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Effects of angiogenin on granulosa and theca cell function in cattle

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Abstract

Angiogenin is a member of the ribonuclease A superfamily of proteins that has been implicated in stimulating angiogenesis but whether angiogenin can directly affect ovarian granulosa or theca cell function is unknown. Therefore, the objective of these studies was to determine the effect of angiogenin on proliferation and steroidogenesis of bovine granulosa and theca cells. In experiments 1 and 2, granulosa cells from small (1 to 5 mm diameter) follicles and theca cells from large (8 to 22 mm diameter) follicles were cultured to evaluate the dose-response effect of recombinant human angiogenin on steroidogenesis. At 30 and 100 ng/ml, angiogenin inhibited ($P < 0.05$) granulosa cell progesterone production and theca cell androstenedione production but did not affect ($P > 0.10$) granulosa cell estradiol production or theca cell progesterone production, and did not affect numbers of granulosa or theca cells. In experiments 3 and 4, granulosa and theca cells from both small and large follicles were cultured with 300 ng/ml of angiogenin to determine if size of follicle influenced responses to angiogenin. At 300 ng/ml, angiogenin increased large follicle granulosa cell proliferation but decreased small follicle granulosa cell progesterone and estradiol production and large follicle theca cell progesterone production. In experiments 5 and 6, angiogenin stimulated ($P < 0.05$) proliferation and DNA synthesis in large follicle granulosa cells. In experiment 7, 300 ng/ml of angiogenin increased ($P < 0.05$) CYP19A1 messenger RNA (mRNA) abundance in granulosa cells but did not affect CYP11A1 mRNA abundance in granulosa or theca cells and did not affect CYP17A1 mRNA abundance in theca cells. We conclude that angiogenin appears to target both granulosa and theca cells in cattle, but additional research is needed to further understand the mechanism of action of angiogenin in granulosa and theca cells, as well as its precise role in folliculogenesis.

Keywords

angiogenin (ANG); granulosa cells; theca cells; IGF1; tumor necrosis factor α (TNFα)

Introduction

Angiogenesis or the formation of blood vessels from pre-existing blood vessels is controlled by many different angiogenic factors (Lee *et al*., 1999; Ucuzian *et al*., 2010). Angiogenesis

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is involved in a variety of physiological processes such as wound repair and embryological development (Redmer and Reynolds, 1996; Przybylski, 2009; Appelmann *et al*., 2010), and is under the influence of anti-angiogenic and pro-angiogenic molecular mediators. One of the pro-angiogenic factors is angiogenin (ANG), a member of the ribonuclease A superfamily of 10 to 28 kDa proteins ascribed as a group of enzymes that have inherent substrate specificity and differing functional capacities (for review see: Tello-Montoliu *et al*., 2006; Gupta *et al*., 2013; Premzl, 2014). However, unlike many of the RNase A superfamily members, ANG has been shown to have its own specific membrane receptor that mediates its effects (Hatzi and Badet, 1999; Xu *et al*., 2001). Angiogenin was first isolated from medium conditioned by human colon carcinoma (HT-29) cells (Fett *et al*., 1985) and later found in normal human serum (Shapiro *et al*., 1987), bovine milk (Maes *et al*., 1988) and other mammalian serum (Bond *et al*., 1993). Specific angiogenic effects of ANG are multifaceted (Gao and Xu, 2008) and includes induction of endothelial cell proliferation (Hu *et al*., 1997; Tsuji *et al*., 2005), tumor cell adhesion (Soncin *et al*., 1994), and tumor cell invasion (Gho *et al*., 2002).

In domestic animals, ovarian vasculature surrounding follicles increases during normal follicular development (Yamada *et al*., 1995; Jiang *et al*., 2002, 2003; Moonmanee *et al*., 2013), but the molecular mechanisms that initiate ovarian angiogenesis are not well defined. In the bovine and human ovary, using *in situ* hybridization, *ANG* messenger RNA (mRNA) was localized in granulosa cells and oocytes (but not theca cells) of secondary and tertiary follicles, luteal cells of developing corpora lutea, and vascular endothelial and smooth muscle cells (Lee *et al*., 1999; Koga *et al*., 2000). Malamitsi-Puchner *et al*. (2001) reported that follicular fluid levels of ANG were significantly greater in human follicles yielding mature *v*. immature oocytes. Moreover, follicular fluid concentrations of ANG (ranging from 100 to 800 ng/ml) and progesterone were positively correlated in women (Koga *et al*., 2000). Therefore, we hypothesized that ANG, in addition to its angiogenic effects, would stimulate steroidogenesis and increase proliferation of both granulosa and theca cells. Therefore, the objectives of this study were to determine the effect of ANG on granulosa and theca cell proliferation and steroid production *in vitro*.

Materials and methods

Hormones and reagents

The hormones and reagents used in cell culture were: ovine FSH (NIDDK-oFSH-20; activity: 175 X NIH-FSH-S1 U/mg) and ovine LH (NIDDK-oLH-26; activity: 1.0 X NIH-LH-S1 U/mg) from the National Hormone & Pituitary Program (Torrance, CA, USA), recombinant human ANG and IGF-1 (IGF1) from R&D Systems (Minneapolis, MN, USA), testosterone from Steraloids (Wilton, NH, USA), fetal calf serum (FCS) from EquiTech-Bio Inc. (Kerrville, TX, USA), and collagenase and DNase from Sigma-Aldrich Corp. (St. Louis, MO, USA). Human ANG and bovine ANG proteins share 66% amino acid homology (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

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Cell culture

Ovaries from non-pregnant beef cows were collected from a local slaughterhouse, and based on surface diameter, granulosa cells were collected from small (1 to 5 mm) and large (8 to 22 mm) follicles as previously described (Langhout *et al.*, 1991; Stewart *et al.*, 1995; Spicer *et al.*, 2008). Small and large follicles were bisected after aspiration of follicular fluid and granulosa cells separated from the theca interna via blunt dissection, and the theca interna was torn into small pieces, rinsed with basal serum-free medium (1 : 1 Dulbecco's modified Eagle's medium and Ham's F-12; 0.12 mM gentamycin, 2.0 mM glutamine, and 38.5 mM sodium bicarbonate; all obtained from Sigma-Aldrich Corp.) and enzymatically digested for 1 h at 37°C as previously described (Stewart *et al.*, 1995; Spicer and Chamberlain, 1998; Spicer *et al.*, 2008). Briefly, after digestion, the non-digested tissue was filtered out, and theca cells were then centrifuged at 50 × **g** for 5 min, supernatant discarded and pellet washed with serum-free medium. The purity of theca cells prepared this way was >90% (Spicer and Stewart, 1996; Spicer *et al.*, 2008). The theca and granulosa cells were re-suspended in serum-free media containing collagenase and DNase at 1.25 and 0.5 mg/ml, respectively, to prevent cell clumping before plating.

Viable cells (2×10^5 in 25 to 45 μl of medium/well) were plated in each well of 24-well Falcon multiwell plates (Becton Dickinson, Lincoln Park, NJ, USA) containing 1 ml of basal medium with 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 and the various treatments (see below) applied in serum-free medium (1 ml per well) for 48 h with a medium change at 24 h. After 48 h, medium was collected for steroid quantification via radioimmunoassays (RIA) and cells were collected for cell enumeration via Coulter counting (see below). The concentrations of LH, FSH and IGF1 were selected based on previous studies (Spicer and Stewart, 1996; Spicer and Chamberlain, 1998; Spicer *et al.*, 2002). Because steroid production in this culture system is maximized with combined gonadotropins and growth factor treatment, only weakly responsive to gonadotropins alone, and not responsive to growth factors alone (Stewart *et al.*, 1995; Spicer and Chamberlain, 1998; Spicer *et al.*, 2002), gonadotropins were used in combination with IGF1 for most experiments. The concentrations of ANG used for experiments were selected based on studies indicating that concentrations of ANG in bovine plasma (Bond and Vallee, 1988; Chang *et al.*, 1997) and human follicular fluid (Koga *et al.*, 2000; Malamitsi-Puchner *et al.*, 2001; Kawano *et al.*, 2003) range between 0.4 and 800 ng/ml.

Experimental design

Experiment 1 was designed to determine the dose-response effects of ANG on steroidogenesis and number of granulosa cells of small follicles. Granulosa cells from small follicles were selected for this experiment because we have shown that they grow and respond well to growth factor treatments (Spicer *et al.*, 2002). Granulosa cells from small follicles were cultured for 48 h in the presence of 10% FCS, and then cells were washed and incubated in serum-free medium in the presence of 0, 30 or 100 ng/ml of ANG without or with 30 ng/ml of FSH and/or 30 ng/ml of IGF1 for 48 h. Medium was changed every 24 h and contained 500 ng/ml of testosterone as an estrogen precursor (Spicer and Chamberlain,

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1998). Cells were counted and medium samples were collected for progesterone (P4) and estradiol (E2) determinations via RIA (see below).

Experiment 2 was designed to determine the dose-response effects of ANG on steroidogenesis and number of theca cells of large follicles. Theca cells from large follicles were selected for this experiment because we have shown that they grow and respond well to growth factor treatments (Spicer *et al.*, 2008). Theca cells from large follicles were cultured for 48 h in the presence of 10% FCS, and then cells were washed and incubated in serum-free medium in the presence of 0, 30 or 100 ng/ml of ANG without or with 30 ng/ml of LH and/or 30 ng/ml of IGF1 for 48 h. Medium was changed every 24 h. Cells were counted and medium samples were collected for P4 and androstenedione determinations via RIA (see below).

Experiment 3 was designed to compare the effects of high dose (i.e. 300 ng/ml) ANG on steroidogenesis and number of granulosa cells isolated from small and large follicles. Because experiment 1 showed minor effects of 30 and 100 ng/ml of ANG on small follicle granulosa cell numbers or steroid production, studies were conducted using 300 ng/ml of ANG with both small and large follicle granulosa cells. Cells from small and large follicles were cultured for 48 h in the presence of 10% FCS, and then cells were washed and incubated in serum-free medium in the presence of 0 or 300 ng/ml of ANG without or with 30 ng/ml of FSH and/or 30 ng/ml of IGF1 for 48 h. Medium was changed every 24 h and contained 500 ng/ml of testosterone as an estrogen precursor (Spicer and Chamberlain, 1998). Cells were counted and medium samples were collected for P4 and E2 determinations via RIA (see below).

Experiment 4 was designed to compare the effects of high dose (i.e. 300 ng/ml) ANG on steroidogenesis and number of theca cells isolated from small and large follicles. Because experiment 2 showed minor effects of 30 and 100 ng/ml of ANG on large follicle theca cell numbers or steroid production, studies were conducted using 300 ng/ml of ANG with both small and large follicle theca cells. For this experiment, small follicle theca cells were obtained from follicles 3 to 6 mm in diameter as previously described (Spicer *et al.*, 2008). Cells from small and large follicles were cultured for 48 h in the presence of 10% FCS, and then cells were washed and incubated in serum-free medium in the presence of 0 or 300 ng/ml of ANG without or with 30 ng/ml of LH and/or 30 ng/ml of IGF1 for 48 h. Medium was changed every 24 h. Cells were counted and medium samples were collected for P4 and androstenedione determinations via RIA (see below).

Experiment 5 was designed to test the effect of ANG on large follicle granulosa cell numbers. Cells were cultured for 48 h in 10% FCS medium, and then treated for an additional 24 h or 48 h with either 0 or 300 ng/ml of ANG in the presence of 10% FCS. Separate cultures were terminated at 0, 24 and 48 h post-treatment, and cells were counted. Dose of ANG was selected based on results from experiments 1 and 3.

Experiment 6 was designed to determine the effect of ANG on large follicle granulosa and theca cell proliferation as measured by ^{3}H-thymidine incorporation into DNA. Because experiments 1 to 4 showed an effect of ANG on cell numbers only in the presence of IGF1,

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this experiment evaluated the effect of ANG in the presence of IGF1. After 48 h in 10% FCS, cells were serum-starved for 24 h by culturing in serum-free medium, medium changed, and then cells cultured for an additional 40 h in serum-free medium with either 0 or 300 ng/ml of angiogenin in the presence of 1 μCi of ^{3}H-thymidine and 30 ng/ml of IGF1 to assess DNA synthesis as previously described (Spicer and Aad, 2007; Spicer *et al.*, 2008).

Experiment 7 was conducted to determine the effect ANG on steroidogenic gene expression in granulosa and theca cells. Granulosa cells from small follicles and theca cells from large follicles were cultured for 48 h in 10% FCS, medium changed, and then cells cultured for an additional 24 h in serum-free medium with either no treatment or 300 ng/ml of ANG. Granulosa cells were concomitantly treated with 30 ng/ml of FSH and IGF1 and theca cells were treated concomitantly with 30 ng/ml of LH and IGF1. Doses of FSH, LH and IGF1 were selected based on previous studies (Spicer *et al.*, 2002; Lagaly *et al.*, 2008). Gonadotropins were added to all treatments because IGF1 alone has little or no effect on steroid production (Lagaly *et al.*, 2008; Spicer *et al.*, 2008). After 24 h of treatment, cells were lysed in 0.5 ml of TRIzol for RNA extraction (see below) and quantification of *CYP11A1, CYP19A1* or *CYP17A1* mRNA.

RNA extraction and quantification

Total RNA was extracted using TRIzol reagent protocol (Life Technologies, Carlsbad, CA, USA), and RNA was quantitated by spectrophotometry at 260 nm using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) as previously described (Voge *et al.*, 2004; Aad *et al.*, 2006; Schreiber and Spicer, 2012). Quantification of gene expression was conducted by fluorescent one-step real-time PCR as previously described (Spicer and Aad, 2007; Grado-Ahuir *et al.*, 2011). The bovine *CYP11A1, CYP17A1* and *CYP19A1* primer and probe sequences and information are described by Lagaly *et al.* (2008). The internal standard was 18S ribosomal RNA. Data analysis was done using the comparative threshold cycle (C_t) method as previously described (Voge *et al.*, 2004; Aad *et al.*, 2006). Briefly, the C_t was determined by subtracting the 18S C_t value from the target gene unknown value. For each target gene and within each experiment, the C_t was determined by subtracting the higher C_t (the least expressed unknown) from all other C_t values. Fold changes in target gene mRNA abundance were calculated as being equal to $2^{- C_t}$.

Determination of steroid concentrations and cell numbers

Medium was collected from individual wells and frozen at −20°C for subsequent determination of concentrations of P4, E2 and/or androstenedione via RIA as previously described (Langhout *et al.*, 1991; Stewart *et al.*, 1995; Spicer and Chamberlain, 1998). The intra- and interassay coefficients of variation were 5.6% and 11% for the progesterone RIA, 6.5% and 12% for the estradiol RIA, and 4.1% and 7.3% for the androstenedione RIA.

Numbers of cells were determined via Coulter counting as previously described (Langhout *et al.*, 1991; Stewart *et al.*, 1995; Spicer *et al.*, 2008) and used to calculate steroid production on a ng or pg per 10^5 cell basis. Briefly, cells were gently washed twice with 0.9% saline (500 μl; w/v), exposed to 500 μl of trypsin solution (0.25%, w/v) for 20 min at 25°C, scraped

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from each well, and counted using a Coulter counter (model Z2; Beckman Coulter Inc., Miami, FL, USA).

Cell aggregates were disrupted via pipetting the cell suspension back and forth through a 500 μl pipette tip three to five times, and diluted in 9 ml of 0.9% saline (w/v) before counting.

Statistical analysis

Data are presented as the least squares means (± SE) of measurements from three or more individual pools of large and small follicle granulosa/theca cells used as experimental replicates. Each biological replicate (i.e. pool of cells) was conducted on independent pools of cells. Each pool of small follicle granulosa cells were collected from 10 to 30 ovaries (n = 5 to 15 cattle) yielding 6 to 8 ml of follicular fluid. Each of the large follicle granulosa/theca cell pools was obtained from 7 to 10 follicles from at least five animals. Small follicle theca cells were obtained from 6 to 20 ovaries (n = 3 to 10 animals). Within each replicated experiment, treatments were applied to each pool of cells in duplicate or triplicate culture wells. Steroid production was expressed as ng or pg/10^5 cells per 24 h, and cell numbers at the termination of each experiment were used for this calculation. Specific differences in cell numbers and steroid production among treatments were determined via ANOVA using GLM procedure of SAS (Statistical Analysis System, Cary, NC, USA) and Fisher's protected least significant difference procedure (Ott, 1977). Significance was declared at $P < 0.05$.

Results

Experiment 1: dose response of ANG on cell numbers and steroidogenesis of small follicle granulosa cells

Treatment of granulosa cells with IGF1 alone increased ($P < 0.05$) cell numbers by 54% to 73% (Table 1), however none of the doses of ANG (i.e. 30 or 100 ng/ml) affected ($P > 0.10$) control or IGF1-induced granulosa cell numbers (Table 1). Alone FSH had no effect ($P > 0.05$) on cell numbers but FSH significantly enhanced the IGF1-induced increase ($P < 0.001$) in cell numbers (Table 1). Dose of ANG had no significant effect on E2 production (Figure 1a). FSH and IGF1 synergized to stimulate ($P < 0.01$) E2 production by 6.6-fold, and ANG had no significant effect on this FSH plus IGF1-induced E2 production (Figure 1a); alone neither FSH nor IGF1 affected ($P > 0.10$) E2 production. Both IGF1 and FSH increased P4 production and 100 ng/ml of ANG reduced ($P < 0.05$) the FSH plus IGF1-induced P4 production by 16% (Figure 1b).

Experiment 2: dose response of ANG on steroidogenesis of large follicle theca cells

Treatment of theca cells with IGF1 increased ($P < 0.05$) cell numbers by 31% to 56% (Table 1). Neither LH nor ANG (30 or 100 ng/ml) affected ($P > 0.10$) theca cell numbers induced by IGF1 (Table 1). LH increased ($P < 0.05$) both androstenedione and P4 production, and ANG (30 and 100 ng/ml) decreased ($P < 0.05$) IGF1 plus LH-induced androstenedione production by 22% to 23%, but ANG had no effect ($P > 0.10$) on basal, LH-induced or IGF1-induced androstenedione production (Figure 2a). None of the doses of ANG affected ($P > 0.10$) P4 production by theca cells (Figure 2b).

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Experiment 3: effect of high-dose of ANG on steroidogenesis of small and large follicle granulosa cells

Angiogenin had no effect ($P > 0.10$) on numbers of small follicle granulosa cells, but increased ($P < 0.05$) cell numbers in FSH + IGF1-treated large follicle granulosa cells (Table 2). Treatment with FSH and IGF1 increased ($P < 0.05$) cell numbers by 2.3-fold and 4.8-fold above controls in small and large follicle granulosa cell cultures, respectively. Angiogenin decreased ($P < 0.05$) P4 production by 12% in small follicle granulosa cells treated with FSH + IGF1 and by 30% in control (untreated) large follicle granulosa cells (Table 2). In small follicle granulosa cells, FSH and IGF1 stimulated ($P < 0.01$) E2 production by 5.7-fold, and ANG decreased this hormone-induced E2 production by 27% (Table 2). In large follicle granulosa cells, ANG had no effect ($P > 0.10$) on E2 production (Table 2). To compare across granulosa cell experiments, data (basal and FSH plus IGF1 treatment) from experiments 1 and 3 were expressed as fold of controls and shown in Supplementary Figure S1.

Experiment 4: effect of high-dose ANG on steroidogenesis of small and large follicle theca cells

LH decreased ($P < 0.05$) small follicle theca cell numbers induced by IGF1 but had no effect ($P > 0.10$) on numbers of large follicle theca cells (Table 3). Angiogenin (300 ng/ml) restored this decrease induced by LH (Table 3) in small follicle theca cells, and increased ($P < 0.05$) numbers of IGF1-treated large follicle theca cells by 23% but had no effect on LH + IGF1-treated large follicle theca cells (Table 3). Angiogenin had no effect ($P > 0.10$) on P4 production by small follicle theca cells (Table 3), but decreased ($P < 0.05$) P4 production in IGF1-treated large follicle theca cells by 17% (Table 3). In both small and large follicle theca cells, ANG had no effect ($P > 0.10$) on IGF1-induced or LH + IGF1-induced androstenedione production (Table 3). To compare across theca cell experiments, data (basal and LH plus IGF1 treatment) from experiments 2 and 4 were expressed as fold of controls and shown in Supplementary Figure S2.

Experiment 5: effect of ANG on granulosa and theca cell proliferation induced by 10% FCS

After 2 days of treatment, ANG (300 ng/ml) further enhanced ($P < 0.05$) proliferation of large follicle granulosa cells stimulated by 10% FCS (Figure 3a). Granulosa cells grew 2.4- and 2.7-fold between day 0 and 2 in Control and ANG-treated cultures, respectively ($P < 0.05$; Figure 3a). Cell numbers in ANG-treated cells did not differ ($P > 0.10$) from controls on day 1 of treatment (Figure 3a).

Experiment 6: effect of ANG on granulosa and theca cell proliferation induced by IGF1

Treatment of bovine granulosa cells from large follicles with 300 ng/ml of ANG increased ($P < 0.05$) IGF1-induced ^{3}H-thymidine incorporation into DNA by 2.6-fold (Figure 3b). Angiogenin (300 ng/ml) had no effect ($P > 0.10$) on large follicle theca cell proliferation as measured by ^{3}H-thymidine incorporation into DNA (Figure 3b).

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Experiment 7: effect of ANG on steroidogenic gene expression in granulosa and theca cells

Treatment of 300 ng/ml of ANG to granulosa cells from small follicles and theca cells from large follicles had no effect ($P > 0.10$) on *CYP11A1* mRNA abundance (Figure 4). However, ANG at 300 ng/ml increased ($P < 0.05$) *CYP19A1* mRNA abundance in granulosa cells and had no effect ($P > 0.10$) on *CYP17A1* mRNA abundance in theca cells (Figure 4).

Discussion

Results of the present study revealed that ANG stimulated large follicle granulosa cell proliferation and inhibited small follicle granulosa cell P4 and E2 production and large follicle theca cell P4 and androstenedione production, whereas ANG had no effect on large follicle theca cell proliferation.

For the first time, the present experiments showed that presumed physiological concentrations of ANG (i.e. 30 to 300 ng/ml) were bioactive in both granulosa and theca cell cultures. Although not known for cattle, follicular fluid levels of ANG in women range from 0.4 to 800 ng/ml (Koga *et al.*, 2000; Malamitsi-Puchner *et al.*, 2001; Kawano *et al.*, 2003). Within this concentration range, ANG inhibited granulosa and theca cell steroidogenesis but stimulated granulosa cell proliferation, suggesting a possible regulatory role in follicular development. We hypothesize that ANG, while stimulating blood vessel development, may also promote proliferation of large follicle granulosa cells, while slowing differentiation of small follicle granulosa cells. Presumably, this ANG-induced suppression of small follicle differentiation would allow small follicles to grow and develop its vasculature before they are able to respond to increased gonadotropins during the preovulatory period.

Angiogenin effects on numbers of granulosa or theca cells have not been reported previously and varied with dose, cell type and hormone treatment. At 30, 100 and 300 ng/ml, ANG had no significant effect on numbers of small follicle granulosa cells or numbers of large follicle theca cells. However, at 300 ng/ml, ANG increased proliferation of large follicle granulosa cells in the presence of IGF1 and 10% FCS. These results indicate a change in ANG response as follicles develop. A previous study indicated that ANG stimulates ^{3}H-thymidine incorporation into human umbilical venous endothelial cells grown in medium containing 5% FCS and 5 ng/ml of basic fibroblast growth factor (Hu *et al.*, 1997; Liu *et al.*, 2001). Cell survival and anti-apoptotic effects of ANG have been reported in other cell models (Li and Hu, 2012; Saikia *et al.*, 2014). Thus, effects of ANG on follicular atresia should be investigated. Also, further research will be required to determine the mechanisms by which differentiated granulosa cells from large follicles are more responsive to the stimulatory effect of ANG on proliferation than less differentiated granulosa cells of small follicles, but likely involve changes in the IGF1 intracellular response system, and/or a change in numbers of ANG receptors.

In the present study, ANG had weak inhibitory effects on P4 production by small follicle granulosa cells concomitantly treated with FSH and IGF1, a condition in which cyclic adenosine monophosphate is dramatically elevated (Davoren *et al.*, 1985; Zhang *et al.*, 2000). In untreated large follicle granulosa cells and in IGF1-treated large follicle theca

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cells, ANG also weakly inhibited P4 production. However, these small decreases in P4 production were not accompanied with any change in abundance of *CYP11A1* mRNA suggesting that the mechanism for P4 inhibition by ANG is not mediated via a change in *CYP11A1* expression. Nonetheless, the present studies indicate for the first time that ANG, in addition to its purported role in inducing angiogenesis, may regulate steroidogenesis in granulosa and theca cells. In small follicle granulosa cells, the paradoxical increase in *CYP19A1* mRNA induced by ANG while E2 production remained unchanged at the same dose of ANG will require further elucidation. ANG did not affect E2 production by large follicle granulosa cells indicating that its effect on steroidogenesis in large follicle granulosa cells is exclusive to P4 production. In large follicle theca cells, 30 and 100 ng/ml of ANG decreased LH plus IGF1-induced androstenedione production but when tested at 300 ng/ml, ANG had no effect on androstenedione production or abundance of *CYP17A1* mRNA, suggesting abi-phasic response of theca cells to ANG. However, this inhibitory effect of ANG on androstenedione production was weak (i.e. 22% to 23% inhibition) and theca cell P4 production was only inhibited by 17% in large follicle theca cells treated with 300 ng/ml of ANG, suggesting a minor role for ANG in regulating theca cell steroidogenesis. Immunohistochemical staining and *in situ* hybridization in cattle showing that theca cells localize ANG protein but not *ANG* mRNA provides further support of the idea that ANG produced by granulosa cells communicates with theca cells (Lee *et al.*, 1999). Further studies will be required to elucidate the developmental cell-specific effects of ANG on ovarian cell proliferation and steroidogenesis.

The mechanism of action and intracellular signaling pathway of ANG that affect granulosa and theca cell mitosis is unknown. In a recent study, ANG-induced proliferation of human glioblastoma U87MG cells was reported to act via a nuclear factor-κB pathway (Xia *et al.*, 2015). In another study, the protein kinase B/Akt pathway was reported to be induced by ANG in human umbilical vein endothelial cells (Kim *et al.*, 2007). Moreover, Saikia *et al.* (2014) showed that ANG-generated transfer RNA halves (tiRNAs) and survival of mouse embryonic cortical neuronal cells involves a cytochrome c interaction that inhibits apoptosome formation and activity. Whether any of these pathways are sensitive to ANG in bovine granulosa and theca cells will require further study.

In summary, results of the present study provide new evidence for ANG-dependent regulation of proliferation and steroidogenesis in granulosa and thecal cells that may influence follicle development. In particular, results indicate that ANG inhibits steroidogenesis of undifferentiated (small follicles) granulosa cells and stimulates mitogenesis of differentiated (large follicle) granulosa cells. The steroidogenic effects of ANG on large follicle theca cells although weak were also inhibitory. We conclude that ANG may target granulosa and theca cells in cattle, stimulating proliferation and inhibiting steroidogenesis, but additional research is needed to understand the mechanism of action of ANG in granulosa and theca cells, as well as its precise role in folliculogenesis.

Supplementary Material

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Implications

Angiogenin is a member of the ribonuclease A superfamily of proteins implicated in inducing angiogenesis. The present study provides new evidence for angiogenin-dependent regulation of follicle development. In particular, results indicate that angiogenin inhibits steroidogenesis of undifferentiated (small follicles) granulosa cells and stimulates mitogenesis of differentiated (large follicle) granulosa cells. The steroidogenic effects of angiogenin on large follicle theca cells were also inhibitory. Further studies will be required to elucidate cell-specific effects of angiogenin on ovarian cell proliferation and steroidogenesis during follicular development.

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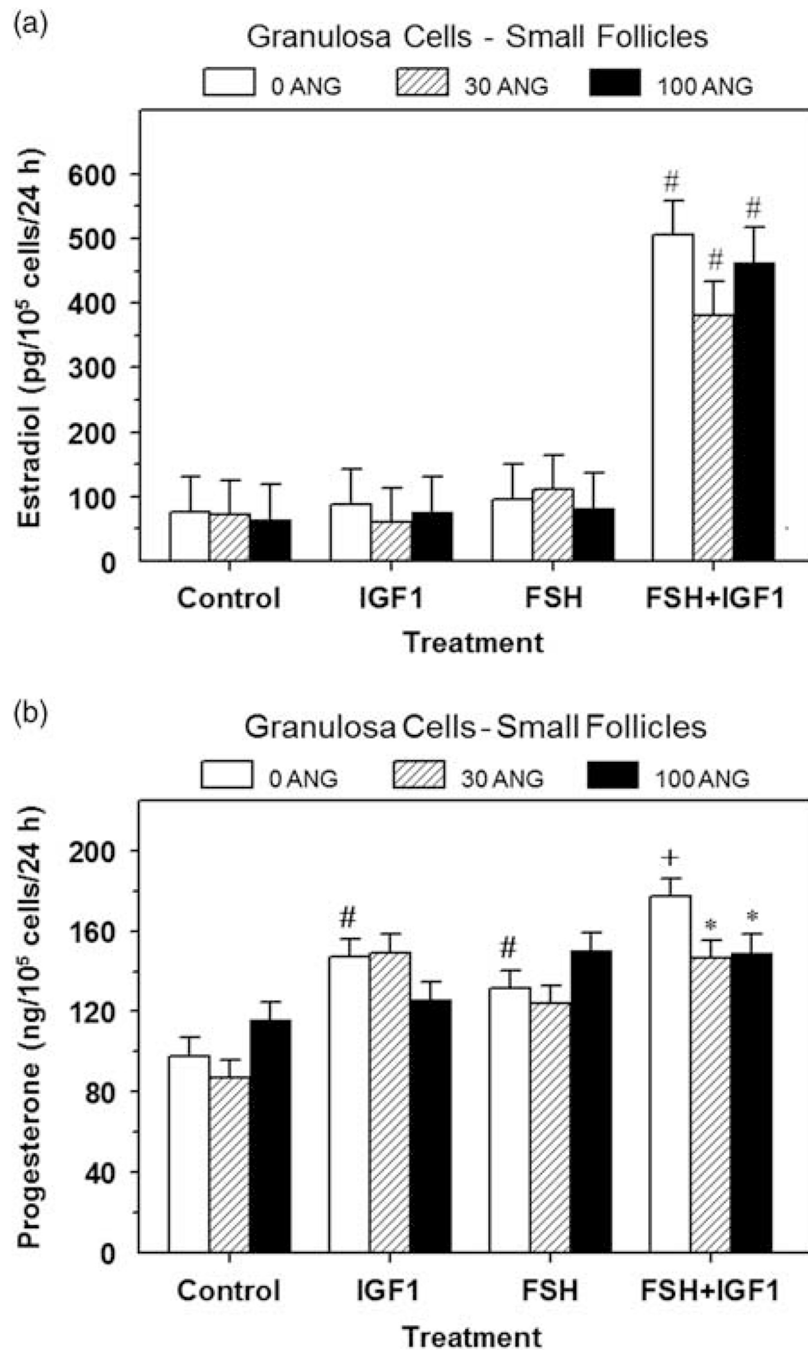

Figure 1.

Effect of angiogenin on basal, FSH- and IGF1-induced estradiol (a) and progesterone (b) production by small-follicle granulosa cells (experiment 1). Cells were cultured for 48 h as described in 'Materials and methods' section, and then treated for an additional 48 h with 0, 30 or 100 ng/ml of angiogenin (ANG) and: Control (no IGF1 or FSH), IGF1 (30 ng/ml), FSH (30 ng/ml) or FSH plus IGF1. Values are means ± SEM of three separate experiments ($n = 6$). *Within a panel and treatment, mean differs ($P < 0.05$) from its respective 0 ANG

mean. [#]Within a panel, mean differs ($P < 0.05$) from its respective Control mean. [+]Within a panel, mean differs ($P < 0.05$) from its respective IGF1 alone or FSH alone treatment mean.

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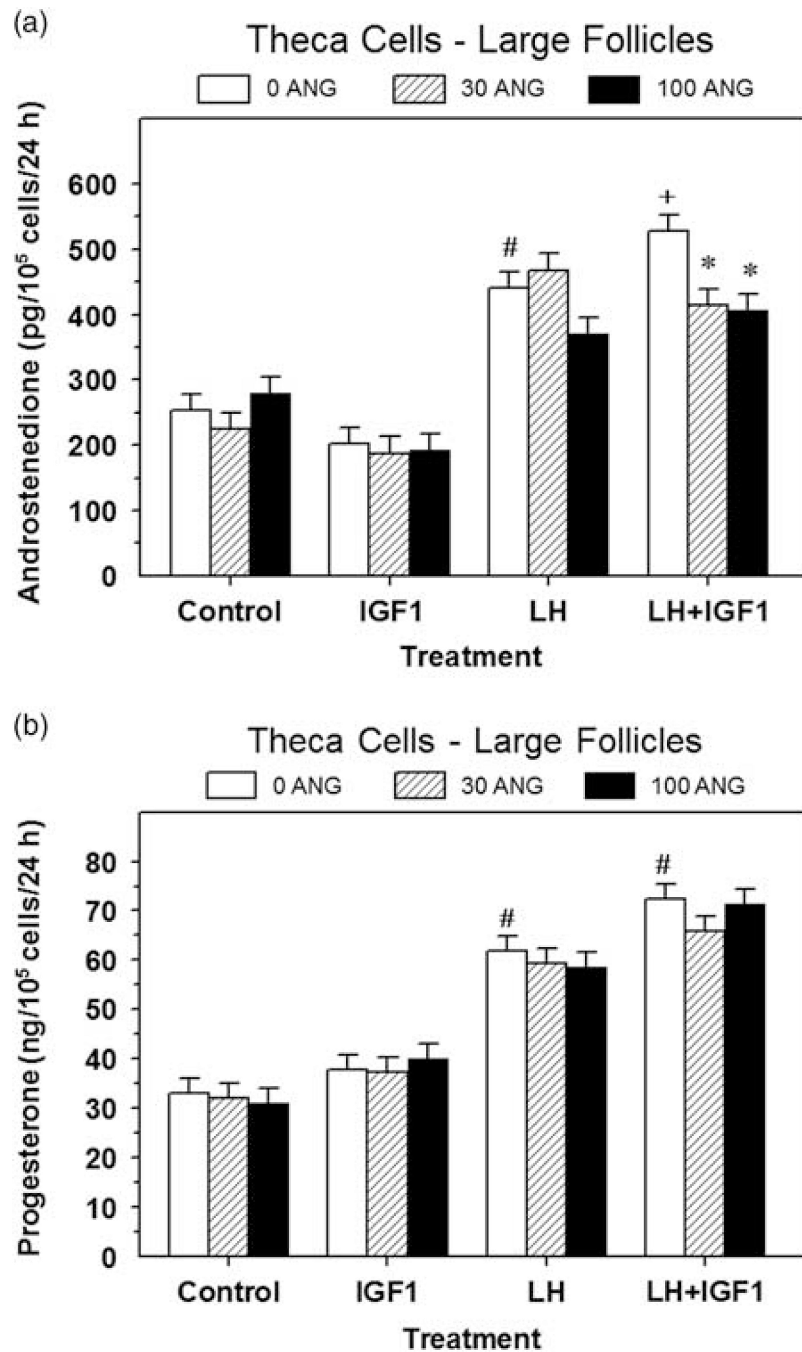

Figure 2.

Effect of angiogenin on basal, LH- and IGF1-induced androstenedione (a) and progesterone (b) production by large follicle theca cells (experiment 2). Cells were cultured for 48 h as described in 'Materials and methods' section, and then treated for an additional 48 h with 0, 30 or 100 ng/ml of angiogenin (ANG) and: Control (no IGF1 or LH), IGF1 (30 ng/ml), LH (30 ng/ml) or LH plus IGF1. Values are means ± SEM of three separate experiments ($n = 6$). *Within a panel and treatment, mean differs ($P < 0.05$) from its respective 0 ANG

mean. [#]Within a panel, mean differs ($P < 0.05$) from its respective Control mean. [+]Within a panel, mean differs ($P < 0.05$) from its respective LH alone treatment mean.

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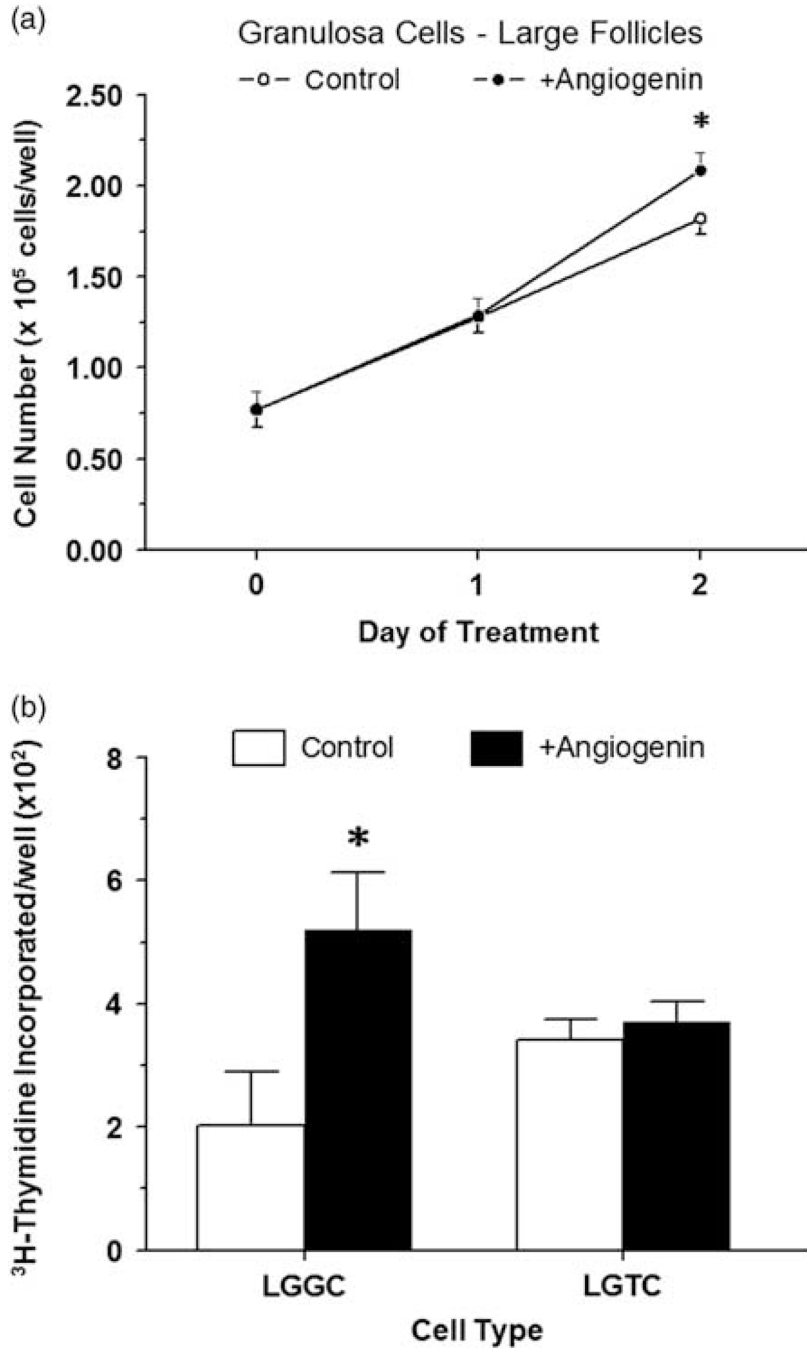

Figure 3.

Effect of angiogenin on proliferation of large follicle granulosa cells induced by 10% FCS (a) (experiment 5) and ^{3}H-thymidine incorporation into large follicle granulosa and theca cells (b) treated with IGF1 (experiment 6). (a) Cells were cultured for 48 h as described in 'Materials and methods' section, and then treated for an additional 48 h with 0 or 300 ng/ml of angiogenin in the presence of 10% FCS; cells were enumerated via Coulter counting. (b) Cells were cultured for 48 h as described in 'Materials and methods' section, serum-starved for 24 h, and then treated for an additional 40 h with ^{3}H-thymidine and IGF1 (30 ng/ml) and

either 0 or 300 ng/ml of angiogenin. *Within a panel, asterisk (*) indicates mean differs ($P < 0.05$) from its respective control (0 ANG) mean. Values are means ± SEM of three separate experiments ($n = 6$).

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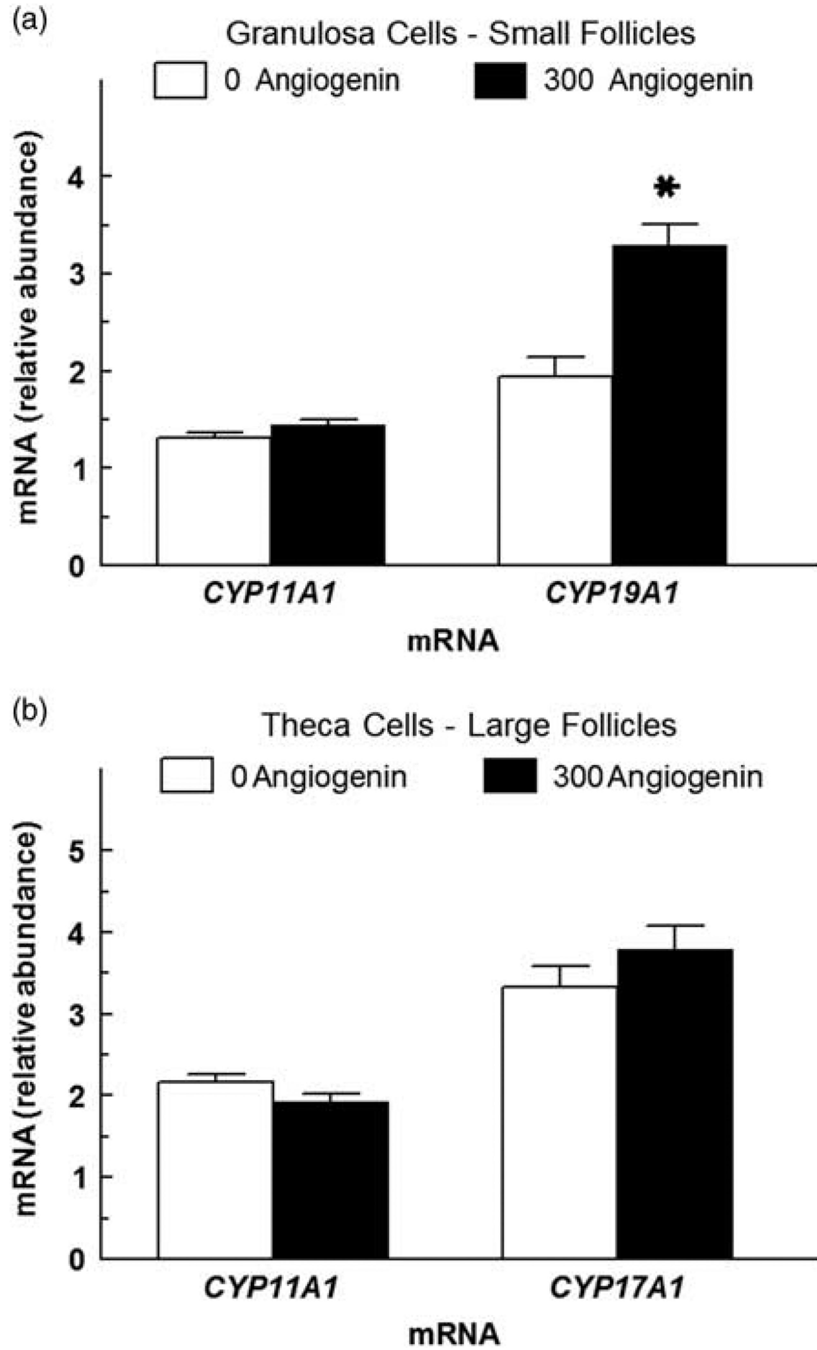

Figure 4.

Effect of angiogenin (ANG) on steroidogenic enzyme gene expression in granulosa and theca cells (experiment 7). (a) Effect of effect of ANG (300 ng/ml) on *CYP19A1* (aromatase) and *CYP11A1* (side-chain cleavage enzyme) mRNA abundance in small follicle granulosa cells. (b) Effect of ANG (300 ng/ml) on *CYP17A1* and *CYP11A1* mRNA abundance in large follicle theca cells. Granulosa cells from small follicles and theca cells from large follicles were cultured for 48 h in the presence of 10% FCS, and then treated in serum-free medium with ANG for 24 h. Granulosa cells were also concomitantly treated

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with 30 ng/ml of IGF1 and 30 ng/ml of FSH, and theca cells were concomitantly treated with 30 ng/ml of IGF1 and 30 ng/ml of LH. Values are means of three separate experiments (± SEM) and normalized to constitutively expressed 18S ribosomal RNA. *Mean differs ($P < 0.05$) from its respective control (0 ng/ml of ANG).

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Table 1

Effect of recombinant human angiogenin on granulosa and theca cell numbers in experiments 1 and 2

Experiments	FSH or LH	IGF1	Angiogenin (ng/ml) (cell numbers ($\times 10^5$/well))			
			0	30	100	SEM[1]
Experiment 1 – GC FSH	–	–	1.10[a]	1.03[a]	1.02[a]	0.08
Experiment 2 – TC LH	–	–	0.78[a]	0.85[a]	0.83[a]	0.07
Experiment 1 – GC FSH	+	–	1.07[a]	1.29[a]	1.24[a]	0.08
Experiment 2 – TC LH	+	–	0.84[a]	0.77[a]	0.93[a]	0.07
Experiment 1 – GC FSH	–	+	1.69[b]	1.74[b]	1.76[b]	0.08
Experiment 2 – TC LH	–	+	1.20[b]	1.18[b]	1.24[b]	0.07
Experiment 1 – GC FSH	+	+	2.15[c]	2.29[c]	2.42[c]	0.08
Experiment 2 – TC LH	+	+	1.21[b]	1.20[b]	1.22[b]	0.07

Granulosa cells (GC) from small (1 to 5 mm; experiment 1) and theca cells (TC) from large (8 to 22 mm; experiment 2) bovine follicles were cultured as described in ‘Materials and methods’ section, and treated for 48 h with FSH (for GC), LH (for TC) and/or IGF1 (0 or 30 ng/ml) and 0, 30 or 100 ng/ml of angiogenin.

[a,b,c]Within an experiment, means without a common letter differ ($P < 0.05$).

[1]SEM for $n = 6$.

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Table 2

Effect of 2-day treatment of 300 ng/ml of angiogenin (ANG) on basal and IGF1 plus FSH-induced progesterone production, estradiol production and numbers of granulosa cells from small (1 to 5 mm) and large (8 mm) bovine follicles of experiment 3

Size of follicle	Treatment	Dose of ANG (ng/ml)	Progesterone (ng/10^5 cells/24 h)	Estradiol (ng/10^5 cells/24 h)	Cell number (×10^5 per well)
Small	Control	0	58.0[a]	0.05[a]	0.64[a]
Small	Control	300	52.0[a]	0.05[a]	0.69[a]
Small	FSH+IGF1	0	85.6[c]	0.29[c]	1.46[b]
Small	FSH+IGF1	300	75.3[b]	0.21[b]	1.61[b]
SEM[1]			2.6	0.01	0.07
Large	Control	0	203.0[b]	0.58[a]	0.15[a]
Large	Control	300	142.0[a]	0.36[a]	0.20[a]
Large	FSH+IGF1	0	215.0[b]	2.63[b]	0.75[b]
Large	FSH+IGF1	300	188.0[b]	2.26[b]	0.82[c]
SEM[1]			13.0	0.27	0.02

[a,b,c]Within a column, means without a common superscript differ ($P<0.05$).

[1]SEM for $n = 6$.

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Table 3

Effect of 2-day treatment of 300 ng/ml of angiogenin (ANG) on IGF1- and IGF1 plus LH-induced progesterone production, androstenedione production and numbers of theca cells from small (3 to 6 mm) and large (8 mm) bovine follicles of experiment 4

Size of follicle	Treatment	Dose of ANG (ng/ml)	Progesterone (ng/10^5 cells/24 h)	Androstenedione (ng/10^5 cells/24 h)	Cell number (×10^5 per well)
Small	+IGF1	0	42.9^{a}	1.15^{a}	1.17^{b}
Small	+IGF1	300	42.0^{a}	1.07^{a}	1.16^{b}
Small	LH +IGF1	0	205.3^{b}	31.2^{b}	0.90^{a}
Small	LH +IGF1	300	190.4^{b}	28.0^{b}	1.11^{b}
SEM[1]			6.0	0.9	0.04
Large	+IGF1	0	28.8^{b}	1.06^{a}	1.13^{a}
Large	+IGF1	300	24.0^{a}	0.91^{a}	1.28^{b}
Large	LH +IGF1	0	44.0^{c}	4.00^{b}	1.21^{ab}
Large	LH +IGF1	300	40.8^{c}	3.94^{b}	1.20^{ab}
SEM[1]			1.6	0.17	0.05

[a,b,c] Within a column, means without a common superscript differ ($P < 0.05$).

[1] SEM for $n = 9$.