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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 (GC) of cystic vs. normal follicles of cattle. The present experiments were designed to determine if *GPR34* mRNA in 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 non-lactating 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 post-ovulation. 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, two presumed ligands of GPR34, L- α -lysophosphatidylserine (LPPS) and LPP-ethanolamine, increased ($P < 0.05$) GC numbers and estradiol production by twofold 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.

Keywords

G protein coupled receptor; granulosa cell; follicular development; cattle

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1. Introduction

Among transmembrane proteins, G protein-coupled receptors (GPR) 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 five 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 P2Y₁₂-like receptors (e.g., ADP receptors P2Y₁₂), the UDP-glucose receptor P2Y₁₄ and several orphan receptors [1,4,9]. *GPR34*, an orphan receptor of the P2Y₁₂-like receptor group [4,8,10], was confirmed as a lysophosphatidylserine 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 (GC) of cystic ovarian follicles as opposed to normal dominant follicles [13], and an increase in *GPR34* mRNA in bovine 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 (i.e., TNF α 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 (E₂), FSH and IGF1, and that GC *GPR34* gene expression changes during normal follicular growth. Because infected *GPR34* deficient mice exhibit greater TNF α 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

Non-lactating 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. Following 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 in order to obtain sufficient quantities of RNA, follicular fluid from follicles 1 to 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 TNF α from R&D Systems (Minneapolis, MN, USA); testosterone from Steraloids (Wilton NH); cortisol and prostaglandin E₂ (PGE₂) from Sigma-Aldrich Corp. (St. Louis, MO); fetal calf serum (FCS) from Equitech-Bio, Inc. (Kerrville, TX); and L- α -lysophosphatidylserine (LPPS) and L- α -lysophosphatidylethanolamine (LPPE) from Avanti Polar Lipids, Inc. (Alabaster, AL).

Ovaries from non-pregnant beef heifers were collected from a local slaughterhouse, and based on surface diameter, GC were collected from small (1 to 5 mm; SMGC) and large (8 to 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 E₂ concentrations than small follicles and have GC that are more differentiated (i.e., 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.) prior to 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), 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 to 110 μ l of medium) were plated on 24-well Falcon multiwell plates (Becton Dickinson, Lincoln Park, NJ, USA) in 1.0 ml of medium containing 10% FCS (v/v). Cells were cultured at 38.5 °C in 10% FCS (v/v) for the first 48 h with a medium change at 24 h. Cells were then washed twice with serum-free medium (0.5 ml) and the various treatments (see below) were applied in serum-free medium for either 24 h or 48 h with a medium change at 24 h. At the end of treatment, medium was collected and either cells counted or TRI Reagent (0.5 ml) was added to the wells for collection of cellular RNA (see below).

Exp. 2 was designed to test the effects of TNF α , IGF1, and TNF α plus IGF1 on *GPR34* mRNA expression in SMGC and LGGC. Cells were cultured as described above and treatments applied for 24 h. Treatments were as follows: Control (no additions), IGF1 (30 ng/ml), TNF α (30 ng/ml), and TNF α plus IGF1. Because a previous study reported an increase in *GPR34* mRNA in bovine GC after IGF1 treatment [13], IGF1 was evaluated, and TNF α was evaluated because infected *GPR34*-deficient mice exhibited greater concentrations of TNF α in spleen cells than WT control mice [4]. Doses of IGF1 and TNF α were selected based on previous studies showing that these doses significantly alter steroidogenesis [17,18,31,32]. After 24 h of treatment, cells were lysed in TRI Reagent for RNA extraction.

Exp. 3 was designed to test the effects of IGF1, IL-2 and IL-6 on *GPR34* mRNA expression in SMGC. Cells were cultured as described in Exp. 2 except that cells were not treated with FSH. Treatments were as follows: Control (no additions), IGF1 (30 ng/ml), IL-2 (30 ng/ml) plus IGF1, or IL-6 (30 ng/ml) plus IGF1. The cytokines IL-2 and IL-6 were evaluated because infected *GPR34*-deficient mice exhibited greater concentrations of IL-2 but not IL-6 in spleen cells than WT control mice [4], and both IL-2 and IL-6 have been shown to inhibit bovine GC and theca cell steroidogenesis [16–18]. Doses of IGF1, IL-2 and IL-6 were selected based on previous studies showing that these doses significantly alter bovine GC steroidogenesis [15–18,33,34]. After 24 h of treatment, cells were lysed in TRI Reagent for RNA extraction.

Exp. 4 was designed to test the effects of cortisol, PGE2, SHH, WNT3A, and/or ANG on *GPR34* mRNA expression in SMGC. These intrafollicular factors were tested because of their recent implication in cystic follicle development [13] and for ovarian IGF1 stimulation [20,35]. In particular, we found that gene expression of the PGE2 receptor, ANG, Indian hedgehog and a WNT3A binding protein (i.e., secreted frizzled-related protein 4) was elevated in GC of cystic follicles [13]. The SMGC were cultured as described above with treatments applied for 24 h as follows (all treatments included 30 ng/ml of IGF1 but no FSH): Control, cortisol (300 ng/ml), PGE2 (300 ng/ml), SHH (500 ng/ml), WNT3A (300 ng/ml), and ANG (300 ng/ml). Doses of cortisol, IGF1, SHH, PGE2, ANG, and WNT3A were selected based on previous studies showing that these doses significantly alter GC function [20,31,32,36,37]. After 24 h of treatment, medium was aspirated and cells were lysed in TRI Reagent for RNA extraction.

Exp. 5 was designed to test the effect FSH on *GPR34* mRNA expression in SMGC and LGGC. Cells were cultured as described above and treatments applied for 24 h. Treatments were as follows: Control (no additions) or FSH (30 ng/ml). Dose of FSH was selected based on previous studies showing that this doses significantly alters steroidogenesis [17,18,31,32]. After 24 h of treatment, cells were lysed in TRI Reagent for RNA extraction.

Exp. 6 was designed to evaluate the functional role of GPR34 by comparing the effect of two lysophospholipids that are presumed ligands of GPR34 [11,12] on IGF1-induced steroid production and cell numbers in SMGC. Cells were cultured as described in Exp. 2 with four treatments applied for 48 h with a medium change at 24 h as follows (all treatments included 500 ng/ml of testosterone as an estrogen precursor and 30 ng/ml of FSH): 0, 0.3, 1.0 or 3.0 μ g/ml of either LPPS or LPPE in the presence of IGF1 (30 ng/ml). Doses of the lysophospholipids and IGF1 were based on previous studies [34,38], and FSH was added to all treatments because IGF1 alone has little or no effect on steroid production [29,34]. After the second 24-h treatment period, medium was collected for steroid RIA and cells were counted.

Exp. 7 was designed to evaluate if IGF1 treatment alters the effect of LPPS or LPPE on steroid production and cell numbers in SMGC. Cells were cultured as described in Exp. 2 with six treatments applied for 48 h with a medium change at 24 h as follows (all treatments included 500 ng/ml of testosterone as an estrogen precursor and 30 ng/ml of FSH): 0, 0.3 or 1.0 μ g/ml of either LPPS or LPPE in the absence or presence of IGF1 (30 ng/ml). The two lower doses of the lysophospholipids used in Exp. 5 were selected because it was reasoned that an increase in receptors would not only enhance the response to lower doses but also reveal ligand preference. After the second 24-h treatment period, medium was collected for steroid RIA and cells were counted.

2.3 Radioimmunoassays (RIA) and Cell Counting

The progesterone (P4) RIA were conducted as previously described [29,33]. Intra-assay coefficient of variation (CV) averaged $7.7 \pm 1.5\%$. The E₂ RIA were conducted as previously described [29,33]. Intra-assay CV averaged $7.4 \pm 1.4\%$. For each set of replicated experiments and steroid analysis, all samples were run in a single RIA. To determine cell numbers, culture medium was removed from wells, cells were washed, trypsinized and counted on a Coulter counter (Z2 Coulter® Particle Count and Size Analyzer; Beckman Coulter, Hialeah, FL) as previously described [29,30].

2.4 RNA extraction and quantitative RT-PCR

RNA was extracted using TRI-Reagent (Life Technologies, Gaithersburg, MD) according to manufacturer's instructions. The concentration of mRNA was determined using a NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE) as previously described [22,39]. The abundance of *GPR34* mRNA was quantified in duplicate using fluorescent quantitative single-step RT-PCR using an ABI Prism® 7500 sequence detection system (Applied Biosystems, Foster City, CA). The housekeeping gene used in this study was *18S* rRNA (Applied Biosystems) as previously described [39,40], and run in duplicate for all samples. The *GPR34* primers and probes were designed using Primer Express software (Applied

Biosystems) and the sequences have been published [13]. Target gene dual labeled probes (FAM-TAMRA) and the *18S* probe (VIC) for TaqMan polymerase were obtained from Applied Biosystems. Furthermore, RT-PCR products were run on agarose gels to verify the length and size of the expected target gene, and the same RT-PCR cDNA samples were sequence to verify the amplified product. The data analysis was conducted using the comparative threshold cycle (Ct) method as previously described [29,39,40]. Briefly, the Ct was determined by subtracting the *18S* Ct value from the *GPR34* unknown value. Within each experiment, the Ct was determined by subtracting the higher Ct (the least expressed unknown) from all other Ct values. Fold changes in *GPR34* mRNA abundance were calculated as being equal to 2^{-Ct} . The CV for the Ct of *GPR34* and *18S* mRNA duplicates averaged $3.3 \pm 0.8\%$ and $1.6 \pm 0.5\%$, respectively.

2.5 Statistical analyses

The analysis of the *in vivo* Exp. 1 data aimed to determine if *GPR34* mRNA abundance changes in GC during different periods of follicular development and to detect its relationship with steroidogenesis. Data were analyzed via factorial ANOVA with MIXED procedures of SAS for Windows (version 9.2, SAS Institute Inc., Cary, NC) and are presented as means ($\pm$ SEM) of measurements. Main factors in this split unit model were days post-ovulation (main unit factor; early, days 3-4, and late, days 5-6, growing phase of the first dominant follicle), follicle group (split unit factor; $n = 3$) based on follicle size (small or large) and their follicle estrogenic status (E_2 active: $E_2 > P_4$ concentrations or E_2 inactive: $E_2 < P_4$ concentrations), and their interactions. Random effect of cow nested in day was used as the error term for day effect, and follicle group by cow nested in day was used as the error term for follicle group effect and the follicle group by day interaction. Data from *in vitro* experiments are presented as the least squares means ($\pm$ SE) of measurements from three or more replicated experiments. Specific differences in variables measured (i.e., relative fold mRNA abundance, steroid production, and cell numbers) among treatments were determined via ANOVA using GLM procedure using SAS for Windows (version 9.2, SAS Institute Inc.) and Fisher's protected least significant difference procedure [41]. Data were tested for homogeneity of variance using Hartley's F max test [41]. To correct for heterogeneity of variance (when necessary), data were analyzed after transformation to natural $\ln(x + 1)$.

3. Results

3.1 Exp. 1: In vivo changes in GPR34 mRNA in GC during follicle growth

No significant ($P > 0.10$) day effect or interaction existed between follicle group and day, but a significant follicle-group effect was observed. Specifically, abundance of *GPR34* mRNA in GC was 2.8- to 3.8-fold greater ($P < 0.05$) in large and small E_2 -inactive (follicular fluid $E_2 < P_4$) follicles than in large E_2 -active (follicular fluid $E_2 > P_4$) follicles (Fig. 1A). When only F1 and F2 follicles were evaluated, rank but not day or their interaction tended ($P = 0.10$) to affect abundance of *GPR34* mRNA such that *GPR34* mRNA abundance tended to be greater ($P = 0.10$) in F2 than F1 follicles on day 3-4 post-ovulation but not on day 5-6 post-ovulation (Fig. 1B). Statistical analysis of the *18S* Ct (average = 14.8 ± 0.2) revealed no significant effect of day, follicle group or their interaction.

3.2 Exp. 2: Effects of TNF α and IGF1 on GPR34 mRNA in SMGC and LGGC

In SMGC, IGF1 treatment increased ($P < 0.05$) basal *GPR34* mRNA abundance by 2.2-fold, whereas TNF α had no effect ($P > 0.05$) on *GPR34* mRNA abundance (Fig. 2A). However, TNF α attenuated ($P < 0.05$) the stimulatory effect of IGF1 on *GPR34* mRNA abundance in SMGC (Fig. 2A). In LGGC, both TNF α and IGF1 treatment alone decreased ($P < 0.05$) basal *GPR34* mRNA abundance by 47% and 45%, respectively (Fig. 2B). Furthermore, a combined treatment with TNF α and IGF1 caused a further significant (76%) decrease in abundance of *GPR34* mRNA in LGGC (Fig. 2B). Statistical analysis of the *18S* Ct (average = 10.1 ± 0.1) revealed no significant effect of treatment.

3.3. Exp. 3: Effects of IGF1, IL-2 and IL-6 on GPR34 mRNA in SMGC and LGGC

In SMGC, similar to Exp. 2, IGF1 treatment increased ($P < 0.05$) basal *GPR34* mRNA abundance by 2.1-fold, whereas IL-2 completely blocked and IL-6 only slightly attenuated the stimulatory effect of IGF1 on *GPR34* mRNA abundance (Fig. 3A). In LGGC, IGF1 treatment decreased ($P < 0.05$) basal *GPR34* mRNA abundance by 60%, whereas IL-2 and IL-6 had no significant effect on this inhibition (Fig. 3B). Statistical analysis of the *18S* Ct (average = 16.3 ± 0.4) revealed no significant effect of treatment.

3.4. Exp. 4: Effect of cortisol, PGE2, SHH, WNT3A, and ANG on GPR34 mRNA in SMGC

In SMGC, cortisol, SHH and WNT3A did not affect ($P > 0.10$) *GPR34* mRNA abundance, however, PGE2 and ANG treatment decreased ($P < 0.05$) *GPR34* mRNA abundance by 19% and 21%, respectively, in SMGC (Fig. 4). Statistical analysis of the *18S* Ct (average = 11.3 ± 0.2) revealed no significant effect of treatment.

3.3. Exp. 5: Effect of FSH on GPR34 mRNA in SMGC and LGGC

In SMGC and LGGC, FSH treatment had no effect ($P > 0.10$) on *GPR34* mRNA abundance (data not shown). Relative abundance of *GPR34* mRNA in control and FSH-treated cells averaged 8.1 and 8.2 ± 0.4 in SMGC, and 12.5 and 17.8 ± 0.5 in LGGC, respectively. Statistical analysis of the *18S* Ct (average = 16.3 ± 0.4) revealed no significant effect of treatment.

3.6. Exp. 6: Effect of LPPS and LPPE on cell numbers and E₂ and P₄ production induced by IGF1 in SMGC

Treatment with LPPS and LPPE dose-dependently increased cell numbers in FSH plus IGF1-treated SMGC (Fig. 5A). The highest dose (i.e., 3 $\mu\text{g/ml}$) of LPPS and LPPE increased cell numbers by 2.2- and 2.0-fold, respectively. Treatment with LPPS and LPPE dose-dependently increased ($P < 0.05$) E₂ production in FSH plus IGF1-treated SMGC (Fig. 5B). The highest dose (i.e., 3 $\mu\text{g/ml}$) of LPPS and LPPE increased E₂ production by 3- and 5-fold, respectively. Treatment with LPPS but not LPPE treatment increased ($P < 0.05$) P₄ production in FSH plus IGF1-treated SMGC (Fig. 5C).

3.7. Exp. 7: Effect of IGF1 on SMGC response to LPPS and LPPE

Cells treated with FSH alone did not respond to LPPS or LPPE whereas SMGC treated with FSH plus IGF1 responded to both LPPS and LPPE (Fig. 6). Specifically, treatment with 1.0

µg/ml (but not 0.3 µg/ml) of LPPS and both 0.3 and 1 µg/ml of LPPE increased GC numbers in FSH plus IGF1-treated SMGC but neither LPPS nor LPPE had an effect ($P > 0.10$) on GC numbers in FSH-treated SMGC (Fig. 6A). The highest dose (i.e., 1 µg/ml) of LPPS and LPPE tested increased ($P < 0.05$) IGF1-induced cell numbers by 1.43- and 1.34-fold, respectively. Treatment with IGF1 and FSH increased ($P < 0.05$) cell numbers by 1.96-fold above FSH-treated controls. Treatment with 1.0 µg/ml of LPPS and LPPE increased ($P < 0.05$) E₂ production in FSH plus IGF1-treated SMGC by 1.64- and 1.41-fold, respectively (Fig. 6B). Treatment with IGF1 and FSH increased ($P < 0.05$) E₂ production by 8.2-fold above FSH-treated controls (Fig. 6B). Neither LPPS nor LPPE had an effect ($P > 0.10$) on P₄ production in control or IGF1-treated SMGC (Fig. 6C). Treatment with IGF1 and FSH increased ($P < 0.05$) P₄ production by 2.2-fold above FSH-treated controls (Fig. 6C).

4. Discussion

The current study is the first to characterize changes in *GPR34* mRNA in GC during follicle growth as well as show that hormone responsive *GPR34* mRNA in GC changes with follicle size. Because we observed that *GPR34* mRNA abundance was less in large EA than large EI follicles and less in F1 than F2 follicles on day 3-4 (but not day 5-6), *GPR34* may be involved in regulating selection of the dominant follicle.

In the present study, IGF1, TNFα, and PGE₂ decreased *GPR34* mRNA abundance in LGGC. Interestingly, all of these factors have been related with the process of angiogenesis in the ovulatory follicle [18,42,43]. As a follicle grows and develops, it acquires increased mRNA abundance of *LH receptors* and *aromatase* in GC, and increased levels of E₂ and free IGF1 in follicular fluid [30,44,45]. Importantly, LH is required to induce follicular rupture, angiogenesis and formation of the corpus luteum [42]. Since IGF1, PGE₂, and TNFα concentrations in follicles increase in response to LH to increase angiogenesis prior to ovulation and decrease *GPR34* mRNA abundance, we hypothesize that overexpression of *GPR34* may prevent ovulation of the dominant follicle, which would be in agreement with our previous finding that greater *GPR34* mRNA abundance is found in cystic follicles in comparison to healthy dominant follicles [13]. Our study also suggests that the IGF1 effect on *GPR34* mRNA abundance shifts from stimulatory in SMGC to inhibitory in LGGC. The reason for this shift in response to IGF1 is unknown and will require further study. Differences in responsiveness of SMGC and LGGC is not without precedence. For example, we have shown that TNFα inhibits steroidogenesis of SMGC but has no effect on LGGC [18], and that LGGC are more responsive to FSH than are SMGC [34]. Perhaps the stimulatory effect of IGF1 on *GPR34* mRNA in small follicles allows for a more robust E₂ and proliferation response to increases in free IGF1 concentrations. Additional work will be required to verify these suggestions.

Because *GPR34*-deficient mice immunized with BSA antigen exhibited greater concentrations of cytokines (i.e., TNFα and IL-2) in spleen cells than WT control mice [4], we evaluated the effect of cytokines on *GPR34* expression in GC. The cytokines we tested were TNFα, IL-2 and IL-6 because these cytokines also inhibit IGF1-induced GC steroidogenesis and are involved with systemic inflammation and the regulation of immune cells [15–18]. In the present study, all three cytokines (i.e., TNFα, IL-2 and IL-6) attenuated

IGF1-stimulated *GPR34* mRNA expression in SMGC. Moreover, TNF α had inhibitory effects on basal and IGF1-inhibited *GPR34* mRNA expression in LGGC, and indicate that regardless of cell type, TNF α and IGF1 regulate *GPR34* expression through independent mechanisms. As mentioned, inhibitory effects of TNF α , IL-2 and IL-6 on IGF1-induced GC effects are not unique to *GPR34*, as these cytokines also inhibit bovine GC steroidogenesis [15–18]. Increased abundance of *LH receptor* mRNA and aromatase activity characterizes the main difference in LGGC vs. SMGC used for our in vitro experiments [24,30], and as follicles develop during the early follicular wave in cattle in response to the secondary FSH surge, intrafollicular E₂ and free IGF1 concentrations increase [25,44,45]. Abundance of *GPR34* mRNA was severalfold greater in GC of small and large EI follicles than in large EA follicles, and GC of F2 follicles tended to have greater abundance of *GPR34* mRNA than F1 follicles on day 3-4 (but not day 5-6) post-ovulation. Based on the present in vitro results, IGF1 and cytokines are likely the main hormones/ intraovarian factors that are eliciting these differences.

We also tested SHH, WNT3A, ANG and PGE2 because previous studies showed that these factors may also be involved in cystic follicle formation [13] and significantly alter ovarian cell function [32,34,36,37]. In SMGC, SHH and WNT3 had no effect on *GPR34* mRNA expression in the present studies. In contrast, ANG and PGE2 were the only factors that showed inhibitory effects on *GPR34* mRNA expression in SMGC. Follicular fluid PGE2 concentrations increase only during late preovulatory follicular growth [46,47], and thus, are not likely involved in the *GPR34* expression differences observed between EA and EI follicles in the present study. Similarly, ANG immunoreactivity increases as follicles develop from primordial to tertiary in cattle [37], but whether intrafollicular ANG levels change during growth of dominant follicles is unknown. Because ANG had weak inhibitory (i.e., 20% decrease) effects on *GPR34* mRNA, an intrafollicular change of ANG is not likely the cause for the severalfold greater level of *GPR34* mRNA observed in F2 vs. F1 follicles on day 3-4 or in EI vs. EA follicles of the present study. Similarly, because both PGE2 and ANG were inhibitory to *GPR34* expression, greater abundance of *PGE2 receptor* and *ANG* mRNA previously reported in cystic follicles [13] are not likely the cause of the greater abundance of *GPR34* mRNA observed in cystic follicles [13]. Moreover, we found that cortisol had no effect on *GPR34* mRNA abundance in SMGC. Previous studies evaluating other Class 1 GPRs indicate that PGE2 has no effect on expression of chemokine (C-C motif) receptor 6 (CCR6) [48] and that dexamethasone induced mRNA expression of the *P2Y₂ receptor* in human microvascular endothelial cells [49]. Additional research will be required to clarify the physiological significance of PGE2 and ANG regulated *GPR34* mRNA in GC.

Bioactive lysophospholipids such as LPPS and LPPE regulate a plethora of cellular activity in many cell types [6,9–12,50]. Although GPR34 was recently identified as a GPR for LPPS [11,50], studies by Ritscher *et al.* [12] revealed that agonist specificity of GPR34 is species-dependent and that multiple determinants are involved in this process. In particular, Ritscher and colleagues [12] demonstrated that LPPS has activity at GPR34 and indicated that it may not be the only natural agonist for GPR34. In human breast cancer cells, LPPE-induced Ca²⁺ increases whereas in human ovarian cancer cells it did not, suggesting there may also be cell-dependent specificity of GPR ligands [51]. Potential ligands to the lysophosphatidyl-

serine receptor, LPPS and LPPE enhanced bovine SMGC proliferation in FSH plus IGF1-treated SMGC suggesting that *GPR34* may play a functional role in growth of antral follicles in cattle. Interestingly, overexpression of *GPR34* in human lymphoma B cells and human cervical cancer (HeLa) cells increases cell proliferation [14]. Also, elevated *GPR34* expression levels were associated with human gastric adenocarcinomas and its overexpression increased cell invasion in vitro [52]. In the present study, LPPS and LPPE increased E₂ production by several fold in FSH plus IGF1-treated SMGC, suggesting that *GPR34* may play an important role in GC E₂ production as well as GC proliferation, if indeed LPPS and LPPE are *GPR34* ligands in bovine GC. We also found that IGF1 treatment increased the GC proliferation and E₂ response to LPPS and LPPE, supporting the notion that IGF1-induced *GPR34* mRNA enhances the response to *GPR34* ligands in SMGC. A proliferation and E₂ response to LPPS and LPPE was not observed in SMGC treated with FSH alone. Because LPPS and LPPE may bind to other GPR receptors in other cell types [4,53,54], further research will be required to determine if *GPR34* is the sole receptor for LPPS and LPPE in bovine GC. In Exp. 6, both LPPS and LPPE enhanced FSH-induced P4 production and LPPS but not LPPE weakly stimulated P4 production in FSH plus IGF1-treated SMGC, whereas in Exp. 7 neither LPPS nor LPPE had a significant effect on P4 production. Thus, results of Exp. 6 and 7 suggest that the *GPR34* effects are more directed toward cell proliferation and E₂ production than toward P4 production. Further work will be required to more clearly elucidate the physiological role of *GPR34* and their ligands in GC function.

In conclusion, the present study revealed that abundance of *GPR34* mRNA was severalfold greater in GC of small and large EI follicles than in large EA follicles, and that GC of F2 follicles tended to have greater abundance of *GPR34* mRNA than F1 follicles on day 3-4 post-ovulation. The hormones that had the largest effect on GC *GPR34* expression were IGF1 and PGE₂ and the cytokines TNF α and IL-2. The reason for the opposing effects of IGF1 on *GPR34* mRNA in SMGC and LGGC is unknown and will require further study. We hypothesize that IGF1 may promote early small antral follicle development via increased expression of *GPR34*. For the first time, we have identified the lysophospholipid receptor *GPR34* as a developmentally and hormonally regulated gene in GC, the presumed ligands of which enhance IGF1-induced GC proliferation and E₂ production.

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Abbreviations

ANG	Angiogenin
E₂	estradiol
FCS	fetal calf serum
GC	granulosa cell(s)
IGF1	insulin-like growth factor 1
IL-2	interleukin-2
IL-6	interleukin-6
LG	large follicle(s)
P4	progesterone
PGE2	prostaglandin E2
SHH	Sonic hedgehog
SM	small follicle(s)
TNFα	tumor necrosis factor alpha
WNT3A	wingless-type mouse mammary tumor virus integration site family member 3A

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Highlights

- GPR34 mRNA abundance is decreased in estrogen-active follicles.
- GPR34 mRNA was regulated by cytokines and hormones including IGF-I.
- GPR34 ligands significantly altered steroid production by granulosa cells.
- GPR34 may be involved in follicular growth via regulation of steroid production.

Fig. 1.

Effect of estrogen status (i.e., group), rank and day post-ovulation on abundance of granulosa cell *GPR34* mRNA in bovine follicles (Exp 1). Granulosa cells from small (1 to 5 mm; SMGC) and large (> 5 mm; LGGC) follicles were collected, RNA isolated and quantitative RT-PCR used to quantify mRNA levels. Panel A: Large follicles were further classified as estrogen-active (estradiol > progesterone (P4) in follicular fluid; EA) or estrogen-inactive (estradiol < P4 in follicular fluid; EI). All small follicles were EI. ^{ab}Means without a common letter differ ($P < 0.05$). Panel B: The largest two follicles (F1, largest; F2, second largest) were compared across days; means (F1 vs. F2) on Day 3 tended to differ ($P < 0.10$). Values are normalized to constitutively expressed *18S* ribosomal RNA; n = number of samples.

Fig. 2.

Effect of IGF1 and $TNF\alpha$ on *GPR34* mRNA abundance in small- and large-follicle granulosa cells (Exp 2). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 24 h with: Control (no additions), IGF1 (30 ng/ml), $TNF\alpha$ (30 ng/ml) or $TNF\alpha$ plus IGF1. Values are means $\pm$ SEM of three separate experiments ($n = 6$) and normalized to constitutively expressed *18S* ribosomal RNA. ^{abc}Within follicle size group, means without a common letter differ ($P < 0.05$).

Fig. 3.

Effect of IGF1, IL-2 and IL-6 on small- and large-follicle granulosa cell *GPR34* mRNA abundance (Exp 3). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 24 h with: Control (no additions), IGF1 (30 ng/ml), IGF1 plus IL-2 (30 ng/ml) or IGF1 plus IL-6 (30 ng/ml). Values are means $\pm$ SEM of three separate experiments ($n = 6$) and normalized to constitutively expressed *18S* ribosomal RNA. ^{ab}Means without a common letter differ ($P < 0.05$).

Fig. 4.

Effect of cortisol (cort), prostaglandin E2 (PGE2), sonic hedgehog (SHH), wingless-3A (WNT3A), and angiogenin (ANG) on *GPR34* mRNA abundance in granulosa cells collected from small follicles (Exp. 4). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 24 h with 30 ng/ml of IGF1 and either Control (no addition), cortisol (300 ng/ml), PGE2 (300 ng/ml), SHH (500 ng/ml), WNT3A (300 ng/ml), or ANG (300 ng/ml). Values are means $\pm$ SEM of three separate experiments ($n = 6$) and normalized to constitutively expressed *18S* ribosomal RNA. ^{a,b}Means without a common letter differ ($P < 0.05$).

Fig. 5.

Response of small-follicle granulosa cells (SMGC) to lysophospholipids (Exp. 6). Panel A: Effect of 0.3, 1.0 and 3.0 $\mu\text{g/mL}$ of L- α -lysophosphatidylserine (LPPS) or L- α -lysophosphatidylethanolamine (LPPE) on numbers of SMGC cultured in the presence of FSH plus IGF1 (30 ng/ml). Panel B: Effect of 0.3, 1.0 and 3.0 $\mu\text{g/mL}$ of LPPS or LPPE on FSH plus IGF1-induced estradiol production by SMGC. Panel C: Effect of 0.3, 1.0 and 3.0 $\mu\text{g/mL}$ of LPPS or LPPE on FSH plus IGF1-induced progesterone production by SMGC. Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 48 h with 30 ng/ml of FSH and IGF1 (30 ng/ml) and various lysophospholipids at 0, 0.3, 1.0 or 3.0 $\mu\text{g/mL}$. All cells were treated concomitantly with 500 ng/ml of testosterone. Values are means $\pm$ SEM of three separate experiments ($n = 9$). ^{a,b,c,d} Within a panel, means without a common letter differ ($P < 0.05$).

Fig. 6.

Effect of IGF1 on the response of small-follicle granulosa cells (SMGC) to lysophospholipids (Exp. 7). Panel A: Effect of 0.3 and 1.0 $\mu\text{g/mL}$ of L- α -lysophosphatidylserine (LPS) or L- α -lysophosphatidylethanolamine (LPE) cultured with or without IGF1 on numbers of SMGC treated with FSH. Panel B: Effect of 0.3 and 1.0 $\mu\text{g/mL}$ of LPS or LPE on either FSH alone or FSH plus IGF1-induced estradiol production by SMGC. Panel C: Effect of 0.3 and 1.0 $\mu\text{g/mL}$ of LPS or LPE on either FSH alone or FSH plus IGF1-induced progesterone production by SMGC. Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 48 h with FSH (30 ng/ml) and/or IGF1 (30 ng/ml) and either LPS or LPE at 0, 0.3 or 1.0 $\mu\text{g/mL}$. All cells were treated concomitantly with 500 ng/ml of testosterone. Values are means $\pm$ SEM of three separate experiments ($n = 9$). ^{a,b,c,d} Within a panel, means without a common letter differ ($P < 0.05$).