

Regulation of Angiotensin Type 2 Receptor in Bovine Granulosa Cells

Valério M. Portela, Paulo B. D. Gonçalves, Angela M. Veiga, Edmir Nicola, José Buratini, Jr., and Christopher A. Price

Laboratório de Biotecnologia e Reprodução Animal (V.M.P., P.B.D.G.), Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil; Departamento de Fisiologia (J.B.), Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo 18618-000 Brazil; and Centre de recherche en reproduction animale (V.M.P., A.M.V., E.N., C.A.P.), Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Quebec, Canada J2S 7C6

Angiotensin II (AngII) is best known for its role in blood pressure regulation, but it also has documented actions in the reproductive system. There are two AngII receptors, type 1 (AGTR1) and type 2 (AGTR2). AGTR2 mediates the noncardiovascular effects of AngII and is expressed in the granulosa cell layer in rodents and is associated with follicle atresia. In contrast, expression of AGTR2 is reported to occur only in theca cells in cattle. The objective of the present study was to determine whether AngII also plays a role in follicle atresia in cattle. RT-PCR demonstrated AGTR2 mRNA in both granulosa and theca cells of bovine follicles. The presence of AGTR2 protein was confirmed by immunofluorescence. Abundance of AGTR2 mRNA in granulosa cells was higher in healthy compared with atretic follicles, whereas in theca cells, it did not

change. Granulosa cells were cultured in serum-free medium, and treatment with hormones that increase estradiol secretion (FSH, IGF-I, and bone morphogenetic protein-7) increased AGTR2 mRNA and protein levels, whereas fibroblast growth factors inhibited estradiol secretion and AGTR2 protein levels. The addition of AngII or an AGTR2-specific agonist to granulosa cells in culture did not affect estradiol secretion or cell proliferation but inhibited abundance of mRNA encoding serine protease inhibitor E2, a protein involved in tissue remodeling. Because estradiol secretion is a major marker of nonatretic granulosa cells, these data suggest that AngII is not associated with follicle atresia in cattle but may have other specific roles during follicle growth. (*Endocrinology* 149: 5004–5011, 2008)

THE RENIN-ANGIOTENSIN SYSTEM (RAS) consists of the enzyme renin that converts angiotensinogen to angiotensin I (AngI), and angiotensin converting enzyme that cleaves the decapeptide AngI to the octapeptide AngII, which is considered to be the major bioactive peptide of the RAS (1). A local RAS has been described in several organ systems, including the ovary (1). To date, most data point to a role for AngII in the induction of ovulation. AngII infusion induced ovulation in perfused rabbit ovaries, and AngII antagonists inhibited ovulation in rabbits, rats, and cattle (2–6). AngII stimulated prostaglandin secretion from rabbit ovaries (2, 4) and bovine preovulatory follicles (7); this may be the mechanism through which AngII induces ovulation, because mice deficient in prostaglandin synthase 2 fail to ovulate (8).

AngII acts through two distinct transmembrane receptors. The effects of AngII on blood pressure regulation are mediated through the AngII type 1 receptor (AGTR1, also known as AT1), whereas other actions are generally mediated through the type 2 receptor (AGTR2, also known as AT2) (1). There appear to be considerable species differences in the function and location of AngII receptors in the ovary. In

rabbits, receptors are mostly AGTR2 and expressed in granulosa cells of preovulatory follicles, consistent with the role of AngII in ovulation (2). In rats, AGTR2 is also localized to granulosa cells but only in atretic follicles (9–11), which suggests that AngII is involved in the process of atresia. In support of this, AngII inhibited estradiol secretion from cultured rat and rabbit granulosa cells (12, 13), and AngII binding to AGTR2 is increased during atresia of rat granulosa cells *in vitro* (14). However, in cattle, theca but not granulosa cells are reported to contain AngII binding sites, predominantly AGTR2 (7, 15, 16).

The role of AngII in bovine follicle growth/atresia is unknown, although it may play a role in follicle atresia because prorenin concentrations are four to five times higher in atretic compared with healthy follicles (17). The apparent lack of AGTR2 in bovine granulosa cells is particularly intriguing because most signs of early atresia involve changes to granulosa rather than theca cells (18, 19). Therefore, the objectives of the present study were to measure AGTR1 and AGTR2 mRNA abundance in bovine granulosa and theca cells in atretic and nonatretic follicles and to determine whether AGTR1 and AGTR2 mRNA levels are regulated in granulosa cells. A further objective was to identify potential actions of AngII in granulosa cells using a nonluteinizing culture model (20).

Materials and Methods

Follicles

Ovaries were obtained from an abattoir and transported to the laboratory in saline on ice. Follicles 7–14 mm in diameter were dissected

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Abbreviations: AGTR1, AngII type 1 receptor; AngI, angiotensin I; BMP7, bone morphogenetic protein-7; E:P, estradiol to progesterone ratio; FGF2, fibroblast growth factor-2; FGFR, FGF receptor; RAS, renin-angiotensin system; SerpinE2, serine protease inhibitor E2; TTBS, Tris-Tween buffered saline.

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from the ovaries, and follicular fluid was aspirated, centrifuged, and frozen for steroid assay. The antral cavity was repeatedly flushed with cold saline, and granulosa cells were recovered by centrifugation at $1200 \times g$ for 1 min and pooled with the follicular fluid pellet. The remaining granulosa cells adhering to the follicle wall were removed by gently scraping with a blunt Pasteur pipette, and the theca layer was removed with forceps and washed in saline by passing repeatedly through a 1-ml syringe. The samples were collected into Trizol (Invitrogen, São Paulo, Brazil) and homogenized with a Polytron. Total RNA was extracted immediately according to the Trizol protocol.

Follicles were grouped by estradiol to progesterone ratio (E:P) of more than 1 (healthy) or less than 1 (atretic) in follicular fluid as described (21). Cross-contamination of theca and granulosa cells was tested by detection of mRNA encoding cytochromes P450 aromatase (Cyp19) and 17α -hydroxylase (Cyp17) in each sample by PCR as described (22). PCR was performed beyond the linear range for each gene (30 cycles), and theca samples with visibly detectable Cyp19 mRNA or granulosa samples with visibly detectable Cyp17 mRNA were excluded from the analysis.

Granulosa cell culture

The granulosa cell culture system was based on that described by Gutiérrez et al. (23) with slight modifications (24). All materials were obtained from Invitrogen Life Technologies (Burlington, Ontario, Canada) except where otherwise stated. Briefly, follicles were dissected from ovaries of abattoir origin and those with obvious signs of atresia (avascular theca or debris in antrum) were discarded. Follicles were separated into small (2–5 mm diameter), medium (6–8 mm), and large (>8 mm) size groups; 2- to 5-mm follicles are poorly differentiated and express low levels of Cyp19, 6- to 8-mm follicles are estrogenic but do not display markers of dominant follicles (LH receptors in granulosa cells), and healthy follicles more than 8 mm represent dominant follicles (18). Cells were harvested by repeatedly passing bisected follicle walls through a pipette, washed twice by centrifugation at $219 \times g$ for 20 min each, and suspended in supplemented α -MEM (25). Cell viability was estimated with 0.4% Trypan Blue stain. Cells were seeded into 24-well tissue culture plates (Sarstedt, Montreal, Quebec, Canada) at a density of 1×10^6 viable cells per well in 1 ml medium. Cultures were maintained at 37°C in 5% CO₂ in air for 6 d, with 700 μ l medium being replaced every 2 d.

To assess hormone regulation of AGTR1 and AGTR2 mRNA, cells from small follicles (2–5 mm) were cultured with graded doses of FSH or IGF-I. Under these conditions, the cells retain an estrogenic phenotype and do not luteinize (20); in contrast, cells from large follicles undergo luteinization and do not represent granulosa cells from growing follicles (26). In separate cultures, cells were cultured with IGF-I (10 ng/ml) with or without bone morphogenetic protein-7 (BMP7; 50 ng/ml) or fibroblast growth factor-2 (FGF2; 10 ng/ml); these treatments have been demonstrated to alter estradiol secretion in this model (27). We then tested the effect of graded doses of FGF7 or FGF10, because these factors have also been shown to alter granulosa cell function (25, 28). Medium samples were collected on d 6 and stored at –20°C until assay. For PCR assay, cells were lysed in Trizol for extraction of RNA and DNA. To measure AGTR2 protein, cells were collected into TES buffer (10 mM Tris-HCl, 1 mM EDTA, 250 mM sucrose) containing a protease inhibitor cocktail (Complete Mini; Roche, Mississauga, Ontario, Canada) for Western blotting.

To assess the potential role of AngII in differentiating granulosa cells, cells from small follicles (2–5 mm) were cultured in serum-free medium with or without FSH (1 ng/ml), and graded doses of AngII (human AngII; Sigma-Aldrich, Oakville, Ontario, Canada) were added with medium changes on d 2 and 4 of culture. Cultures were also performed with the AGTR2 agonist CGP-42112A (Sigma-Aldrich). Medium was harvested for steroid assay and Western blotting, and cells were harvested for RNA extraction. In separate cultures, the effect of AngII on the number of viable cells at the end of culture was assessed with the MTT assay (R&D Systems, Minneapolis, MN).

Nucleic acid extraction and semiquantitative RT-PCR

Total RNA and DNA were extracted using Trizol according to the manufacturer's instructions. Total RNA was quantified by absorbance

at 260 nm. Total RNA (1 μ g) was treated with deoxyribonuclease (Promega, Madison, WI) and reverse transcribed with Omniscript reverse transcriptase (Qiagen, Mississauga, Ontario, Canada). Bovine-specific primers were used for amplifying AGTR1 (sense, 5'-AAATACATTC-CCCCAAAGGC-3'; antisense, 5'-TGTGGCTTTGCTTTGTTGAG-3') (7), AGTR2 (sense, 5'-TTTGGCTACTCTTCTCTCTGG-3'; antisense, 5'-CATACTTCTCAGGTGGGAAAGC-3') (7), and serine protease inhibitor E2 (Serpine2) (26). Variability in mRNA amounts was assessed by amplifying cyclophilin A (PPIA) (29) in follicle samples or histone H2AFZ (sense, 5'-AGCGTATTACCCCTGGTCAC-3'; antisense, 5'-CCAGGCATCCTTTAGACAGT-3') in cell culture samples. An aliquot (2 μ l for AT1 and AT2 and 0.5 μ l for Serpine2) of the cDNA template was amplified by PCR using 0.25 μ l (2.5 U) *Taq* polymerase (Amersham Pharmacia Biotech Inc., Oakville, Ontario, Canada) in 20 μ l PCR buffer (Amersham) containing 0.1 mM dNTP mix and 0.2 μ M specific primers. After an initial denaturation step for 3 min at 94°C, target cDNA was amplified with a denaturation step at 94°C for 30 sec (AGTR1, AGTR2, and Serpine2) or 45 sec (H2AFZ), annealing for 45 sec at 64°C (AGTR2), 62°C (Serpine2), 60°C (AGTR1 and PPIA), or 58°C (H2AFZ) for 30 sec, and elongation at 72°C for 1 min. All reactions were terminated with a final elongation at 72°C for 5 min. Semiquantitative RT-PCR was validated for each gene product by sequencing amplicons and titrating input RNA amounts and cycle number. Reactions were performed for 28 cycles for AGTR1 and 29 cycles for AGTR2 and H2AFZ.

The PCR products were separated on 2% agarose gels stained with 0.001% ethidium bromide and visualized under UV light. Quantification of band intensity was performed with NIH Image software. Target gene mRNA abundance was expressed relative to H2AFZ (cell culture) or PPIA (*in vivo* samples) mRNA abundance.

Western blot

AGTR2 protein abundance in cell lysates was analyzed by Western blot. Total cell protein was quantified in a NanoDrop spectrophotometer and samples were subjected to electrophoresis in 12% denaturing polyacrylamide gels and electrotransferred onto nitrocellulose membranes. Equal loading was verified by Ponceau S staining. After blocking for 1 h in Tris-Tween buffered saline (TTBS) plus 0.5% skim milk, blots were incubated with 1:5000 rabbit anti-sheep AGTR2 (catalog no. 19134; Abcam Inc., Cambridge, MA) for 2 h with agitation, followed by three

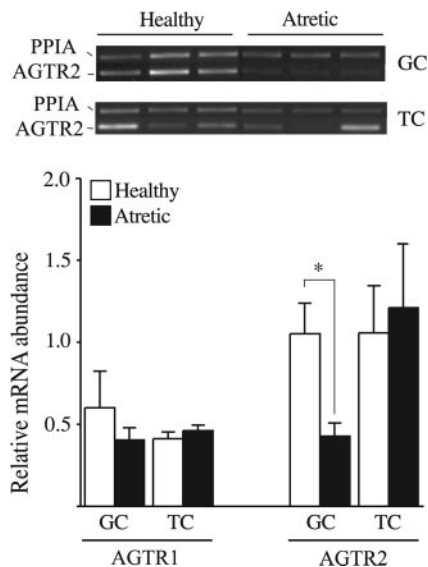


FIG. 1. Relative quantification of mRNA encoding AGTR1 and AGTR2 in theca cells (TC) and granulosa cells (GC) from follicles classified as healthy (E:P > 1; n = 7) or atretic (E:P < 1; n = 8). Steady-state mRNA levels were measured by semiquantitative RT-PCR and expressed relative to the housekeeping gene PPIA. The representative ethidium bromide-stained gel shows PPIA and AGTR2 amplicons from three healthy and three atretic follicles. Data are means $\pm$ SEM. *, $P < 0.05$.

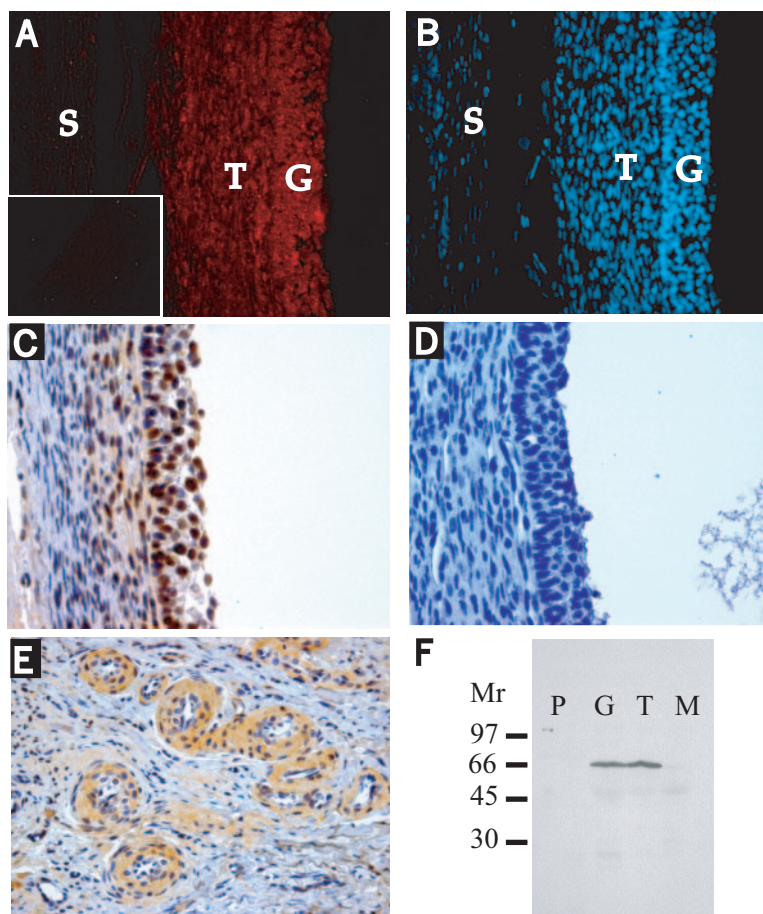


FIG. 2. Immunohistochemical localization of AGTR2 in bovine ovary. A and B, Immunofluorescent detection of AGTR2 in the follicle wall (A) and 4',6-diamidino-2-phenylindole staining in the same follicle (B). The inset in A is an adjacent section processed in the absence of AGTR2 antibody and shows weak background fluorescence. C and D, Colorimetric immunostaining confirms the localization observed by immunofluorescence (C) and absence of staining in the negative control (D). E, Ovarian blood vessels stained strongly for AGTR2. F, Western blot with the same antibody demonstrating a specific band in granulosa and theca cell lysates but not in pancreas or myometrium lysates. G, granulosa; M, myometrium; P, pancreas; S, stroma; T, theca. Photomicrographs were taken at $\times 400$ magnification (A–E).

washes with 0.2% TTBS. The blots were then incubated with 1:10,000 antirabbit-horseradish peroxidase (Amersham) for 1 h with agitation, followed by three washes with 0.2% TTBS. Finally, the blots were incubated in Immobilon Western chemiluminescent horseradish peroxidase (Millipore, Billerica, MA) for 5 min and were exposed to x-ray film for image analysis.

Serine protease inhibitor E2 (Serpine2, also known as protease nexin-1) is secreted by granulosa and not theca cells, and has been correlated with follicle health in cattle (30). We therefore measured the effects of AngII on the secretion of this protein from cultured granulosa cells by Western blot in spent culture medium as described (26).

Immunohistochemistry

To confirm AGTR2 protein localization in bovine follicles *in vivo*, we performed immunohistochemistry with the same antibody used in Western blotting. Ovaries were collected at a local abattoir and fixed in 4% paraformaldehyde, 0.25% picric acid in 0.1 M phosphate buffer, pH 7.3. After deparaffinization and antigen retrieval (boiling in 0.01 M sodium citrate and 0.1 M citric acid for 5 min), sections were blocked in 10% normal goat serum for 1 h, and then AGTR2 antibody (1:300) was applied for 18 h at 4°C. After the primary antibody, slides were washed in PBS and then incubated with biotinylated second antibody for 45 min. After two 5-min washes in PBS, a complex of avidin-peroxidase was applied for 45 min. Positive reactions were revealed by NovaRED (Vector Laboratories, Burlingame, CA) staining. Sections were then washed in distilled water and lightly counterstained with hematoxylin for 10 sec. Control sections were subjected to the same procedure, except that diluted rabbit serum replaced the first antibody.

Additional sections were examined by immunofluorescence, performed as above but using Cy3-conjugated second antibody (Jackson ImmunoResearch, West Grove, PA) and counterstaining with 4',6-diamidino-2-phenylindole. Negative controls were performed in the absence of primary antibody.

Steroid assay

Estradiol and progesterone were assayed in follicular fluid using iodinated tracers and antibodies furnished in the 3rd Generation Estradiol RIA (DSL 39100) and the DSL-3400 Progesterone RIA kits (Diagnostic Systems Laboratories Inc., Webster, TX), respectively. The standard curves and samples were diluted in PBS-gelatin. Intra- and interassay coefficients of variation were 7.4 and 13.5%, respectively, for estradiol and 6.8 and 7%, respectively, for progesterone. The sensitivities of the assays were 0.3 ng/ml for estradiol and 0.2 ng/ml for progesterone. Estradiol was measured in conditioned medium in duplicate as described (31), without solvent extraction. Intra- and interassay coefficients of variation were 6 and 9%, respectively. Progesterone was measured in conditioned medium in duplicate as described (32) with mean intra- and interassay coefficients of variation of 7.2 and 18%, respectively. The sensitivity of these assays was 10 and 4 pg per tube for

TABLE 1. Abundance of AGTR2 mRNA in bovine granulosa cells cultured in serum-free medium for 2, 4, or 6 d in the presence of 1 ng/ml FSH

Follicle size (mm)	Day of culture		
	2	4	6
Small (2–5)	0.53 $\pm$ 0.16	0.60 $\pm$ 0.18	0.83 $\pm$ 0.24
Medium (6–8)	0.47 $\pm$ 0.11	0.41 $\pm$ 0.04	0.49 $\pm$ 0.06
Large (>8)	1.13 $\pm$ 0.18 ^a	1.10 $\pm$ 0.29 ^a	1.96 $\pm$ 0.44 ^a

Steady-state mRNA levels were measured by semiquantitative RT-PCR and expressed relative to the housekeeping gene H2AFZ. Data are means $\pm$ SEM of three independent culture replicates each performed on different occasions.

^a Within day, means of large follicles differ significantly from those of small and medium follicles.

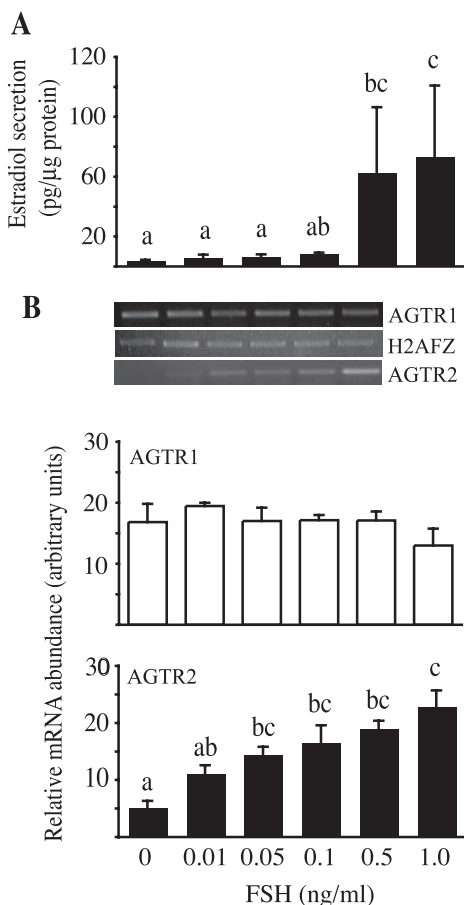


FIG. 3. Effect of FSH on estradiol secretion (A) and AGTR1 and AGTR2 mRNA abundance (B) in bovine granulosa cells *in vitro*. Cells from small follicles (2–5 mm diameter) were cultured in serum-free medium for 6 d with the stated doses of FSH. Steady-state mRNA levels were measured by semiquantitative RT-PCR, and representative gels are shown. Bars with no common letters are significantly different ($P < 0.05$). Data are means $\pm$ SEM of three independent culture replicates performed on different occasions.

estradiol and progesterone, equivalent to 0.3 and 20 ng/ μ g protein, respectively. Steroid concentrations in culture medium were corrected for cell number by expressing per unit mass of cell protein.

Statistical analysis

Treatments were applied to cultures of cells derived from each of three independent pools of ovaries (*i.e.* collected on separate occasions). Data that did not follow a normal distribution (Shapiro-Wilk test) were transformed to logarithms. Homogeneity of variance was tested with O'Brien and Brown-Forsythe tests. ANOVA was performed with JMP software (SAS Institute, Cary, NC) with treatment or follicle group as main effect and culture replicate (where appropriate) as a random variable in the F test. For mRNA analysis, housekeeping gene abundance was included as a covariate in the main effects model. Differences between means were tested with the Tukey-Kramer honestly significant difference test. Data are presented as means $\pm$ SEM.

Results

AngII receptors in bovine follicles

AGTR1 and AGTR2 were first surveyed in theca and in granulosa cells from healthy and atretic follicles of abattoir origin. Healthy follicles (size range 7–14 mm, $n = 7$) contained 1027 ± 529 and 29 ± 3 ng/ml estradiol and progesterone, respectively, and atretic follicles (size range 8–13 mm, $n = 8$) contained 3 ± 1 and 37 ± 7 ng/ml, respectively.

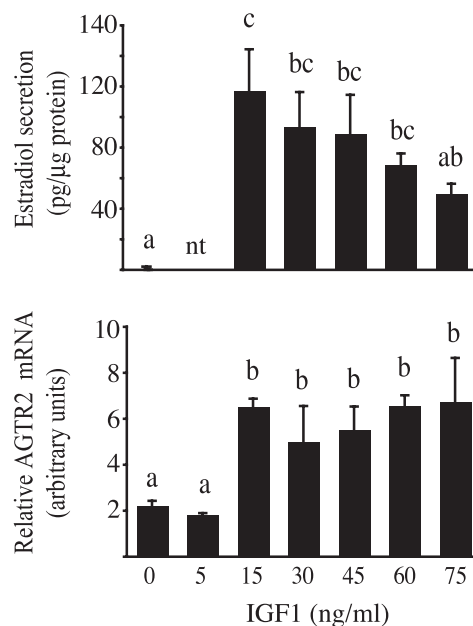


FIG. 4. IGF stimulates estradiol secretion and AGTR2 mRNA abundance in bovine granulosa cells *in vitro*. Cells from small follicles (2–5 mm diameter) were cultured in serum-free medium for 6 d with the stated doses of IGF-I. Steady-state mRNA levels were measured by semiquantitative RT-PCR. Bars with no common letters are significantly different ($P < 0.05$). Data are means $\pm$ SEM of three independent culture replicates performed on different occasions. nt, Not tested.

terone, respectively, and atretic follicles (size range 8–13 mm, $n = 8$) contained 3 ± 1 and 37 ± 7 ng/ml, respectively. Relative AGTR1 and AGTR2 mRNA abundance in theca cells was not different between healthy and atretic follicles (Fig. 1). In granulosa cells, AGTR2 mRNA abundance was significantly higher in healthy compared with atretic follicles, whereas AGTR1 mRNA levels did not differ (Fig. 1). AGTR2 protein was detected by immunohistochemistry in theca and granulosa cells of antral follicles of different sizes (Fig. 2). Strong staining was also observed in the wall of blood vessels, but staining was weak or absent in ovarian stroma or in sections processed without AGTR2 antibody. Staining was specific for AGTR2, because the antibody recognized a single band of the correct size in Western blot (Fig. 2).

Regulation of AGTR2 in granulosa cells

When cells from small, medium, and large follicles were cultured for 6 d in serum-free medium supplemented with 1 ng/ml FSH, the cells from large follicles exhibited greater abundance of AGTR2 mRNA compared with cells from small or medium follicles, but at levels that did not vary with time in culture (Table 1). We then employed a nonluteinizing granulosa cell culture model, using cells from small follicles, to examine the regulation of AGTR2 mRNA. FSH caused a dose-dependent increase in estradiol secretion and AGTR2 mRNA abundance (Fig. 3). Doses higher than 1 ng/ml did not further alter mRNA levels (not shown). AGTR1 mRNA abundance was not affected by FSH (Fig. 3).

IGF-I caused a biphasic increase in estradiol secretion with a diminished effect at the higher doses tested. Granulosa

TABLE 2. Effect of BMP7 on estradiol secretion and abundance of mRNA encoding AGTR1 and AGTR2 in bovine granulosa cells from small follicles (2–5 mm diameter) cultured in serum-free medium

Treatment	Relative mRNA abundance (arbitrary units)		Estradiol (pg/ μ g protein)
	AGTR1	AGTR2	
IGF (10 ng/ml)	10.3 $\pm$ 0.1	8.4 $\pm$ 0.6	85.8 $\pm$ 23.4
IGF (10 ng/ml) + BMP7 (50 ng/ml)	11.6 $\pm$ 0.3	15.8 $\pm$ 1.8 ^a	331.7 $\pm$ 73.5 ^a

Steady-state mRNA levels were measured by semiquantitative RT-PCR and expressed relative to the housekeeping gene H2AFZ. Data are means $\pm$ SEM of three independent culture replicates each performed on different occasions.

^a Means with BMP7 significantly different from those without BMP7.

AGTR2 mRNA abundance was stimulated by IGF-I without apparent down-regulation at higher doses (Fig. 4), and AGTR1 mRNA levels were not altered by IGF-I (not shown). Treatment of IGF-I-stimulated cells with BMP7 increased estradiol secretion and AGTR2 mRNA abundance relative to control cultures and did not affect AGTR1 (Table 2). Regulation of AGTR2 mRNA was reflected in changes in protein, because cellular AGTR2 protein abundance was significantly increased by FSH (1 ng/ml), IGF-I (10 ng/ml), or IGF-I plus BMP7 (50 ng/ml) ($P < 0.05$; Fig. 5).

Finally, we assessed the effects of three members of the FGF family on AGTR expression. FGF2, -7, and -10 inhibited estradiol secretion, and FGF7 and -10 but not FGF2 decreased AGTR2 protein abundance ($P < 0.01$; Fig. 6, A and B). To examine further the effect of FGF2, additional cultures were performed to measure AGTR mRNA. FGF2 did not significantly alter AGTR1 mRNA levels and caused a small but significant decrease in AGTR2 mRNA levels (Fig. 6C).

Effects of AngII on granulosa cells

AngII had no effect on FSH-stimulated estradiol or progesterone secretion or on the number of viable cells but inhibited SerpinE2 secretion at all doses tested (Fig. 7). To determine whether this was a result of altered steady-state

levels of SerpinE2 mRNA, cells were incubated with an effective dose (10 μ M) of AngII, which inhibited SerpinE2 mRNA abundance (6.9 ± 0.5 vs. 3.7 ± 0.1 arbitrary units for 0 and 10 μ M AngII, respectively; $P < 0.05$). The addition of the AGTR2 agonist CGP-42112A to FSH-treated cells inhibited SerpinE2 mRNA levels to the same degree as equimolar concentrations of AngII (Fig. 7). In the absence of FSH, AGTR2 mRNA levels are low (Fig. 3), and under these conditions, AngII did not alter SerpinE2 mRNA compared with treatment with FSH alone.

Discussion

AngII is suspected to play a role in follicular atresia in rodents because AngII inhibits granulosa cell estradiol secretion and AGTR2 is expressed only in the granulosa cells of atretic follicles (11, 13). In cattle, however, AGTR2 was reported only in theca cells (16), suggesting alternative roles for AngII in ruminants. This study provides three new lines of evidence that AngII may promote follicle growth rather than atresia in cattle. First, AGTR2 is expressed in bovine granulosa cells, and at higher levels in healthy compared with atretic follicles. Second, granulosa AGTR2 mRNA abundance is up-regulated by FSH and growth factors that promote follicle growth, and third, AngII does not inhibit estradiol secretion from granulosa cells *in vitro*.

Using RT-PCR, we detected AGTR1 and AGTR2 mRNA in both theca and granulosa cells. Previous studies investigating localization of AngII receptors in the bovine follicle reported [¹²⁵I]AngII binding predominantly or exclusively to the theca cell layer (15, 16). These ligand binding studies may not have been sensitive enough to detect AngII binding in granulosa cells, because no AngII binding was detected in ovarian blood vessels (16), a tissue rich in AngII receptors (33). We verified AGTR2 localization by immunohistochemistry and showed protein in both theca and granulosa layers, in agreement with the PCR data. Blood vessels within the ovarian stroma also stained positive for AGTR2, consistent with studies with sheep uterus (34). AGTR1 mRNA abundance in theca or granulosa cells did not differ between

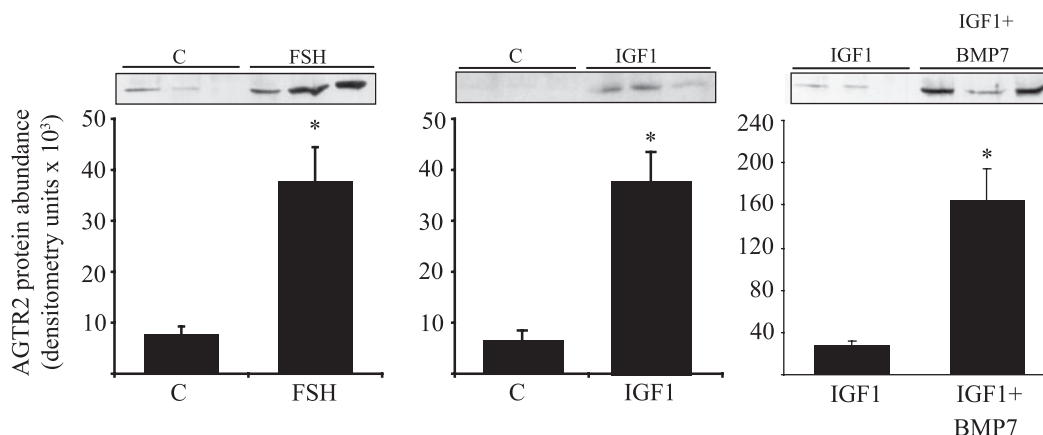


FIG. 5. FSH, IGF-I, and BMP7 stimulate AGTR2 protein abundance in bovine granulosa cells *in vitro*. Cells from small follicles (2–5 mm diameter) were cultured in serum-free medium for 6 d in the absence (C) or presence of FSH (1 ng/ml), IGF-I (10 ng/ml), or IGF-I plus BMP7 (50 ng/ml), and total cell protein was harvested for Western analysis of AGTR2 protein. Note the different scale of the y-axis for IGF-I plus BMP7 data; the blot shown was underexposed relative to other treatments because of the strong signal with BMP7. Bars with asterisks are significantly different from controls ($P < 0.05$). Data are means $\pm$ SEM of three independent culture replicates performed on different occasions.

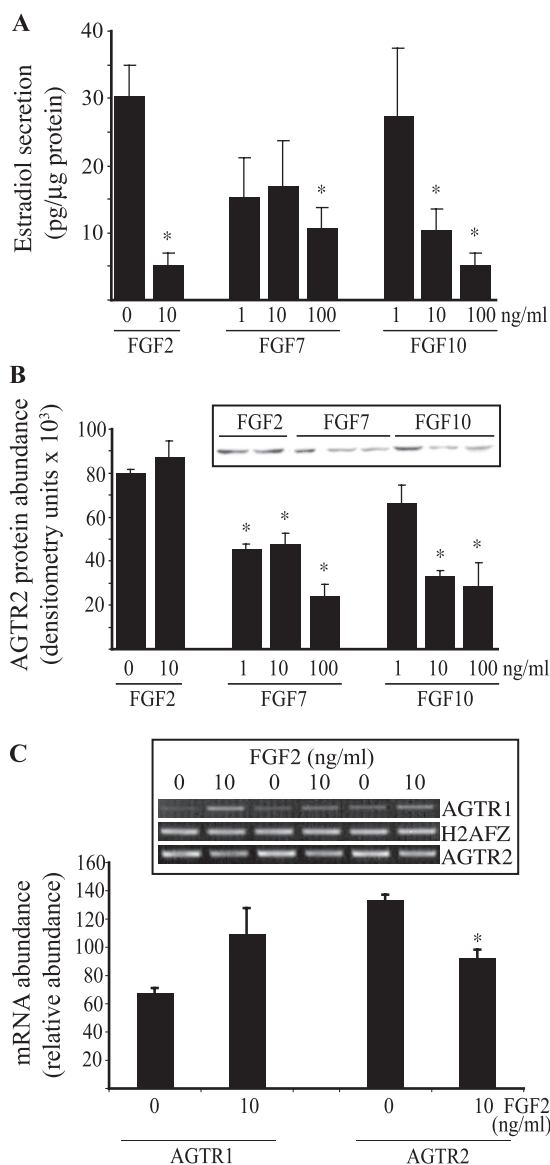


FIG. 6. A and B, The effect of FGF2, FGF7, and FGF10 on estradiol secretion (A) and AGTR2 protein content (B) in bovine granulosa cells *in vitro*. Cells from small follicles (2–5 mm diameter) were cultured in serum-free medium for 6 d in the presence of FSH (1 ng/ml) plus the stated doses of FGF, and total cell protein was harvested for Western analysis of AGTR2 protein. C, The effects of FGF2 on AGTR1 and AGTR2 mRNA levels were measured by RT-PCR. *Inset* in B is a representative Western blot showing samples from one replicate in the same order as in the graph, and *inset* in C shows PCR products for three replicate cultures. Bars with asterisks are significantly different from controls ($P < 0.05$). Data are means $\pm$ SEM of three independent culture replicates performed on different occasions.

healthy and atretic follicles, in agreement with data from bovine theca cells (7), but AGTR2 mRNA abundance was significantly down-regulated in granulosa cells of atretic follicles. This is in contrast to studies with rats that show significant up-regulation of AGTR2 in granulosa cells of atretic follicles (9, 10, 11). This discrepancy may reflect a physiological difference between species or the comparison of rat studies *in vivo* with bovine follicles collected at unknown stages of the estrous cycle. Certainly, there are significant

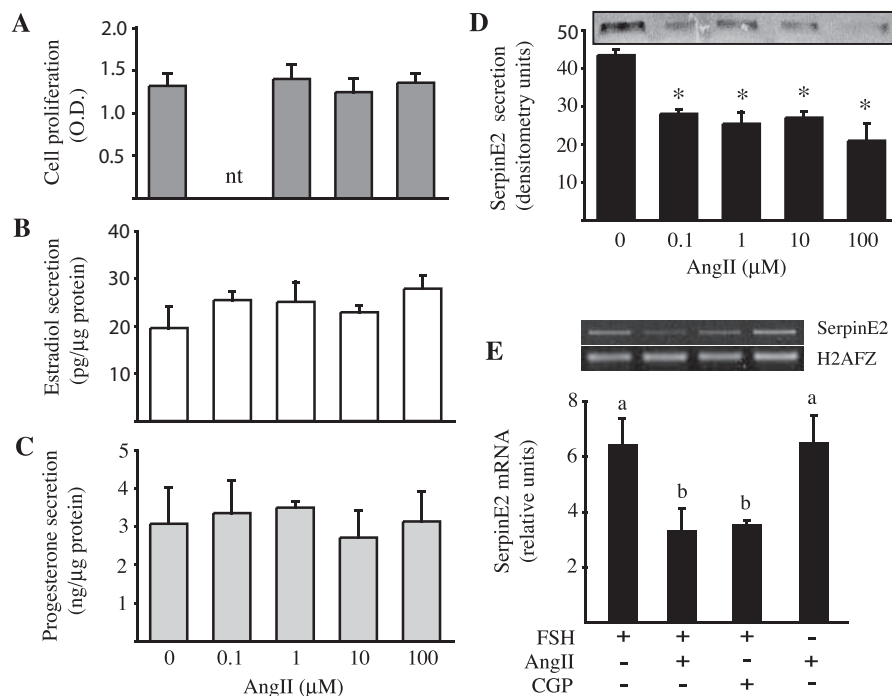
differences in follicle dynamics between cattle and rodents, the development of single *vs.* multiple ovulatory follicles being one of the most obvious (35). The requirement for follicle selection and dominance in cattle could explain alternative or additional actions of paracrine/endocrine factors such as AngII in this species compared with rodents.

Because AGTR2 mRNA levels were affected by follicle health in granulosa but not in theca cells, we used a serum-free granulosa cell culture system to investigate the regulation of AGTR2 mRNA and protein levels. In this culture system, FSH and IGF-I stimulate estradiol secretion and expression of genes encoding steroidogenic enzymes (20, 23, 24, 36) and, as demonstrated here, also stimulate AGTR2 but not AGTR1 mRNA abundance. These results are in agreement with studies showing up-regulation of AGTR2 by IGF-I in a rat fibroblast cell line (37) and vascular smooth muscle cells (38) but are in contrast to studies showing that FSH decreased AngII binding to rat granulosa cells in culture (14, 39). BMP7, another growth factor that stimulates estradiol secretion in this cell model (27), also increased AGTR2 mRNA levels. We are not aware of any other reports describing the effects of BMP7 on AGTR2 mRNA.

The stimulatory effects of FSH, IGF-I, and BMP7 on AGTR2 mRNA levels were reflected in similar stimulation of AGTR2 protein abundance. This coupling between mRNA and protein suggests that receptor protein levels are regulated transcriptionally, although the role of transcription rate and mRNA stability remains to be determined. Bearing in mind that the total cell receptor content does not necessarily reflect the number of receptors available at the cell surface, it would appear that endocrine/paracrine agents that affect AGTR2 mRNA levels are likely to have an impact on AGTR2 signaling. To assess further the role of growth factors, we tested the effect on cell AGTR2 content of three FGFs that have been shown to inhibit estradiol secretion from bovine granulosa cells, FGF2 (40), FGF7 (28), and FGF10 (25). FGF2 did not alter AGTR2 protein levels but decreased mRNA amount. The inhibitory effect on mRNA levels was small (~ 0.7 -fold change) compared with the stimulatory effects of FSH and IGF-I (3- to 4-fold change), which may account for the lack of effect of FGF2 on AGTR2 protein levels. Interestingly, FGF7 and FGF10 both inhibited AGTR2 protein levels. FGF2 and FGF7/10 may have different effects on cells because they activate different receptors. There are five known FGF receptor (FGFR) genes, and at least three of these undergo alternative splicing to give rise to functionally distinct receptors. FGF2 activates FGFR2c, FGFR3c, and FGFR4, whereas FGF7 and FGF10 activate predominantly FGFR2b (41, 42). We are not aware of any other reports of FGF regulation of angiotensin receptors.

The effect of AngII on granulosa cell function is unclear. AngII inhibited progesterone secretion from luteinized porcine granulosa cells (43) but increased progesterone from human granulosa-lutein cells (44). The response to AngII likely depends on the degree of luteinization/differentiation, because AngII inhibited progesterone secretion from human granulosa-lutein cells after 2 d culture but increased secretion after 4 d culture (45). Furthermore, AngII stimulated progesterone secretion from granulosa cells of diethylstilbestrol-treated rats (39) but had no effect on cells from pregnant mare

FIG. 7. Effects of AngII on granulosa cell function. A, Number of viable cells as measured by MTT assay; B and C, estradiol (B) and progesterone (C) secretion as measured by RIA; D, SerpinE2 secretion as measured by Western blot (representative blot shown); E, SerpinE2 mRNA abundance as measured by RT-PCR (representative gel shown). Cells from small follicles (2–5 mm diameter) were cultured in serum-free medium for 6 d in the presence of FSH (1 ng/ml), and the stated doses of AngII were added from d 2. E, Cells were also cultured with FSH plus AngII (10 μ M) or the AGTR2-specific agonist CGP-42112A (CGP; 10 μ M), or with AngII without FSH. Bars with asterisks are significantly different from controls ($P < 0.05$). Bars with no common letters are significantly different ($P < 0.05$). Data are means $\pm$ SEM of three independent culture replicates performed on different occasions. nt, Not tested.



serum gonadotropin-primed rats (46, 47). In the present study, we used a model of nonluteinizing, undifferentiated granulosa cells that represent the granulosa layer of growing follicles before the acquisition of LH receptors (20, 48). The present data show that AngII does not alter steroidogenesis or cell proliferation at this stage of follicle development in cattle.

We also measured the effect of AngII on amounts of SerpinE2 mRNA and secreted protein, because this protease inhibitor is correlated with follicle health (30). AngII has been shown to inhibit SerpinE2 secretion and expression in rat Schwann cells (49) and bovine granulosa cells (present study). The same effect was observed with the AGTR2-specific agonist CGP-42112A. In addition, when AGTR2 but not AGTR1 mRNA levels were reduced by the omission of FSH, AngII did not alter SerpinE2 mRNA levels. Although we cannot rule out an interaction between FSH and AngII that is independent of AGTR2, these data collectively point to AGTR2 as the receptor that mediates the effects of AngII on SerpinE2 secretion.

Previously, we have noted that hormones stimulating estradiol secretion from bovine granulosa cells also stimulate SerpinE2 secretion (27). The present study shows distinct regulation of SerpinE2 that was not accompanied by changes in estradiol secretion and demonstrates a very specific role for AngII. Although SerpinE2 is correlated with follicle health, it is unlikely that the AngII-induced decrease in SerpinE2 observed here resulted in apoptosis of granulosa cells, because 4 d culture with AngII affected neither estradiol secretion nor the number of viable cells. Estradiol secretion from bovine granulosa cells responds to inhibitory signals within 48 h of treatment (50, 51). The role of SerpinE2 in granulosa cell function remains to be elucidated.

In conclusion, the present data show for the first time that bovine granulosa cells express AGTR2 mRNA and protein

and that receptor levels are greater in larger, healthy follicles. This is in contrast to the situation in rats where AGTR2 is expressed only in atretic follicles. In addition, we demonstrate growth factor regulation of AGTR2 mRNA abundance such that endocrine/paracrine factors that increase estradiol secretion also increase AGTR2 mRNA levels. Although AngII did not affect granulosa cell steroidogenesis, it inhibited SerpinE2 protein and mRNA levels. Collectively, these data demonstrate a novel role for AGTR2 signaling in granulosa cells during ovarian follicle growth.

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Address all correspondence and requests for reprints to: C. A. Price, Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Caisse Postale 5000, St-Hyacinthe, Quebec, Canada J2S 7C6. E-mail: christopher.price@umontreal.ca.

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