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Gene Expression of Endothelin-1 in the Porcine Ovary: Follicular Development[1]

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ABSTRACT

We have investigated which follicular compartment and stage of follicular development are associated with endothelin-1 (ET-1) gene expression in the porcine ovary. The localization of mature ET-1 peptide and of its mRNA was determined by immunohistochemistry and by in situ hybridization. Stage of follicular development associated with ET-1 expression was investigated in terms of follicular class and occurrence of atresia. The latter was investigated by determining the occurrence of DNA fragmentation in apoptotic cells on adjacent sections to those used for ET-1 gene expression. Fifteen ovaries from 10 prepubertal pigs stimulated with gonadotropin were collected; a total of 1050 follicles were examined. Specific ET-1 immunoreactivity was restricted to the ovarian vasculature and to the granulosa cell compartment of antral follicles. The pattern of ET-1 mRNA expression was similar to that found for ET-1 immunoreactivity. Primordial, primary, and most secondary follicles did not express ET-1. The theca cell layer did not express ET-1 regardless of follicle developmental stage. ET-1 expression occurred with a significantly greater probability ($P < 0.001$ by the likelihood ratio test) in the granulosa cell compartment of antral follicles than in any other follicle class. Furthermore, in antral follicles, ET-1 expression occurred with a greater likelihood in large antral follicles than in small antral follicles ($P < 0.001$ by the likelihood ratio test). In small antral follicles, only 16.8% expressed ET-1; in contrast, 66.7% of large antral follicles exhibited ET-1 expression. It is interesting that in follicles in which ovulation had already occurred, intense ET-1 expression was found only in the prominent developing vasculature, the other cells present in the luteinized follicle did not display any ET-1 expression. The pattern of ET-1 gene expression observed in this study would be in agreement with our previous suggestion of a plausible physiological role for ET-1 in preventing premature progesterone production by granulosa cells of an antral follicle. The occurrence of atresia and expression of ET-1 in the same follicle was rare. Small and large antral follicles constituted 5.1% and 5.6%, respectively, of the examined follicles in this category. The majority of atretic follicles did not express ET-1 and, conversely, follicles that expressed ET-1 were not atretic. To the best of our knowledge, this is the first report in which large, nonatretic follicles are clearly identified as the population of follicles expressing ET-1. The results of this study delineate the follicular developmental stage and the compartment of when and where ET-1 may be physiologically meaningful.

follicle, follicular development, granulosa cells, ovary, ovulatory cycle

INTRODUCTION

Endothelin-1 (ET-1) appears to have a variety of alternative, additional biological actions in other tissues in addition to its potent vasoconstrictive properties on the vascular system. Several investigators have observed direct ET-1 actions in gonadal cells of several mammalian species. For example, ET-1 stimulated steroidogenesis in perfused ovarian follicles from immature rats treated with eCG [1]. Gonadotropin-stimulated steroidogenesis of cultured porcine [2, 3] and rat [4] granulosa cells was inhibited by ET-1. Endothelin-1 has also been shown to liberate inositol phosphates by rapidly increasing intracellular calcium ion concentrations and activating protein kinase C in swine granulosa cells [3, 5]. Endothelin-1 has been reported to have several direct actions on bovine follicular [6] and luteal cells [7]. On the basis of these observations, the possibility of a gonadal role for ET-1 has been raised [6–10]. It has been hypothesized that ET-1 may play some role in granulosa cell [6, 9] and luteal cell function [7, 8]; however, the specific gonadal role of ET-1 is not known. In this context, determining the ovarian source of ET-1 is a relevant experimental question.

In human and bovine ovaries, expression of ET-1 peptide and its mRNA have been reported; in humans, it is the granulosa cells that have been reported to contain ET-1 mRNA and the mature peptide [11]. In cows, the corpus luteum has been found to express both mRNA and the mature ET-1 peptide [10]. Indeed, it has been hypothesized that locally produced ET-1 may participate in the regression of bovine corpus luteum [7, 12]. In pigs, indirect evidence has implied that granulosa cells may be a gonadal source of ET-1 because its immunoreactivity was demonstrated in the culture medium of these cells [13].

Additional evidence for a gonadal physiological role for ET-1 has been provided by the finding that ET receptors are also expressed in gonadal cells. Messenger RNA encoding endothelin receptors have been demonstrated in granulosa cells of rat [14], cow [15], and human [11], as well as in cells of the bovine corpus luteum [16]. The existence of ET receptors in ovarian cells had been previously suggested because high-affinity and specific binding sites for ET-1 had been reported for rat [4], porcine [9], bovine [7], and human ovaries [17]. There is still some controversy about the type of ET receptor expressed in mammalian ovaries, but there is substantial evidence for the existence of gonadal ET receptors. The functionality of these receptors and the relevance of ET-1 in gonadal physiology are underlined by reports of direct actions of ET on several facets of ovarian granulosa and luteal cell physiology, including steroidogenesis [1–13].

Although granulosa cells appear to be a site of ET-1 synthesis and action, the intraovarian source of ET-1 in porcine ovary has not been properly investigated. Therefore, the objectives of this study were to determine the follicular compartment as well as the stage of follicular development associated with ET-1 gene expression. The localization of the mature ET-1 peptide and of its mRNA in porcine ovary

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was investigated by immunohistochemistry and in situ hybridization. The stage of follicular development associated with ET-1 expression was investigated by examining five types of follicles and by the occurrence of atresia. Gene expression of ET-1 and follicular atresia were investigated by determining the occurrence of apoptosis on adjacent sections to those used for ET-1 immunohistochemistry and in situ hybridization. We report that granulosa cells of nonatretic follicles are the exclusive follicular compartment in which ET-1 expression occurs, and that this expression begins at the antral stage, appears to be maximal in large antral follicles, and declines right after ovulation.

MATERIALS AND METHODS

Ovaries

Prepubertal pigs (65–95 kg body weight) were housed in confinement with ad libitum access to food and water. Sequential administration of eCG and hCG were used to stimulate follicular growth and to induce ovulation as reported elsewhere [18]. Protocols were approved by the Animal Care and Use Committee at West Virginia University. Briefly, gilts received an i.m. injection of 1000 IU eCG (Sigma Chemical Company, St. Louis, MO) followed by an i.m. injection of 750 IU hCG (Sigma). Ovulation occurred 36 h after hCG administration, and ovaries were then collected to obtain follicles at maximum development or after 48 h to observe follicles after ovulation.

Gilts were ovariectomized under tilemine and zolazepan anesthesia (Telazol, A.H. Robins, Richmond, VA). After tracheal intubation, the gilts were ventilated with halothane during ovariectomy. Both ovaries were removed and cut into 1.5 cm^3 pieces. Ovarian fragments were frozen on dry ice and stored at −80°C until sectioning. For sectioning, the frozen tissue was embedded in cryoform (International Equipment Co., Needham, MA), equilibrated at −20°C, and 10-μm sections were prepared using a cryostat. Frozen sections were mounted on gelatin and poly-L-lysine (Sigma)-coated slides, and stored at −80°C until assay.

Immunocytochemistry

A peroxidase-antiperoxidase (PAP) immunostaining procedure was carried out at room temperature (about 27°C, except during primary antibody incubation, which was performed at 4°C) using a Vectastain Elite kit (Vector Laboratories, Burlingame, CA) as described previously [19]. Briefly, microscope slides with mounted frozen ovarian sections were fixed in Sambonis fixative (2% paraformaldehyde, 0.2% picric acid in 150 mM phosphate buffer pH 7.5) for 30 min. The slides were then rinsed three times in Tris-buffered saline (TBS; 50 mM Tris, 1.5% NaCl pH 7.5) for 10 min. Nonspecific binding was reduced by a treatment with 0.3% milk, 1% normal goat serum (NGS; Sternberger Monoclonals Inc., Baltimore, MD) in TBS for 30 min. Slides were washed twice with TBS and incubated overnight with the primary antibody (1:500, 1HC-6901 rabbit anti-ET-1, Peninsula Laboratories, Belmonton, CA). At least two other commercially available ET-1 antibodies were used to corroborate the generality of our observations (rabbit-porcine ET-1 antiserum, Peptide Institute, Osaka, Japan; and monoclonal mouse anti-ET-1 antibody, Affinity Bioreagents, Golden, CO). These additional antibodies yielded similar results. The next day, the slides were washed three times for 10 min with TBS and then incubated for 30 min with the secondary antibody (anti-rabbit immunoglobulin G; Sternberger), applied at a 1:200 dilution in 1% NGS in TBS. To reduce endogenous peroxidase activity, the slides were incubated for 30 min in a 0.3% hydrogen peroxide-methanol solution. After three 10-min washes in TBS, the slides were incubated for 30 min with the rabbit PAP complex (Sternberger; at 1:200 dilution in 1% NGS in TBS). Slides were rinsed in TBS (three 5-min rinses) and treated with peroxidase enhancer solution (Biomedia Corp., Foster City, CA) for 1 min. Slides were immediately treated with 3,3′-diaminobenzidine (DAB) substrate (Biogenex, San Ramon, CA) and incubated for 5 min. Slides were rinsed in distilled water for 5 min; then dehydrated, cleared, and mounted. The stained sections were examined using an Olympus AX70 microscope (Warrendale, PA) equipped with Nomarski differential interference contrast (DIC) objectives. A positive reaction using this protocol is characterized by the deposition of a reddish-brown product at the site of the antibody-antigen-reaction. For photography, some of the luteal sections were counterstained with hematoxylin to facilitate identification of cellular structures. Images were recorded on Kodacolor 200 ASA film (Kodak, Rochester, NY). Control slides included diluted primary antibody that had been preincubated with 10 μM ET-1, normal rabbit serum instead of primary antibody, omission of chromogen, and omission of the secondary antibody. The positive reaction was abolished in all of these control slides.

Complementary DNA Templates to Generate Probes for ET-1 Suitable for In Situ Hybridization

The porcine ET-1 cDNA clone was provided by Dr. M. Yanasigawa from the Howard Hughes Medical Institute at the University of Texas. This clone consists of the full-length ET-1 cDNA subcloned into the *Eco*RI site of pUC118 plasmid [20]. This 1.8-kb insert was excised by *Eco*RI restriction enzyme digestion and used for subcloning in the pGEM-3Z vector (Promega Corp., Madison, WI). The 5′ end of the ET-1 insert went into the *Eco*RI site of the vector. The subcloned prepro-ET-1 cDNA in the pGEM-3Z plasmid consisted of 210 bp of the original prepro-ET-1 cDNA. Sense and antisense riboprobes of 210 bp were synthesized from the DNA templates prepared by digestion of the plasmid DNA with the restriction enzymes, *Hin*dIII or *Eco*RI, respectively. The riboprobes were labeled with digoxigenin-11-uridine-5′-trisphosphate (DIG-11 UTP, Roche, Indianapolis, IN) using an in vitro transcription kit (Ambion, Inc., Austin TX), and purified using a G-50 Micro Column (Amersham Pharmacia Biotech, Piscataway, NJ).

In Situ Hybridization

In situ hybridization was performed as previously described [19] with modifications our laboratory has found suitable for frozen sections of porcine ovaries. Frozen sections (10 μM) were cut in a Zeiss microtome cryostat (HM 505 E, Fisher Scientific, Pittsburgh, PA) and thaw-mounted onto gelatin and chrome alum-coated slides. Slides were air-dried and stored at −80°C until fixation and hybridization. The sections were dried for 30 min at 40°C and then fixed for 5 min in fresh 4% paraformaldehyde in 0.1 M sodium phosphate buffer. Subsequently, the slides containing the fixed tissue were rinsed as follows: twice in 0.1 M phosphate buffer for 5 min, twice in 100 mM glycine in phosphate buffer for 5 min, twice in 0.3% Triton-X-100 in 0.1 M phosphate buffer for 5 min, twice in 0.1 M trietha-

nolamine (pH 8.0) for 10 min, once in 0.1 M triethanolamine with 0.25% acetic anhydride (pH 8.0) for 5 min, and once in standard sodium citrate (1× SSC, 150 mM sodium chloride, and 15 mM sodium citrate pH 7.0) for 30 min. Prehybridization and hybridization buffers contained 50% formamide, 300 mM NaCl, 20 mM Tris (pH 8), 5 mM EDTA, 10 mM sodium phosphate buffer, 1× Denhardts solution, 10 mM dithiothreitol (DTT), and 500 μg/ml yeast transfer RNA. Slides were prehybridized for 2 h at 55°C in a moist chamber.

The labeled riboprobes for in situ hybridization studies, prepared as described earlier, were diluted (200 ng/slide) in hybridization buffer. The slides were incubated and hybridization proceeded at 55°C for 12–16 h in a moist chamber. Following hybridization, the slides were treated with 20 μg/ml RNase A (Ambion) in 10 mM Tris pH 8.0, 1 mM EDTA, and 0.5 M NaCl (i.e., TEN) for 30 min at 37°C to remove all single-stranded RNA, thus leaving only riboprobe-RNA hybrids. The slides were then rinsed in TEN and subjected to consecutive 30-min washes of 2×SSC/1 mM DTT, 1×SSC/1 mM DTT, and 0.5×SSC/1 mM DTT at room temperature. The final wash with 0.5×SSC/1 mM DTT was performed at 60°C for 30 min.

A detection protocol using sheep anti-digoxigenin-alkaline phosphatase (Roche) complex was used. Briefly, the slides were washed twice for 10 min in buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl). A blocking solution (buffer 1 containing 0.1% Triton X-100 and 2% normal sheep serum, NSS) was applied and the slides were incubated for 30 min. Sheep anti-digoxigenin-alkaline phosphatase (1:400 in 0.1% Triton-X 100, 1% NSS in buffer 1) was applied to the slides for 2 h. The slides were washed twice for 10 min in buffer 2 (100 nM Tris-HCL pH 9.5, 125 mM NaCl, 50 mmol $MgCl_2$). The substrate solution (Fast Red, Roche) was prepared by adding one Fast Red tablet per 2 ml of 100 mM Tris HCl pH 8.2. The slides were incubated with 150 μl of substrate until the desired color signal was achieved. The slides were washed in distilled water and covered with crystal mount (Biomedia). Specific controls for the in situ hybridization experiments included 1) hybridization with labeled sense (rather than antisense) probes, 2) pretreatment of slides with RNase to degrade cellular RNA, and 3) hybridization with a labeled antisense probe and a 100-fold molar excess of unlabeled antisense probe. Controls one and two did not contain signal above background. The signal in control three was drastically reduced due to competition for cellular labeling by unlabeled probe.

Determination of Atretic Follicles

Because it is now known that during atresia cells are lost by the apoptotic form of cell death, detection of apoptosis was used to determine follicular atresia. The effectiveness of in situ 3′-end labeling of endonuclease-cleaved DNA on tissue sections to detect apoptosis has been previously shown [21]. Calf thymus terminal transferase (TdT) and biotinylated deoxyuridine trisphosphate (b-UTP, Boehringer-Mannheim, Indianapolis, IN) was used for the in situ 3′-end labeling. Frozen ovarian sections were fixed for 10 min in 10% buffered formalin. After rinsing in TBS, the sections were equilibrated for 10 min at room temperature in TdT buffer (1 mM potassium cacodylate, 125 nM Tris-HCl pH 6.6 containing 2,5 mM $CoCl_2$ and 1.25 mg/ml BSA). The end labeling reaction was carried out at 37°C for 1 h in a humidified chamber with TdT (100 U/ml) and b-dUTP (10 nM/ml) in TdT buffer. Enzyme was absent in the control slides. To quench endogenous peroxidase activity, slides were incubated in 0.3% hydrogen peroxide and methanol solution for 30 min at room temperature. The Vecstain avidin-biotin complex and DAB procedure were used as detection system. The slides were treated with xylene and permount before adding microscope slide coverslips.

Statistics

Data were evaluated by the likelihood-ratio test [22, 23].

RESULTS

Fifteen ovaries collected from 10 pigs were used in these studies; a total of 1050 porcine follicles were examined. Immunoreactivity of ET-1 was restricted to the vasculature and the granulosa cell compartment of antral follicles (Fig. 1, A and D). This immunoreactivity was specific because it was abolished when the primary antibody was preincubated with excess ET-1 or when the primary antibody was omitted (Fig. 1B). Primary antibodies against ET-2 and ET-3 were not effective in generating immunoreactivity in similar areas (data not shown). In the vasculature, ET-1 immunoreactivity was found in Heliceal vessels of the ovarian medulla, as well as in the microvasculature surrounding individual follicles. Both the endothelial and smooth muscle cells of Heliceal vessels were immunoreactive for ET-1 (Fig. 1D).

In ovarian follicles, ET-1 immunoreactivity was observed with a significantly greater likelihood in antral follicles ($P > 0.001$ by the likelihood ratio). Primordial, primary, and most secondary follicles were virtually negative in this assay (Fig. 2C and Table 1). ET-1 immunoreactivity was absent in 113 primordial and 329 primary examined follicles (Table 1). Only 11 out of 257 secondary follicles (4.3%) were positive for the mature ET-1 peptide. Of 232 small antral follicles examined, 39 (16.8%) were ET-1 immunopositive, and 66 out of 99 large follicles showed intense immunoreactivity (Table 1). In antral follicles, the likelihood of observing ET-1 immunoreactivity was significantly greater in large antral follicles than in small ones ($P > 0.001$ by the likelihood ratio). In addition, in small antral follicles, it was common to find that granulosa cells that were farther away from the basement membrane were the ones that had ET-1 immunoreactivity, whereas the ones closer to the basement membrane (i.e., the least differentiated), were not immunoreactive. In contrast, in large antral follicles, immunoreactivity was found throughout the granulosa cell layer (Fig. 2, A and B). The theca cell layer was never immunoreactive, regardless of the developmental stage of the follicle (Figs. 1 and 2).

In 20 follicles in which ovulation had already occurred, intense immunoreactivity was visualized only in the prominent developing vasculature (Fig. 2D, arrows). The other cells that were present in an early luteinized follicle were not immunoreactive for ET-1 (Fig. 2D, arrowheads).

The granulosa cells of large antral follicles with ET-1 immunoreactivity also expressed mRNA that encoded ET-1 (Fig. 3). The specificity of this hybridization is shown in Figure 3A, in which the sense probe was not able to generate a hybridization signal. The theca cells of these follicles did not express any hybridization with the specific ET-1 probe (Fig. 3).

The highest incidence of atresia was observed in small antral follicles; 93 out of 232 follicles examined at this

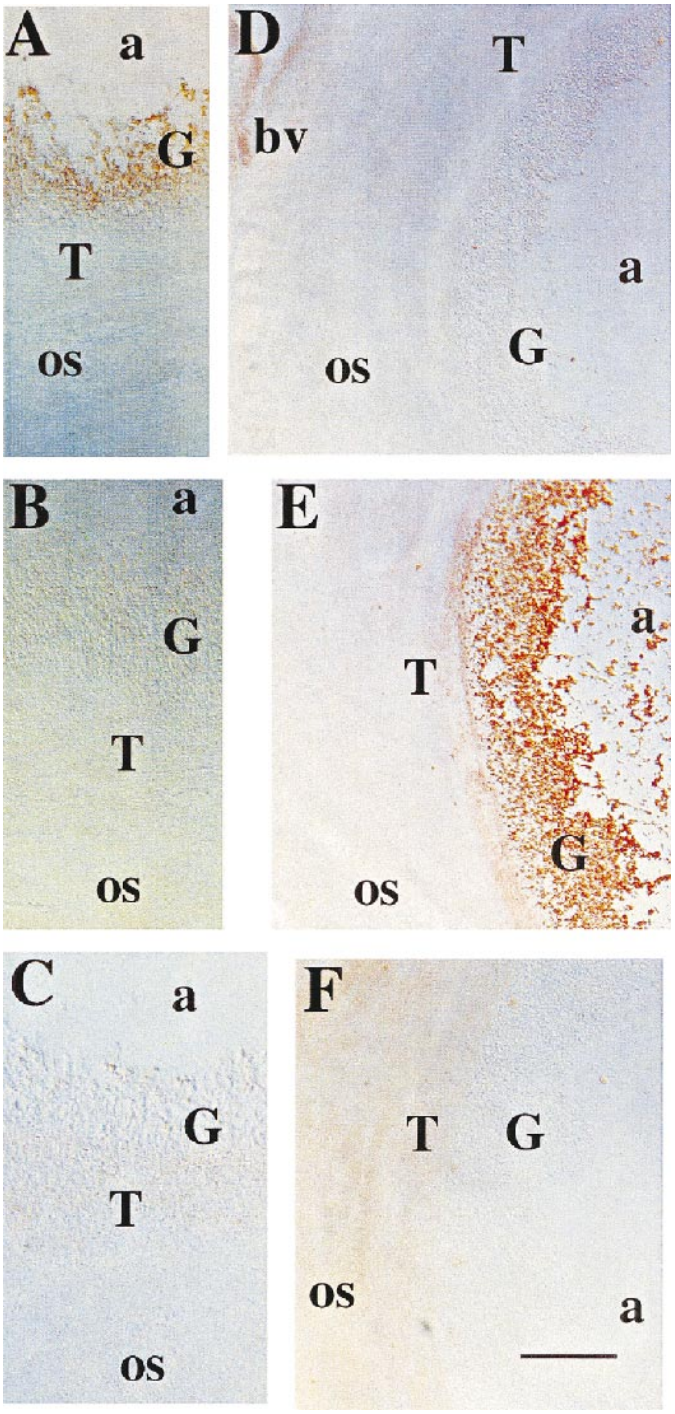


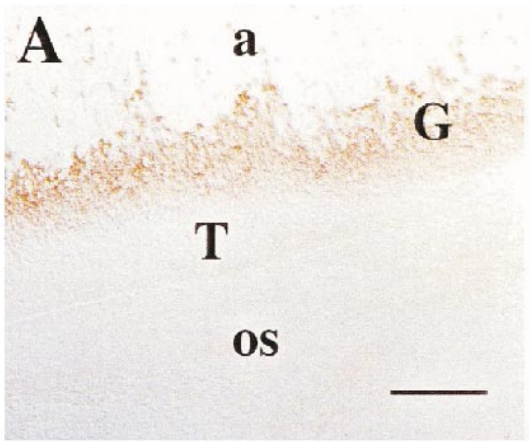
FIG. 1. Immunoreactivity of ET-1 and in situ 3′-end labeling of two large antral follicles. **A, B, C**) Three consecutive sections of a large antral follicle. The section in panel A was immunostained for ET-1 as described in *Materials and Methods*. ET-1 immunoreactivity is present in the granulosa cell (G) layer but absent in the theca cell layer (T) and in the ovarian stroma (os). The specificity of the ET-1 immunostaining shown in **A** is demonstrated in **B** in which the staining was abolished by substitution of the primary antibody with normal rabbit serum. Panel **C** shows a section of this follicle stained with the 3′-end labeling technique described in *Materials and Methods* for detecting DNA fragmentation. The lack of staining in this protocol indicates that this follicle, which expresses the ET-1 gene, is not atretic. **D, E, F**) Three consecutive sections of another large antral follicle. The section shown in **D** was immunostained for ET-1. The only immunoreactivity in this section is seen in the upper left corner of the panel, in the wall of a blood vessel (BV). The section in panel E was tested for DNA fragmentation. The intense red-brown color in the granulosa cell layer of this follicle indicates DNA fragmentation in these cells. No DNA fragmentation is seen in the theca cell layer or in the ovarian stroma of this atretic follicle without ET-1 immunoreactivity. Panel **F** shows that when the terminal transferase enzyme is omitted from the incubation, the color formation is eliminated, demonstrating the specificity of this protocol. Sections were photographed using Nomarski DIC microscopy. Bar in **F** = 200 μm.

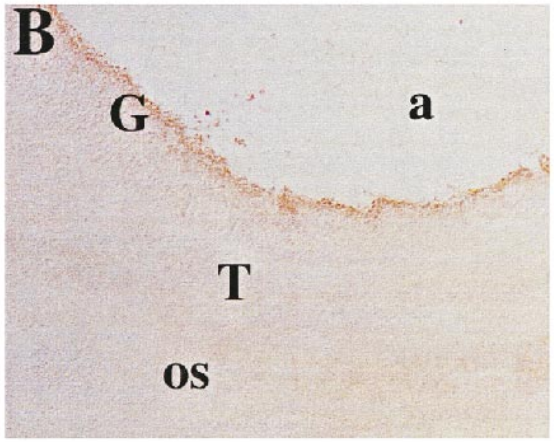
stage, or 40.1%, were undergoing apoptosis (Table 1). Secondary and large antral follicles had an occurrence of atresia of 19.8% and 20.2% respectively (Table 1). In primary follicles, 19 out of 329 or 5.8% were atretic, whereas only 2 out of 113 (1.8%) primordial follicles were undergoing apoptosis (Table). The occurrence of ET-1 immunoreactivity and apoptosis in the same follicle was rare. Small and large antral follicles had a small (5.6% and 5.1%, respectively) subpopulation of follicles in this category. Atretic follicles were usually not immunonegative for ET-1 and vice versa (Table 1). Primordial follicles exhibited the highest percentage (98.2%) of not having ET-1 immunoreactivity or atresia, which progressively declined to 8.8% (8 out of 99) in large antral follicles (Table 1). The specificity of in situ 3′-end labeling of endonuclease-cleaved DNA as a means of detecting apoptosis is shown in Figure 1, E and F. Whereas granulosa cells shown in Figure 1E are heavily apoptotic, the theca cells and the ovarian stroma are not.

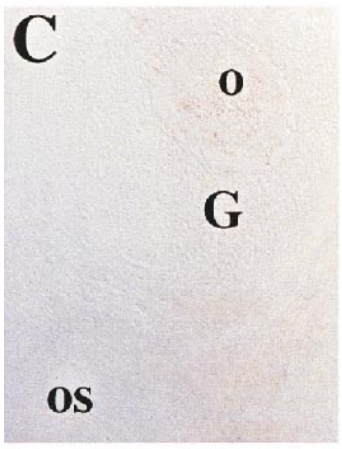
DISCUSSION

In this study we have demonstrated that 1) ET-1 gene expression in ovarian follicles is initiated around the antral stage, it is increased as follicles progress toward the periovulatory stage, and it decreases immediately after ovulation; 2) the granulosa compartment is the exclusive cellular component of the follicle in which ET-1 expression occurs; and 3) the occurrences of atresia and ET-1 gene expression are two events of follicular development that occur in two separate populations of ovarian follicles.

The simultaneous immunodetection and in situ hybridization of ET-1 in the granulosa compartment of the follicle supports our interpretation that gene expression of ET-1 indeed occurs in these cells, and that we are not just detecting the presence of the peptide. The pattern of ET-1 gene expression observed in this study would be in agreement with our previous suggestion of a plausible physiological role for ET-1 in preventing premature progesterone production by the granulosa cells of an antral follicle [5]. The results in the present study expand this idea by determining that this regulation by ET-1 is an autocrine type of integration. In this context, a relevant observation is the recent report by Acosta et al. [15] that found ET-1 to have a dual effect on steroidogenesis; it had an inhibitory effect on progesterone secretion, and a stimulatory action on estradiol secretion in a microdialyzed mature bovine follicle. Although granulosa cells readily produce progesterone in vitro, they primarily secrete estradiol during the follicular phase of the cycle. With progesterone playing such an important role in establishing the endocrinology that regulates the ovarian cycle, its timely synthesis and secretion is of crucial importance. Enodothelin-1 could be one of several local factors that ensures this timely production of progesterone. It is interesting that ET-1 has also been shown to have a negative effect on progesterone production by cells of the bovine corpus luteum [16]. In fact, in cows, it has been suggested that ET-1 mediates the luteolytic effects of prostaglandin $F_{2\alpha}$ [7, 12]. We do not know if steroidogenic cells of the porcine corpus luteum also regain the ability to







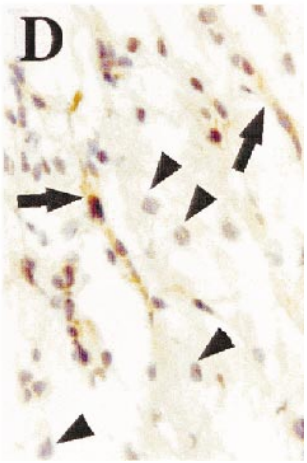


FIG. 2. ET-1 immunoreactivity as a function of follicular development. **A, B, C**) Three follicles immunostained for ET-1 as described in *Materials and Methods.* Staining for ET-1 is indicated by the red-brown color. The labels are granulosa cell (G) layer, theca cell layer (T), ovarian stroma (os), antrum (a), and oocyte (o). Panel **C** shows a very large antral follicle with intense ET-1 immunoreactivity throughout the granulosa cell layer of the follicle. Panel **B** shows ET-1 immunoreactivity in a small antral follicle. The red-brown color is seen in the granulosa cells, but not all the granulosa layer is stained; only the more distal from the basement membrane. Panel **C** shows a secondary follicle in which no immunoreactivity can be detected, indicating lack of ET-1 gene expression. **D**) A postovulatory porcine follicle immunostained for ET-1. Staining for ET-1 is localized to the developing vasculature (arrows). All other cells that resemble the granulosa-derived large luteal cells do not display immunoreactivity for ET-1 (arrowhead). Sections were photographed using Nomarski DIC microscopy. Bar in **A** = 100 μm.

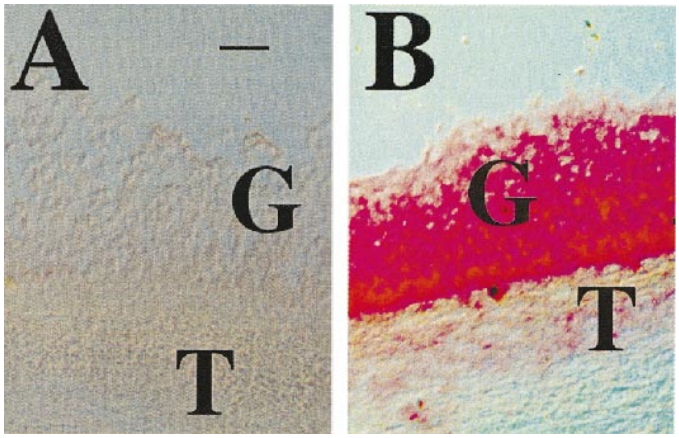


FIG. 3. Detection of ET-1 mRNA by in situ hybridization in sections of porcine follicles with an antisense probe. Red color denotes hybridization of the probe to the target mRNA in the experimental tissue. **A**) Sense RNA probe did not generate any hybridization signal in the tissue. **B**) Intense hybridization signal in the granulosa cell layer of the follicle when antisense RNA probe was used. Ovarian stroma and theca cells did not display any hybridization signal. Sections were photographed using Nomarski DIC microscopy. Bar in **A** = 100 μm.

express ET-1. In bovine corpus luteum, it has been reported that these steroidogenic cells express both the ET-1 and the ET-receptor genes [7, 10, 16]; however, in a previous study, we were able to detect ET-1 binding sites in endothelial cells but not in steroidogenic cells of porcine corpus luteum [9]. Whether this represents a species difference or reflects a technical problem is not yet clear; nevertheless, we have been able to detect ET-1 immunoreactivity in the steroidogenic cells of bovine corpus luteum (unpublished information). Further studies are necessary to clarify this discrepancy. In any case, the fact that ET-1 gene expression was observed in vascular endothelial cells of postovulatory follicles raises the possibility that ET-1 plays a role in the neovascularization that characterizes the differentiating corpus luteum or in the regulation of blood flow in this gland.

An interesting possibility that should be considered is that ET-1 may take part in the local cascade of events that are triggered by the LH surge during ovulation. This possibility appears likely, given the report that ET-1 stimulates prostaglandin secretion [15] and that in vitro, LH stimulates ET-1 release in bovine mature follicles [6].

The large antral follicles studied here could be divided into two main groups, those that had ET-1 expression and those that were atretic. The meaning of this observation is speculative at this time, but future studies should consider some of the following possibilities. This observation could simply mean that atretic follicles lose their ability to produce ET-1 as a secondary consequence of losing LH responsiveness. An alternative possibility is that this observation could indicate that ET-1 gene expression has an antiapoptotic function, and it may be important in the recruitment and selection from a pool of growing follicles; that is, the large antral follicles that express ET-1 represent the follicles that will ovulate. In support of this interpretation, the number of follicles encountered in this group match the number of follicles expected to ovulate with the follicular-stimulating protocol used in this study. However, there were two smaller groups of follicles present in the large antral follicles, a small group with neither ET-1 gene expression nor atresia; and an even smaller group that had both ET-1 gene expression and atresia. It could be argued that the group of large antral follicles with neither ET-1 gene ex-

TABLE 1. ET-1 immunoreactivity and occurrence of atresia in five classes of ovarian follicles.

	ET-1	Atresia only	ET-1 and atresia	Neither
Primordial	0/113	2/113 (1.8%)	0/113	111/113 (98.2%)
Primary	0/329	19/329 (5.8%)	0/329	310/329 (94.2%)
Secondary	11/257 (4.3%)	51/257 (19.8%)	2/257 (0.8%)	193/257 (75.1%)
Small antral	39/232 (16.8%)[a]	93/232 (40.1%)	13/232 (5.6%)	87/232 (37.5%)
Large antral	66/99 (66.7%)[a]	20/99 (20.2%)	5/99 (5.1%)	8/99 (8.8%)

[a] $P < 0.001$ by the likelihood ratio.

pression nor atresia represents the selected group of follicles for ovulation; however, this appears unlikely because the protocol used in this study to stimulate follicular development induces an ovulation rate of about 5–8 follicles per pig. The number of follicles found in this group of large antral follicles was much smaller than predicted from this known ovulation rate. The fact that we observed a very small group of follicles expressing ET-1 and undergoing atresia may just mean that ET-1 gene expression, albeit necessary to prevent apoptosis, is not the only factor involved in preventing it. It is likely that many signaling molecules regulate follicular atresia.

It is interesting that in those follicles in which the granulosa cell layer was clearly apoptotic, the theca cell layer did not show any signs of DNA fragmentation. This observation highlights the possibility that even in these atretic follicles, the theca cell layer may still be performing some activity and that the follicle in general may still perform some function.

To the best of our knowledge, this is the first report in which large, nonatretic follicles are clearly identified as the population of follicles that express ET-1. The results of this study delineate the follicular developmental stage and the compartment in which and when ET-1 may be physiologically meaningful. It is in this cell compartment of these follicles in which a more detailed characterization of the gene regulation, which is necessary for ET-1 signaling, should be carried out in order to understand the details of the potential role of this novel ovarian regulator.

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