab GTPase activating protein 1 is a novel GTPase that may have functions in the ovary. Present in the rat ovary, DBI [11] stimulates steroidogenesis and pregnenolone biosynthesis [12]. Betacellulin is one of the members of the EGF-like growth factor family, which are important mediators of luteinizing hormone (LH) activity [13]. Expression of the abovementioned factors in different types of bovine ovarian follicles and their potential role in the folliculogenesis and ovulation have not been investigated previously.

Among local factors regulating female reproductive functions is lysophosphatidic acid (LPA). Lysophosphatidic acid is a phospholipid that promotes biological actions such as cell proliferation and differentiation [14] and cell-to-cell interactions [15]. In mammals, LPA induces its activity via the most well-known and well-studied high affinity G-protein-coupled receptor (GPCR) types: LPAR1/EDG2, LPAR2/EDG4, LPAR3/EDG7 and LPAR4/P2Y9 [16–18]. LPA is produced by two major enzymes, phospholipase A2 (PLA2) and autotaxin (AX) [19,20]. The role of LPA in reproductive systems has been studied in mice, sows, ewes and cows [3,5,21,22]. In the bovine reproductive tract, LPA is synthesized in the endometrium [23–26], oviduct [27], corpus luteum (CL) [28], and embryo [29]. Moreover, recent studies have demonstrated the follicle-type dependence of LPA in components of the bovine ovarian follicle - thecal [30] and granulosa cells [31].

The goal of this study was to investigate the expression of factors that are potentially involved in growth and development of the ovarian follicle and in ovulation in the thecal and granulosa cells that originate from different ovarian follicle types. We also examined the possible link between the abovementioned factors, LPA synthesis and the expression of LPA receptors in three different types of ovarian follicles.

2. Material & methods

2.1. Ovarian collection

All experimental procedures were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (Agreement No. 34/2012/N). Ovaries, irrespective of the stage of the estrous cycle, were collected from mature cows at a local abattoir and transported to the laboratory within 40 min in sterile phosphate buffered saline (PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2KPO4, 1.8 mM KH2PO4, pH = 7.4), supplemented with 0.4% gentamycin (Sigma; #G-1397)).

2.2. Collection of the experimental material

In the follicular fluid aspirated from antral ovarian follicles (diameter < 5 mm), the levels of E2 and P4 were measured by the RIA method (the DIAsource E2–RIA–CT Kit, KIP0629, DIAsource and the DIAsource PROG–RIA–CT Kit, KIP1458, DIAsource, respectively). Based on the intrafollicular E2:P4 ratio (according to Ireland and Ireland 1994), ovarian follicles were divided into three groups: healthy (E2:P4 > 1), transitional (0.01 < E2:P4 < 1) and atretic (E2:P4 < 0.01). After collection of the follicular fluid, the antral cavity of each follicle was flushed with cold PBS to recover granulosa cells. The theca layer was removed with forceps and washed in PBS by passing repeatedly through a 1-ml syringe. Thecal and granulosa cells obtained from single follicles categorized as healthy (n = 16), transitional (n = 16) or atretic (n = 16) were used for this study. The samples were collected in RNAlater (Sigma, R-0901) and stored at −80 °C until RNA extraction.

2.3. Total RNA extraction, reverse transcription (RT) and real-time PCR

The theca layer was homogenized before RNA extraction. Total RNA was extracted according to the Total RNA Mini isolation kit protocol (A&A Biotechnology, # 031–100). The RNA samples were stored at −80 °C. Before use, RNA content and quality were evaluated by spectrophotometric measurement. The amount of 500 ng of each sample of total RNA was reverse transcribed (according to First Strand cDNA Synthesis Kit for RT-qPCR protocol, Thermo Scientific, #K1642). The expression of mRNA for enzymes responsible for LPA synthesis (phospholipase A2–PLA2 and autotaxin–AX), the receptors for LPA (LPAR1, LPAR2, LPAR3 and LPAR4) and factors of potential significance to the ovulatory process and preovulatory follicle development (PGES, DBI, CD36, TFG, RABGAP1, and BTC) were measured by real-time PCR using specific TaqMan probes.

Real-time PCR was performed with an ABI Prism 7900 (Applied Biosystems, Life Technologies, USA) sequence detection system using the Maxima Probe/ROX qPCR Master Mix (#K0231, Thermo Scientific, USA). The PCR reactions were performed in 384-well plates. The results of mRNA transcription were normalized to beta-actin (ACTB, an internal control) mRNA levels and were expressed as arbitrary units. The housekeeping gene was chosen using the NormFinder software by comparing the following two candidate genes: GAPDH and ACTB [32]. The primers were designed using an online software package (http://frodo.wi.mit.edu/primer3/input.htm). The primer sequences and the sizes of the amplified fragments of all of the transcripts are shown in Table 1. For the relative quantification of mRNA levels, Miner software was used (http://miner.ewindup.info).

2.4. Immunohistochemistry

To immunolocalize factors involved in the ovulation process and follicular development in the bovine ovarian follicle, ovaries with antral follicles were fixed in buffered 4% formaldehyde for immunohistochemistry (according to [33]). The proteins of interest were stained with the use of goat polyclonal antibodies: anti-BTC, anti-DBI, anti-CD36 (dilution for all antibodies 1:100, Santa Cruz Biotechnology, #sc-5800, #sc-23474, #sc-5522, respectively), anti-PGES (dilution 1:50, Santa Cruz Biotechnology, #sc-32589), anti-TFG (dilution 1:150, Abcam, #ab72126) and anti-RABGAP1 (dilution 1:200, Abcam, #ab153992). Negative control sections were incubated with goat irrelevant IgG (concentration 1:100, Santa Cruz Biotechnology, #sc-2028).

2.5. Statistical analysis

Statistical analyses were conducted using GraphPad PRISM v. 6.0 software (GraphPad Software, Inc.). All experimental data are shown as the mean ± SEM, and the differences were considered significantly different at the 95% confidence level ($P < 0.05$). The analyses were performed using one-way ANOVA followed by the Kruskal-Wallis multiple comparison test (Figs. 1 and 2) or correlation analysis followed by Pearson's test (Table 2, Figs. 4–7).

3. Results

3.1. The expression of PGES, BTC, DBI, CD36, RABGAP1 and TFG in the granulosa cells of healthy, transitional and atretic ovarian follicles

The mRNA abundances of all the examined factors was detected in granulosa cells of healthy, transitional and atretic ovarian follicles (Fig. 1). The greatest mRNA expression levels were measured

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