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G protein-coupled receptor 34 in ovarian granulosa cells of cattle: changes during follicular development and potential functional implications

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ABSTRACT

Abundance of *G protein-coupled receptor 34* (*GPR34*) mRNA is greater in granulosa cells (GCs) of cystic vs normal follicles of cattle. The present experiments were designed to determine if *GPR34* mRNA in granulosa cell [GC] changes during selection and growth of dominant follicles in cattle as well as to investigate the hormonal regulation of *GPR34* mRNA in bovine GC in vitro. In Exp. 1, estrous cycles of nonlactating cows were synchronized and then ovariectomized on either day 3–4 or 5–6 after ovulation. *GPR34* mRNA abundance in GC was 2.8- to 3.8-fold greater ($P < 0.05$) in small (1–5 mm) and large (≥8 mm) estrogen-inactive dominant follicles than in large estrogen-active follicles. Also, *GPR34* mRNA tended to be greater ($P < 0.10$) in F2 than F1 follicles on day 3–4 postovulation. In Exp. 2–7, ovaries were collected at an abattoir and GC were isolated and treated in vitro. Expression of *GPR34* was increased ($P < 0.05$) 2.2-fold by IGF1. Tumor necrosis factor (TNF)-α decreased ($P < 0.05$) the IGF1-induced *GPR34* mRNA abundance in small-follicle GC, whereas IGF1 decreased ($P < 0.05$) *GPR34* expression by 45% in large-follicle GC. Treatment of small-follicle GC with either IL-2, prostaglandin E2 or angiogenin decreased ($P < 0.05$) *GPR34* expression, whereas FSH, cortisol, wingless 3A, or hedgehog proteins did not affect ($P > 0.10$) *GPR34* expression. In Exp. 6 and 7, 2 presumed ligands of GPR34, L-a-lysophosphatidylserine (LPPS) and LPP-ethanolamine, increased ($P < 0.05$) GC numbers and estradiol production by 2-fold or more in small-follicle GC, and this response was only observed in IGF1-treated GC. In conclusion, *GPR34* is a developmentally and hormonally regulated gene in GC, and its presumed ligands enhance IGF1-induced proliferation and steroidogenesis of bovine GC.

1. Introduction

Among transmembrane proteins, G protein-coupled receptors (GPRs) constitute one of the largest gene families whose primary function is to transmit extracellular stimuli such as light, hormones, neurotransmitters, peptides, and nucleotides into intracellular signals via interaction of their intracellular domains with heterotrimeric G proteins [1–3]. There are 5 structurally different GPR families of receptors including glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin [1,4,5]. The largest family of rhodopsin-like receptors (class 1 GPRs) is evolutionarily highly conserved [6–8] and includes the $P2Y_{12}$-like receptors (eg, ADP receptors $P2Y_{12}$), the UDP-glucose receptor $P2Y_{14}$, and several orphan receptors [1,4,9]. *GPR34*, an orphan receptor of the $P2Y_{12}$-like receptor group [4,8,10], was confirmed as a lysophosphatidylserine

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receptor in 2012 [11,12]. Originally, *GPR34* was isolated from a human fetal brain cDNA library in 1999 [7].

GPR34 shows a wide-spread expression pattern in murine and human tissues including the ovary [8]. A recent study in cattle discovered that there is an increased abundance of *GPR34* mRNA in granulosa cells (GCs) of cystic ovarian follicles as opposed to normal dominant follicles [13], and an increase in *GPR34* mRNA in bovine granulosa cell [GC] after IGF1 treatment [13] but whether *GPR34* is involved in normal ovarian function is unknown. Understanding how *GPR34* mRNA abundance changes during normal follicular growth may help us understand how it may be involved in formation of cystic follicles. Although homozygous *GPR34*-deficient mice are vital and exhibit normal fertility, they exhibit an inadequate activation of their immune system after antigen and pathogen contact [4]. In particular, *GPR34* was found to be involved in maintaining osmotic homeostasis of glial cells [4], to exhibit greater concentrations of cytokines (ie, TNFα and IL-2) in spleen cells than wild-type (WT) control mice [4], and may play a role in tumor cell growth [14]. We hypothesized that GC *GPR34* gene expression is controlled by reproductive hormones such as estradiol (E_2), FSH, and IGF1, and that GC *GPR34* gene expression changes during normal follicular growth. Because infected *GPR34*-deficient mice exhibit greater TNFα and IL-2 concentrations [4], and because both of these cytokines play a role in regulating bovine follicular steroidogenesis [15–18], we also evaluated the effect of cytokines on *GPR34* expression in GC. Determining the developmental and hormonal regulation of *GPR34* mRNA expression in bovine GC may help us understand how cystic follicles acquire increased *GPR34* mRNA and provide new ways to treat cystic follicles in cattle.

2. Materials and methods

2.1. In vivo exp. 1 design

Nonlactating Holstein cows (n = 16) were used for this experiment as previously described [19]. Briefly, follicle development was monitored daily via ultrasonography using an Aloka 500V with a 7.5-MHz probe. After ovulation, cows were assigned to be ovariectomized either at day 3–4 (early growing phase of the first dominant follicle; n = 8 cows) or day 5–6 (late growing phase of the first dominant follicle; n = 8 cows). Ultrasound examinations were performed daily, continuing until assigned day of ovariectomy. Both ovaries from each cow were removed via lateral incision through the left paralumbar fossa area after local anesthesia (2% lidocaine; 60 to 80 mL s.c. and i.m.). After each ovariectomy, ovaries were identified as right and left, put on ice, and transported to the laboratory where diameters of all follicles ≥5 mm were recorded, and ovarian tissue and fluid collected. The animal experimentation described in this report was approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Follicular fluid from follicles >5 mm in diameter (classified as dominant or large) was aspirated individually and centrifuged to obtain GC, and to obtain sufficient quantities of RNA, follicular fluid from follicles 1–5 mm in surface diameter (small) was pooled within ovaries and then centrifuged to obtain GC as previously described [19–21]. For large follicles, diameters were measured using calipers after dissection from the ovarian stroma. After centrifugation, follicular fluid was aspirated and stored at −20°C, each dissected large follicle was bisected, and the inner wall was scraped, rinsed with Ham's F-12 to remove any remaining GC which were combined with GC collected from follicular fluid. The GC collected from small follicles were pooled and kept separate for each ovary. Cells were lysed in 0.5 mL of TRI Reagent and stored frozen at −80°C until RNA extraction (see below).

2.2. In vitro exp. 2 to 7 designs

The hormones and reagents used in cell culture were: ovine FSH (NIDDK-oFSH-20; activity: 175 X NIH-FSH-S1 U/mg) from the National Hormone and Pituitary Program (Torrance, CA, USA); recombinant human IGF1, angiogenin (ANG), wingless-type mouse mammary tumor virus integration site family member 3A (WNT3A), Sonic hedgehog (SHH), IL-2, IL-6, and recombinant bovine TNFα from R&D Systems (Minneapolis, MN, USA); testosterone from Steraloids (Wilton NH, USA); cortisol and prostaglandin E2 (PGE2) from Sigma-Aldrich Corp. (St. Louis, MO, USA); fetal calf serum (FCS) from Equitech-Bio, Inc (Kerrville, TX, USA); and L-α-lysophosphatidylserine (LPPS) and L-α-lysophosphatidylethanolamine (LPPE) from Avanti Polar Lipids, Inc (Alabaster, AL, USA).

Ovaries from nonpregnant beef heifers were collected from a local slaughterhouse, and based on surface diameter, GC were collected from small (1–5 mm; SMGC) and large (8–22 mm; LGGC) follicles as previously described [20,22]. For SMGC experiments, each pool of cells was derived from cells collected and pooled from at least 10 animals. For LGGC experiments, each pool of cells was derived from cells collected and pooled from at least five animals. The LGGC that were collected from large follicles appeared healthy having good vascularity and moderately transparent follicular fluid. This size classification was based on previous observations indicating that: (1) follicles ≥8 mm in diameter have much greater androstenedione and E_2 concentrations than small follicles and have GC that are more differentiated (ie, greater amount of LH receptors) [21,23,24], (2) selection of dominant follicles occurs at about 8 mm in diameter [25], (3) follicles that are destined to ovulate average 10 ± 2 mm surface diameter [26], and (4) similar classifications have been used previously to inventory follicles during bovine estrous cycles [27,28]. The GC were re-suspended in serum-free medium (1:1 DMEM and Ham's F-12 containing 0.12-mM gentamycin, 2.0-mM glutamine, 38.5-mM sodium bicarbonate, 1.25-mg/mL collagenase, and 0.5 DNase; Sigma-Aldrich Corp.) before plating as previously described [29,30]. Viability of bovine GC from small and large follicles were determined by trypan blue exclusion method using a hemocytometer (American Optical Corporation, Buffalo, NY, USA) and averaged 74% and 50%, respectively, which are within the range of viabilities previously reported for these cells types collected from slaughterhouse tissues [29,30]. Viable cells (2.0×10^5 in 20–110 μL of medium) were plated on 24-well