***In situ* analysis of the changes in expression of ovarian inhibin subunit mRNAs during follicle recruitment after ovulation in pigs**

W. M. Garrett¹, S. O. Mack², R. M. Rohan³ and H. D. Guthrie^{1*}

¹*Germplasm and Gamete Physiology Lab, Agricultural Research Service, United States Department of Agriculture, Beltsville, MD 20705, USA;* ²*Department of Physiology and Biophysics, Howard University, College of Medicine, Washington DC 20059, USA;* and ³*Department of Surgery, Children's Hospital, Boston, MA 02114, USA*

In situ hybridization was used on frozen tissue sections with digoxigenin-labelled antisense riboprobes to inhibin/activin α and β_A subunits to determine whether inhibin/activin subunit mRNA expression was associated with development of growing, steroidogenically active follicles during follicle recruitment after ovulation. Cell proliferation-associated nuclear antigen Ki-67 protein and cytochrome P450 aromatase expression in granulosa cells were determined immunohistochemically and used as markers for granulosa cell proliferation and steroidogenesis, respectively, on days 3, 5 and 7 after the onset of oestrus. The amounts of inhibin/activin α and β_A subunit mRNA and P450 aromatase protein were greater (102, 93, and 238%, respectively; $P < 0.05$) in medium than in small non-atretic follicles and were positively correlated with Ki-67 and with each other. Inhibin/activin α and β_A mRNA, P450 aromatase, and Ki-67 in granulosa cells were reduced by 66–83% ($P < 0.001$) in atretic follicles compared with non-atretic follicles. In addition, inhibin/activin α and β_A mRNA and P450 aromatase in small (1–2 mm) non-atretic follicles decreased ($P < 0.05$) between day 3 and day 7 independently of morphological or biochemical signs of atresia. The pattern of inhibin/activin subunit mRNA expression supports the notion that activin and inhibin have roles in growth and steroidogenesis in follicle recruitment during the early luteal phase of the oestrous cycle.

Introduction

During the follicular phase of the oestrous cycle in pigs, growth of medium size follicles (3–5 mm in diameter) is suppressed until recruitment of follicles is initiated on day 3 of the next oestrous cycle (day 0 = first day of oestrus) (Guthrie *et al.*, 1995a). Initially, the amount of atresia is low, 5% or less on days 3 and 5; however, by day 7, approximately 50% of the follicles have become atretic (Guthrie *et al.*, 1995b; Garrett and Guthrie, 1996). Coincident with increased atresia, there is evidence of decreased granulosa cell proliferation (Garrett and Guthrie, 1996), increased granulosa cell apoptosis, and decreased production of androstenedione and oestradiol (Guthrie *et al.*, 1995b; Garrett and Guthrie, 1996). These changes in the pattern of atresia, follicle replenishment, and steroidogenesis in nonovulatory follicles during the pre- and post-ovulatory periods in pigs are associated with changes in secretion of FSH (Guthrie *et al.*, 1995a) that may be regulated by ovarian inhibin secretion (de Jong, 1988; Findlay, 1994).

Inhibin, a dimeric protein composed of an α subunit and one of two possible β subunits, is produced in the granulosa cells of ovarian follicles, and functions as a negative

regulator of pituitary FSH secretion (de Jong, 1988; LaPol and Hsueh, 1991). Apart from the initial primary role of inhibin as an endocrine regulator of pituitary function, there is evidence that inhibin and activin, a dimer of inhibin/activin (I/A) β subunits, act as intra-ovarian autocrine or paracrine factors, regulating multiple facets of follicular development (Mather *et al.*, 1992; Hillier and Miro, 1993a; Findlay, 1994; Ford and Howard, 1997). Multiple molecular forms of inhibin are detectable in pig follicular fluid during both the follicular and the early luteal phase of the pig oestrous cycle, both in atretic and non-atretic follicles (Guthrie *et al.*, 1997). The predominant forms are less abundant in atretic than they are in non-atretic follicles, possibly as a result of decreased inhibin synthesis.

In the present study, the objective was to determine whether the expression of inhibin α and β_A subunit mRNA was predominant in growing, steroidogenically active follicles compared with atretic, dying follicles during follicle recruitment after ovulation. Non-radioactive *in situ* hybridization was used to detect inhibin α and β_A transcripts. The cell proliferation-associated nuclear antigen, Ki-67, and cytochrome P450 aromatase (P450arom) proteins on ovarian frozen serial sections were detected by immunohistochemistry to use as markers for growing and steroidogenically active follicles, respectively.

*Correspondence.

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Materials and Methods

Animals

Eight untreated crossbred gilts with previous oestrous cycles of 19–21 days were killed by electrical shock and exsanguination between 08:00 and 10:00 h on days 3, 5 and 7 ($n = 3, 2$ and 3 per day, respectively) after the onset of oestrus (first day of oestrus = day 0). Ovaries were recovered and transported to the laboratory on ice in ice-cold PBS. The animals used in this study were treated in accordance with the *NIH Guide for the Care and Use of Experimental Animals*. Experimental protocols were approved by the Beltsville Area Animal Care and Use Committee.

Tissue preparation

After a brief survey of the surface morphology, one ovary from each animal was selected at random and cut into blocks of approximately $2\text{ cm} \times 2\text{ cm} \times 1\text{ cm}$. Blocks of ovarian tissue were immersed in Tissue-Tek OCT compound (Miles Inc., Elkhart, IN) in Peel-A-Way disposable embedding moulds (Polysciences Inc., Warrington, PA) as reported by Garrett and Guthrie (1996), except that the blocks were frozen in a dry ice ethanol bath. Pig liver served as a tissue negative control for *in situ* hybridization of inhibin subunit mRNAs, and was frozen in the same manner as ovarian tissue. All tissue samples were stored at -80°C before cryosectioning. Frozen sections of 8 mm were cut on a Hacker-Bright cryostat (Hacker Instruments, Inc., Fairfield, NJ) and thaw-mounted onto Superfrost Plus precoated slides (Fisher Scientific, Pittsburgh, PA). One block from each ovary was sectioned. A total of 86 follicles was analysed, 53 were classified as small ($< 3\text{ mm}$) and 33 as medium ($3\text{--}5\text{ mm}$). Blocks were trimmed on the cryostat until the cross-sectional diameters of the follicles noted for that block before freezing were reached, then 21 serial sections were cut and mounted. Each immunostaining procedure or 3'-end labelling reaction was performed in duplicate on two separate slides, and a third slide was used as a negative control. *In situ* hybridization for inhibin subunit mRNAs was performed using one slide for the antisense riboprobe, and a second slide for the sense strand negative control.

Immunohistochemistry

The steroidogenic enzyme P450arom was detected on tissue sections using a rabbit polyclonal antibody as described by Garrett and Guthrie (1996). The cell proliferation-associated nuclear antigen Ki-67 was detected with a mouse monoclonal antibody obtained from Novocastra Laboratories Ltd, through Vector Laboratories (Burlingame, CA). The procedure used for immunohistochemical detection of antigens was identical to the procedure described by Garrett and Guthrie (1996). Sections were fixed in Zamboni's fixative (2% (w/v) paraformaldehyde, 0.2% (w/v) picric acid in 150 mmol phosphate buffer l^{-1} , pH 7.5) and the primary antibodies were detected by the peroxidase anti-peroxidase method using reagents from Sternberger Monoclonals Inc.

(Baltimore, MD). The rabbit polyclonal anti-P450arom antibody was used at a dilution of 1:2000, and the mouse monoclonal anti-Ki-67 antibody was used at a dilution of 1:200. Negative control slides were prepared by substitution of either rabbit or mouse IgG for the primary antibodies, and were devoid of any non-specific background staining. One duplicate slide was counterstained with Gill's haematoxylin number 2 (Sigma Chemical Co., St Louis, MO) diluted 1:1 with water. The other duplicate slide and the negative control slide were not counterstained.

Preparation of digoxigenin-labelled riboprobes

An EcoR1/Pst1 fragment corresponding to nucleotides 1–766 of the cDNA encoding the pig α subunit (Mayo *et al.*, 1986) was subcloned into a pGEM-3Z vector (Promega, Madison WI) to prepare the inhibin α sense and antisense riboprobes. A fragment corresponding to nucleotides 952–1295 (Rohan *et al.*, 1991) was subcloned into a Bluescribe vector (Stratagene, La Jolla CA) and this sequence was used for generating sense and antisense inhibin β_A RNA probes. After linearizing the vectors with the appropriate restriction enzymes, digoxigenin-labelled sense and antisense riboprobes were transcribed using a DIG RNA labelling kit (Boehringer Mannheim, Indianapolis, IN). The resulting inhibin α sense and antisense probes were approximately 0.7 kb and the inhibin β_A sense and antisense riboprobes were approximately 0.3 kb .

The cDNAs used to produce the riboprobes in this study were used for northern blot analysis of RNA isolated from pig follicles, liver and lung tissue as described by Guthrie *et al.* (1992). Riboprobe for I/A β_B subunit transcript was not prepared for this study as it has been shown that the β_B subunit transcript is expressed in amounts $\leq 10\%$ of the crossreaction of the β_B subunit mRNA with the β_A cDNA in northern analysis (Guthrie *et al.*, 1992). The α cDNA hybridized to a 1.7 kb mRNA species and the β_A cDNA to a large band at 9.5 kb , with a smaller species prominent at 6.6 kb present in some follicles as reported in swine by Guthrie *et al.* (1992) and in ewes by Rohan *et al.* (1991) (data not shown). No α or β_A subunit mRNA was detected in RNA isolated from liver or lung tissue (data not shown).

In situ hybridization of inhibin subunit mRNA

In situ hybridization of inhibin subunit mRNA with digoxigenin-labelled riboprobes was carried out on frozen tissue sections with modifications of the methods of Schaeren-Wiemers *et al.* (1993) and Panoskaltis-Mortari and Bucy (1995). Pig ovarian or liver sections were fixed for 1 h at room temperature in freshly prepared 4% (w/v) paraformaldehyde, then rinsed three times for 5 min each in PBS. After the last rinse, the sections were covered with hybridization solution (45% (v/v) deionized formamide, 25 mmol $\text{NaPO}_4\text{l}^{-1}$, pH 6.5, $5 \times \text{SSC}$, $1 \times$ Denhardt's reagent, and $250\text{ }\mu\text{g}$ sheared denatured herring sperm DNA ml^{-1} ; Amresco, Solon, OH) and prehybridized for 2 h at 55°C in a sealed box humidified with $2 \times \text{SSC}$. The prehybridization solution was aspirated off, and

replaced with fresh hybridization solution containing either 125 ng digoxigenin-labelled inhibin α ml⁻¹ or 250 ng digoxigenin-labelled inhibin β_A antisense riboprobes ml⁻¹. The sections were covered with a siliconized cover slip, and the slides were hybridized overnight at 55°C in a sealed box humidified with 2 × SSC. The next day, the coverslips were removed by soaking in 2 × SSC at room temperature, and unbound probe was removed by stringency washes at 55°C in 2 × SSC, twice for 15 min each, 0.2 × SSC, once for 15 min, and in 0.1 × SSC, twice for 15 min each. After the stringency washes, the slides were rinsed for 5 min in 50 mmol Tris buffered saline (TBS) l⁻¹ (pH 7.6), then blocked for 30 min with 5% (v/v) normal goat serum in TBS. Hybridized probes were immunodetected by an overnight incubation at 4°C with an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Boehringer Mannheim) at a dilution of 1:500. The slides were rinsed three times for 5 min each in TBS, then treated for 10 min with alkaline phosphatase detection buffer (100 mmol Tris-HCl l⁻¹, 100 mmol NaCl l⁻¹, 50 mmol MgCl₂ l⁻¹, pH 9.5). Final colour development was accomplished with the binary alkaline phosphatase chromogen, nitro blue tetrazolium chloride (NBT, 375 µg ml⁻¹) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 188 µg ml⁻¹) in alkaline phosphatase detection buffer containing 1 mmol levamisole l⁻¹. Colour development was allowed to proceed for 2 h and 20 min for the inhibin α probe, and for 1 h and 50 min for the I/A β_A probe. The colour reaction was terminated by rinsing the slides in Tris EDTA buffer (10 mmol Tris l⁻¹ and 1 mmol EDTA l⁻¹). Finally, the slides were rinsed in deionized water, air dried, and the sections were covered with Crystal Mount (Biomedica Corp., Foster City, CA).

In situ 3'-end labelling

In situ 3'-end labelling of endonuclease cleaved DNA in apoptotic cells was performed as described by Garrett and Guthrie (1996) using calf thymus terminal transferase (TdT) and biotinylated deoxyuridine triphosphate (b-dUTP), both obtained from Boehringer Mannheim. Sections were fixed for 10 min in 10% (v/v) buffered formalin, and the end-labelling reaction was carried out at 37°C for 1 h in a humidified chamber with TdT (100 iu ml⁻¹) and b-dUTP (10 nmol ml⁻¹) in TdT buffer (200 mmol sodium cacodylate l⁻¹, 25 mmol Tris-HCl l⁻¹, pH 6.6, containing 2.5 mmol CoCl₂ l⁻¹ and 0.25 mg BSA ml⁻¹). Incorporated b-dUTP in apoptotic cells was detected with a 1:2000 dilution of Streptavidin-Peroxidase (Boehringer Mannheim). Negative controls were prepared by omission of terminal transferase in the reaction mixture, and were devoid of background staining. One duplicate slide was counterstained with Gill's haematoxylin number 2 diluted 1:1 with water. The other duplicate slide and the negative control slide were not counterstained.

Evaluation of immunostaining, in situ hybridization and 3'-end labelling

Stained slides were observed on a Zeiss Axioskop (Carl Zeiss, Inc., Thornwood, NY) microscope and the intensity of

the immunostaining or *in situ* hybridization signal was determined by absorbance of acquired video images. The video camera from an Alpha Innotech IS-1000 digital imaging system (Alpha Innotech Corporation, San Leandro, CA) was attached to the microscope and images were acquired at ×40 magnification. The spot densitometry module of the IS-1000 software was used to draw four polygons within the granulosa cell layer of each follicle, and a background polygon was drawn in the stroma adjacent to the follicle being measured. The background value was automatically subtracted from the absorbance values obtained from the granulosa cell measurements. Four measurements of average pixel value were taken on the basis of a 256 level grey scale, where 0 represented white and 255 represented black, for each follicle. The average of these four measures was used for statistical analysis.

In situ 3'-end labelling was used to define atresia status as an independent variable to evaluate the effects of atresia on follicular function. *In situ* 3'-end labelling has been shown to be expressed in cells that exhibit the histological appearance of apoptosis (Garrett and Guthrie, 1996) as described by Kerr and Harmon (1991). Apoptotic cells were defined as cells containing nuclei with condensed chromatin that were either margined into masses aligned with the nuclear membrane or shrunk into a misshapen mass, or cells with chromatin fragmented into multiple densely stained masses that were clustered together among adjacent non-apoptotic cells. Apoptotic bodies were discrete membrane-bound structures, possibly originating from more than one cell, that were located among viable cells or sloughed into the follicular antrum. Follicles were assessed on a four-point scale based on the classification system of Hay *et al.* (1976). Follicles classified as non-atretic (assigned a value 0) had no clear sign of atretic change, having a membrana granulosa that was compact with closely opposed cells, and only occasional labelled apoptotic cells or apoptotic bodies. Early atretic follicles (first degree atresia) contained moderate numbers of labelled apoptotic cells or apoptotic bodies distributed along the antral border of the membrana granulosa and were assigned a value of 1. Moderately atretic follicles (second degree atresia) had greater numbers of labelled cells and apoptotic bodies widely distributed in the membrana granulosa and were assigned a value of 2. Late atretic follicles (third degree atresia) contained numerous labelled granulosa cells or widespread disintegration of membrana granulosa with many apoptotic bodies in the antrum and were assigned a value of 3. Only two follicles were classified as being moderately atretic so they were placed in the late atretic group.

Statistical analysis

Analysis of variance was performed using the MIXED procedure in release 6.12 of the Statistical Analysis System software for personal computers (SAS, 1997). The Repeated option was used for each dependent variable to model the covariance structure among follicles within gilts. If correlation among follicles was found, compound symmetry covariance structure (variances and correlations equal) was

Table 1. Effects of follicle size on expression of inhibin α and inhibin β subunit mRNAs and P450 aromatase and cell proliferation-associated nuclear antigen Ki-67 in non-atretic follicles on days 5 and 7 of the oestrous cycle in pigs

Follicle diameter (mm)	Number of follicles	Inhibin α	Inhibin β	P450 aromatase	Ki-67
1–2	30	16.3 $\pm$ 2.6 ^a	28.7 $\pm$ 3.9 ^a	9.2 $\pm$ 3.5 ^a	47.7 $\pm$ 4.4
3–5	8	31.6 $\pm$ 4.4 ^b	55.5 $\pm$ 7.5 ^b	29.7 $\pm$ 8.1 ^b	42.1 $\pm$ 10.3

Values are least squares mean optical density units $\pm$ SEM.

^{ab}Means with no superscript letter in common differ significantly by LSMEANS PDIFF procedure ($P \leq 0.05$).

Table 2. Effects of atresia on expression of inhibin α and inhibin β subunit mRNAs and P450 aromatase and cell proliferation-associated nuclear antigen Ki-67 in all follicles on days 3, 5 and 7 of the oestrous cycle in pigs

Atresia stage	Number of follicles	Inhibin α	Inhibin β	P450 aromatase	Ki-67
Non-atretic	56	20.9 $\pm$ 1.7 ^a	36.8 $\pm$ 2.7 ^a	12.7 $\pm$ 1.6 ^a	47.8 $\pm$ 2.1 ^a
Early atretic	6	14.8 $\pm$ 5.1 ^{ab}	22.5 $\pm$ 8.2 ^{ab}	13.2 $\pm$ 4.9 ^a	24.2 $\pm$ 6.5 ^b
Late atretic	24	6.2 $\pm$ 2.5 ^b	6.2 $\pm$ 4.1 ^b	4.2 $\pm$ 2.5 ^b	14.7 $\pm$ 3.2 ^{bc}

Values are least squares mean optical density units $\pm$ SEM.

^{abc}Means with no superscript letter in common differ significantly by LSMEANS PDIFF procedure ($P \leq 0.05$).

Table 3. Effects of day of the oestrous cycle in pigs on expression of inhibin α and inhibin β subunit mRNAs and P450 aromatase and cell proliferation-associated nuclear antigen Ki-67 in non-atretic small (1–2 mm) follicles

Day	Number of follicles	Inhibin α	Inhibin β	P450 aromatase	Ki-67
3	18	20.6 $\pm$ 2.8 ^a	36.7 $\pm$ 5.0 ^a	9.0 $\pm$ 1.8	46.3 $\pm$ 4.7 ^a
5	11	14.3 $\pm$ 3.1 ^{ab}	22.9 $\pm$ 5.4 ^{ab}	8.1 $\pm$ 2.0	39.7 $\pm$ 5.1 ^{ab}
7	19	9.7 $\pm$ 2.3 ^b	17.5 $\pm$ 4.1 ^b	4.6 $\pm$ 1.5	29.6 $\pm$ 3.9 ^b

Values are least squares mean optical density units $\pm$ SEM.

^{ab}Means with no superscript letter in common differ significantly by LSMEANS PDIFF procedure ($P \leq 0.05$).

used in the repeated statement for the final analysis of variance. Three separate groupings of data were required because follicle atresia status and the number of small and medium follicles varied considerably among animals. The effect of follicle atresia status on expression of the dependent variables I/A α and β_A , P450arom, and Ki-67 was determined by a one-way analysis of a fixed effects model for all 86 follicles using orthogonal contrasts to test for linear and quadratic regression of the dependent variables on atresia status. The effect of day of the oestrous cycle was determined by a one-way analysis of a fixed effects model for all 48 small non-atretic follicles using orthogonal contrasts to test for linear and quadratic regression of the dependent variables on day. The effects of follicle size class was determined for non-atretic follicles, pooled over days 5 and 7, ($n = 38$) using a one-way analysis of a fixed effects model. Correlation analysis was used to test the degree to which the expression of inhibin α and the expression of I/A β_A in all follicles were associated with each other and with the expression of P450arom and Ki-67.

Results

Both I/A antisense riboprobes hybridized specifically to the granulosa cells of antral follicles (Fig. 1a,c). The

complementary sense strand probes used as negative controls failed to hybridize to any tissue structure (Fig. 1b,d). No specific hybridization signal for either probe was observed in preantral follicles and ovarian stroma (data not shown).

The strongest hybridization signal for the inhibin α probe was found in the mural granulosa cells along the basement membrane of the follicle (Fig. 1a), whereas the hybridization signal for the I/A β_A probe was distributed uniformly across the granulosa cell layer (Fig. 1c). Sixteen follicles were negative for expression of one or both of the I/A subunit transcripts; of these, nine were negative for both, six were negative for β_A and positive for α , and one was positive for β_A and negative for α . Thirteen of the 16 follicles negative for one or both of the I/A subunit transcripts were late atretic. Hybridization of either probe to the thecal cell layer of these early luteal phase follicles was difficult to discern and was, at best, extremely faint.

The P450arom peptide was localized to the granulosa cell layers of both small and medium follicles, being more abundant in non-atretic than in atretic follicles. However, 24 follicles were negative for P450arom; 21 were atretic, and ten were also negative for one or both I/A transcripts. As reported by Garrett and Guthrie (1996), the thecal layer of early luteal phase follicles did not express P450arom (Fig. 2c). The granulosa cells of preantral follicles did not express

Fig. 1. Test of hybridization specificity of inhibin subunit riboprobes. Hybridizable anti-sense strands of inhibin α (a) and inhibin β_A (c) and the non-hybridizable sense strands of inhibin α (b) and inhibin β_A (d) were incubated with pig ovarian tissue sections. (e) Tissue section analysed for P450arom and counterstained with haematoxylin for better demonstration of follicle morphology. Only the anti-sense strands hybridized specifically with inhibin α or inhibin β_A mRNA on the tissue sections. GC: granulosa cell; T: theca interna. Scale bar represents 100 μm .

P450arom (data not shown). The cell proliferation-associated nuclear antigen Ki-67 was localized in cell nuclei of both the granulosa and thecal cell layers of non-atretic follicles, and some early atretic follicles (Fig. 2d). Granulosa cells of 12

follicles were negative for Ki-67 and all of these were atretic. Although not quantified in this study, Ki-67 labelling was also detected in preantral follicles with multi-laminate granulosa cells.

As non-atretic follicles increased in size on days 5 and 7, expression of inhibin α , I/A β_A , and P450arom increased ($P < 0.01$) being 102, 93 and 238% greater, respectively, in medium non-atretic than in small non-atretic follicles (Table 1). Expression of Ki-67 was unaffected by follicle size. The correlation analysis of inhibin α , I/A β_A , P450arom, and Ki-67 expression, from which the effects of day and follicle size were partialled, showed that there was a strong linear relationship between I/A α and β_A subunits ($r = 0.816$, $P = 0.0001$), and of both subunits with P450arom (α : $r = 0.669$, $P = 0.0001$; β_A : $r = 0.620$, $P = 0.0001$) and with Ki-67 (α : $r = 0.571$, $P = 0.0001$; β_A : $r = 0.594$, $P = 0.0001$).

Inhibin α subunit mRNA, I/A β_A subunit mRNA, P450arom, and Ki-67 were all reduced (70, 83, 66 and 69%, respectively) ($P < 0.001$) in late atretic compared with non-atretic follicles (Table 2). All four variables showed a negative linear regression on atresia status. In small non-atretic follicles, inhibin α mRNA, I/A β_A mRNA, and Ki-67 decreased 53, 52 and 36%, respectively ($P < 0.02$) between days 3 and 7 (Table 3). Expression of P450arom also

decreased between days 3 and 7, but the change was not statistically significant.

Discussion

The novel finding of this study is that expression of I/A subunit genes increases during the period of increasing growth and steroidogenesis that occurs during follicle recruitment after ovulation in pigs. In cattle, as many as four waves of recruitment, selection and dominance can occur during the oestrous cycle (for review, see Bao and Garverick, 1998). However, in pigs, only a first wave of follicle recruitment after ovulation can be studied because follicle selection and dominance are suppressed or cannot be detected until the follicular phase of the cycle (Guthrie *et al.*, 1995a). Many studies have shown that expression of I/A subunit genes is localized to the granulosa cells of antral follicles (Torney *et al.*, 1989; Engelhardt *et al.*, 1993; Braw-Tal, 1994; Tisdall *et al.*, 1994). However, little is known about the

Fig. 2. Effect of follicle atresia on the expression of inhibin subunit mRNAs, P450arom and the cell proliferation antigen, Ki-67 in pig follicles. The small follicle on the right in each figure was designated as atretic on the basis of granulosa cell *in situ* 3'-end labelling and did not express inhibin α (a) or inhibin β_A (b) subunit mRNAs, or P450arom (c), and expressed reduced Ki-67 labelling (d), while the healthy follicle on the left expressed both mRNAs and P450arom. The follicle on the right is in an early stage of atresia because many of the cells are still proliferating, but at a reduced rate compared with the non-atretic follicle. Scale bar represents 100 μ m.

relationship between I/A subunit gene expression and cell proliferation or the amount of steroidogenesis in recruited or growing follicles. In the present study, the positive correlation of I/A subunit mRNA expression with Ki-67 antigen and P450arom expression indicates that production of inhibin or activin, or both, may be associated with, or exert paracrine control over, granulosa cell proliferation and steroidogenesis. In addition, on days 1 and 3 of altrenogest-synchronized follicle selection, inhibin α and I/A β_A subunit mRNAs were positively correlated with follicle size and the concentration of oestradiol in follicular fluid (Guthrie *et al.*, 1992).

In the present study, most follicles (81% of those examined) expressed both subunits. Although I/A subunits are coded by separate genes (LaPolta and Hsueh, 1991), the abundance of inhibin α and I/A β_A subunit transcripts were highly correlated in follicles expressing both subunits during the early luteal phase of the oestrous cycle (present study) and the early follicular phase of altrenogest-synchronized pigs (Guthrie *et al.*, 1992). This finding indicates that, in pigs, inhibin α and I/A β_A expression is tightly co-regulated at these stages. However, during other stages of follicular development in pigs (Guthrie *et al.*, 1992; present study), sheep (Engelhardt *et al.*, 1993; Braw-Tal, 1994; Tisdall *et al.*, 1994), cattle (Torney *et al.*, 1989) and rats (Woodruff *et al.*, 1988), expression of I/A subunits is discordant. Typically, expression of mRNA encoding α subunit is present throughout folliculogenesis, from the early antral to the preovulatory stage of development (Woodruff *et al.*, 1988; Torney *et al.*, 1989; Engelhardt *et al.*, 1993; Braw-Tal, 1994; Tisdall *et al.*, 1994). In contrast, expression of the β_A subunit is restricted to a smaller population of follicles, absent in preantral and early antral follicles and decreasing as follicles reach the preovulatory stage, especially after the preovulatory LH surge. These characteristics of I/A gene expression are consistent with the notion that production of the β_A subunit is rate limiting to activin and inhibin dimer formation (Woodruff *et al.*, 1988; Rodgers *et al.*, 1989).

Most observations *in vitro* support the role of activin as an autocrine regulator of granulosa cells promoting gonadotrophin-supported development, supporting granulosa cell differentiation during preantral and early antral stages and preventing premature luteinization and terminal differentiation during later stages of antral follicle development (Findlay, 1994; Li *et al.*, 1995). Activin has mitogenic effects on cultured granulosa cells (Miro and Hillier, 1996), and can act as a morphogen, by stimulating antrum formation in cultured preantral follicles (Li *et al.*, 1995). Evidence for an anti-atretic effect of activin is its ability to attenuate expression of transcripts encoding insulin-like growth factor binding proteins 4 and 5 in granulosa cell culture (Choi *et al.*, 1997). However, data from studies *in vivo* indicate that the injection of activin into follicles was atretogenic (Woodruff *et al.*, 1990). On the basis of the inhibitory effect of activin on steroidogenesis in cultured pig granulosa cells, it was proposed that activin was atretogenic (Ford and Howard, 1997); however, these results may also be explained by the blocking of terminal granulosa cell differentiation before the preovulatory LH surge. There is convincing evidence for a stimulatory role for activin on

steroidogenesis; activin significantly augments the stimulatory action of FSH on P450arom activity in rat and primate granulosa cells (Miro *et al.*, 1991; Hillier and Miro, 1993b).

In the present study, the amounts of inhibin subunit mRNAs, P450arom, and Ki-67 were progressively reduced as follicles became increasingly more atretic. The transcripts for both inhibin subunits have been shown to be significantly lower in non-ovulatory follicles sampled during the follicular phase (Guthrie *et al.*, 1992), and inhibin immunoblot activity is reduced in atretic compared with non-atretic follicles during the early luteal or follicular phase (Guthrie *et al.*, 1997). Thus, in pigs, the present results, together with the general reduction of follicular fluid steroid concentration (Guthrie *et al.*, 1994, 1995b), steroidogenic enzyme mRNAs and proteins (Tilly *et al.*, 1992; Garrett and Guthrie, 1996, 1997), and gonadotrophin receptors observed in atretic as compared with non-atretic follicles, support the concept of a general decrease in follicle function during the early stages of the atretic process (for review, see Tsafiriri and Braw, 1984).

The explanation for the increased incidence of atretic follicles and the reduced biosynthetic activity even in non-atretic follicles may be related to changes in plasma FSH concentration. During the follicular phase in pigs, the growth of small and medium follicles and the secretion of FSH remain suppressed until the preovulatory LH surge (Guthrie *et al.*, 1995a). Plasma FSH concentrations then increase transiently on days 2 and 3 of the next cycle (periovulatory FSH release) (Hasegawa *et al.*, 1988; Guthrie *et al.*, 1995a), which may initiate follicle recruitment (Guthrie *et al.*, 1995a). Subsequent atresia and decreased expression of I/A α and β_A genes may be a consequence of FSH withdrawal after the periovulatory FSH surge (Hasegawa *et al.*, 1988; Guthrie *et al.*, 1995a).

The present results show that increased expression of I/A α and β_A subunit mRNAs in granulosa cells was correlated with granulosa cell proliferation and follicle growth during recruitment. These observations are consistent with the concept of a positive effect of I/A subunit synthesis in the ovary of pigs on follicle growth and steroidogenesis during follicle recruitment after ovulation.

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