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Expression of Follistatin and Inhibin/Activin Subunit Genes in Porcine Follicles

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ABSTRACT

Expression of the follistatin (FS) and inhibin/activin (I/A) α, βₐ, and βₜ subunit genes in porcine ovarian follicles was evaluated by reverse transcriptase polymerase chain reaction and/or RNase protection procedures to establish changes during the final stages of follicular development. For the I/A α and βₜ subunits, expression increased (p < 0.05) as follicles progressed to the mid-stage of the follicular phase. The βₐ subunit was expressed in lower concentrations, and all three I/A subunits showed a marked reduction (p < 0.01) in expression by the late stage of follicular development. In contrast to this pattern, FS showed a marked reduction (p < 0.01) in expression by the late stage of follicular development, and continued to decline in advanced follicles (after estrus). The predominant mRNA encoded for FS-315, and the ratio of mRNA for FS-315 to mRNA for FS-288 did not differ significantly during the late stages. Within an animal, concentration of FS mRNAs was related more to stage of the follicular phase than to follicular size. Follistatin concentration of FS changed in a manner similar to that observed for expression of its gene. We conclude that expression of the FS gene and translation of its mRNA decrease as follicles approach ovulatory status.

INTRODUCTION

Follistatin (FS) is a monomeric protein originally isolated from bovine and porcine follicular fluids and was shown to be an inhibitor of FSH secretion [1–3]. Molecular cloning and sequence analysis of FS cDNAs and genes from pigs [4, 5], humans [6], and rats [7] revealed that the FS gene is highly conserved in these species, and there are two forms of FS mRNA generated by alternative splicing [4].

The predicted protein sequences of the secreted forms consist of 288 (FS-288) and 315 (FS-315) amino acids. The FS-315 differs from FS-288 by having an extra 27 amino acids at the carboxyl terminus. The FS gene is a single copy of approximately 6.0 kilobases (kb) consisting of five exons and an initial exon encoding the signal peptide [4].

One biological function of FS, inhibition of FSH secretion, is similar to that of inhibin; but FS is structurally quite different, and its potency is weaker than that of inhibin. FS binds both activin [8] and inhibin [9] through a common β subunit. This binding inhibits activin’s stimulation of FSH secretion [9]. Although FS mRNA has been found in a variety of tissues, including ovary, testis, and pituitary [4, 10–12], regulatory effects within these tissues are poorly understood. Northern blot hybridization of porcine ovarian poly(A)⁺ RNA showed a single band of 2.5 kb, but work from other laboratories [11, 13, 14] using total RNA from rat and bovine tissues, revealed two bands of approximately 2.5 kb and 1.6 kb, respectively. Two major (ca. 2.7 kb and 1.5 kb) and one minor (0.5 kb) mRNA for FS were detected in sheep [15].

Activins and inhibins were initially isolated from porcine and bovine follicular fluids as gonadal proteins with the capacity to stimulate and inhibit, respectively, FSH secretion and its synthesis in anterior pituitary glands. Inhibins are dimeric proteins consisting of a common α subunit and one of two β subunits, and activins are also dimeric proteins but are composed of only the β subunits [16, 17]. The transcripts for these subunits have been detected in gonads and several extragonadal tissues. In porcine follicles, only mRNA for the α and βₐ subunits were detected by dot-blot analysis [18].

How FS gene expression is regulated during porcine follicular development remains to be characterized. A better understanding of such regulation will provide insight into the control of follicular selection and growth. The major objectives of the study reported here were 1) to determine how the expression of the FS gene differs in follicles of different sizes within the same animal, and of the same size but at different stages of the follicular phase of the estrous cycle, and 2) to determine the ratio of mRNA species coding for FS-315 to mRNA species coding for FS-288 as influenced by follicular size or stage of the follicular phase.

MATERIALS AND METHODS

Collection of Ovarian Follicles and Follicular Fluid

Females of mixed breeds (7–9 mo old) were evaluated for estrous behavior twice daily with a sexually mature male. All females had experienced at least one estrus cycle before they were slaughtered at random from 17 to 20 days after estrus (early to mid stages of the follicular phase, n = 12) or > 24 h after estrus (late stage of the follicular phase, n = 5). A jugular blood sample was obtained 24 h before and at slaughter for determination of plasma estradiol and progesterone concentrations [19, 20]. At slaughter, females were electrically stunned, and ovaries were obtained quickly and placed into cold saline. Follicles larger than 3 mm in diameter were dissected, with diameter determined with calipers. Each follicle was stored individually in liquid nitrogen except four to six that were representative of mean diameter for each animal. From these, follicular fluid was collected by aspiration and evaluated within a single assay for estradiol concentration [19].

Follicular fluid samples from an independent population of primiparous, crossbred females (> 1 yr old) were evaluated within a single assay for FS concentration by RIA [21]. Ovaries were obtained at slaughter, and follicular fluid was obtained by aspiration of the 16–20 largest antral fol-
laries per female. Porcine FS, a mixture of three molecular-
weight forms, was the reference preparation. Estradiol con-
centrations were determined for follicular fluid, and sows were
grouped according to criteria established in the cluster
analysis (see Statistical Analysis section). The 27 females
in the early phase of follicular development lacked active
corpora lutea, had a mean follicular diameter of < 5.5 mm,
and had follicular fluid estradiol concentrations of < 100
ng/ml. The 18 females in the mid-follicular phase had a
mean follicular diameter of ≥ 5.5 mm, had follicular fluid
concentrations of estradiol of > 100 ng/ml, and had not
exhibited behavioral estrus. Sixteen females that were des-
nignated as being in the late phase of follicular develop-
ment had exhibited behavioral estrus and had follicular fluid
estradiol concentrations of < 150 ng/ml.

Primers Used in Quantitative Reverse Transcriptase
Polymerase Chain Reaction (RT-PCR) and Subcloning

On the basis of the reported nucleotide sequence [5],
primers 5'-FS, 3'-FS1, and 3'-FS2 corresponding to nucleo-
tides 1000–1029 (exon 3), 1837–1866 (exon 4), and
1939–1968 (exon 5) of the porcine FS gene were synthe-
sized by the DNA Synthesis Laboratory of University of
Nebraska. The primer sequences for the FS and β-actin
genes were the same as those described previously [22].
The expected RT-PCR product amplified with 5'-FS and
3'-FS1 is 216 base pairs (bp) from both RNA species, and
the product amplified with 5'-FS and 3'-FS2 is 318 bp from
FS-315. Using these specific primers, amplified PCR prod-
ucts from cDNA that had been synthesized by reverse tran-
scription were subcloned, sequenced, and determined to cor-
respond to the predicted region of each gene [22, 23].

End-Labeling of Oligonucleotide Primers and Preparation
of Riboprobe

Oligonucleotide DNA primers were labeled as described
[24, 25]. After linearization of plasmids pFS-9, ps-9, pβh-21,
 pβn-5, and pβ-actin with Eco RV or HindIII, sense and
antisense riboprobes were transcribed with DNA-dependent
SP6 or T7 RNA polymerase in the presence of [α-

32P]uridine triphosphate (UTP) under the conditions de-
scribed [24, 25]. The DNA templates were removed by in-
cubation with RNase-free DNase I at 37°C for 15 min.

RNA Isolation and Quantitative RT-PCR

The 66 representative follicles with a variety of diamete-
rs were randomly chosen from the frozen follicles with
at least one from each of the 17 gilts and consisted of 13
from group I, 42 from group II, and 11 from group III.
Total cellular RNA was isolated from individual follicles
by guanidine isothiocyanate extraction and CsCl centri-
fugation as described by Chirgwin et al. [26] and was treated
with RNase-free DNase I at 37°C for 30 min before
RT-PCR or RNase protection assay. Reverse transcription
and quantitative PCR were performed [22, 24] for every
gene studied. The amount of mRNA of each sample was
normalized for porcine β-actin RNA, which was done si-
multaneously under the same conditions except with dif-
ferent primers.

RNase Protection Assay

Hybridizations and RNase digestions were performed with
an RPA-II kit (Ambion, Inc., Houston, TX) [24, 25].
The numbers of follicular RNA samples included in the
RNase protection assays were 5, 10, and 9 for early, mid,
and late stages of the follicular phase with mean follicular
diameters of 4.4 ± 0.2, 6.2 ± 0.3, and 9.3 ± 0.7 mm,
respectively. Size of selected follicles represented the pre-
dominant size for each female. For every riboprobe used,
two yeast RNA control tubes, one containing RNase A/T1
(negative control) and the other containing no RNase A/T1
(positive control), were always included in the assay.

Statistical Analysis

Data were analyzed with procedures CLUSTER and
ANOVA (analysis of variance) in the Statistical Analysis
System (SAS) statistical package [27–29] and are presented
as mean ± SEM. Correlation coefficient (r) was used to
measure the similarities of different females because it
could eliminate the effects on clustering of unit employed
in each hormone measurement or follicle statistic. With the
RT-PCR data, female nested within group (stage of the fol-
licular phase) was used as error to test effect of group. To
evaluate effect of size classification across groups, the data
set was restricted to follicles ≤ 5 mm or > 7 mm. For
evaluation of size classification within group, the data set
was restricted to groups II or III, and follicle classification
within gilt was the error term. When an F-test was signif-
icant, specific means were compared by using the Bonfer-
roni procedure [29].

RESULTS

Classification of Animals

On the basis of plasma estradiol and progesterone con-
centrations on the day before and the day of follicle col-
lection, plus mean and standard error of follicle number (> 3
mm in diameter) for each animal, three clustering tech-
niques—average linkage, complete linkage, and centroid-
hierarchical methods—were employed to group animals
used in the study. Similar groupings resulted with each of
these methods and classified the gilts into three groups, i.e.,
early (group I), mid (group II), and late (group III) stages
of the follicular phase. Follicular statistics and steroid con-
centrations of the groups are summarized in Table 1. In
group I, follicles were smaller (ca. 4.6 mm in diameter) and
had lower estradiol concentrations than follicles observed
in the other two groups. In group II, follicular size was

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Follicle number</th>
<th>Follicle diameter (mm)</th>
<th>Follicular fluid Estradiol (pg/ml)</th>
<th>ΔE2* (pg/ml)</th>
<th>ΔP4* (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4</td>
<td>20.8 ± 2.2</td>
<td>4.6 ± 0.2</td>
<td>14 ± 9</td>
<td>1.3 ± 0.6</td>
<td>-3.8 ± 1.6</td>
</tr>
<tr>
<td>II</td>
<td>8</td>
<td>20.1 ± 1.8</td>
<td>6.5 ± 0.2</td>
<td>334 ± 66</td>
<td>11.6 ± 1.6</td>
<td>-0.1 ± 0.2</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>15.0 ± 1.6</td>
<td>7.4 ± 0.3</td>
<td>52 ± 28</td>
<td>-8.8 ± 1.6</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

* Changes in plasma E2 and P4 concentrations during the 24 h before collection of follicles.
FIG. 1 Increase in RT-PCR product with number of PCR cycles. Under the conditions given in the Materials and Methods, RNA from individual follicles was reverse transcribed, and cDNA mixture equivalent to 0.2 μg total RNA was subjected to various cycles of amplification (A), or aliquots of the cDNA mixture equivalent to 0.0–0.4 μg RNA were amplified for 25 cycles (C) with the primers 5’-FS and 3’-FS1. Reaction products were resolved by gel electrophoresis and visualized by ethidium bromide staining. The amounts of radioactivity recovered from the excised gel bands were plotted against the amplification cycles (B) or the input RNA (D). M represents 100-bp molecular ladder.

intermediate between groups I and III, and follicular fluid estradiol concentrations were the highest of the three groups. Follicles of group III were the largest, and because they were obtained > 24 h after onset of estrus and their estradiol concentrations were low; presumably, they had experienced at least a portion of the preovulatory release of LH [30].

Determination of RT-PCR Conditions

Using the same strategy described previously [22], optimal PCR conditions were determined for each set of primers reported in this study. The rates of amplification were exponential between 15 and 30 cycles for both sets of primers. After 30 cycles, the rates decreased and approached a plateau at 35 cycles (see example for primers 5’-FS and 3’-FS1 in Fig. 1, A and B). On the basis of the exponential accumulation of PCR products of the FS gene with both sets of primers, 25 cycles of amplification were selected for the quantitative RT-PCR analysis. Also, aliquots of cDNA mixture equivalent to 0.0–0.4 μg total RNA were amplified for 25 cycles under the same conditions (see Fig. 1, C and D). It was found that 0.2 μg of total RNA was required to give a detectable signal on the ethidium bromide-staining agarose gels. Conditions were established as optimal for both sets of primers.

Expression of Different Forms of FS mRNA (RT-PCR)

Expression of FS-315 mRNA decreased significantly as females progressed from the early to the late stages of antral development (p < 0.01; Fig. 2). Follicles from group II females (n = 42) were intermediate in their mRNA concentrations relative to follicles from groups I (n = 13) and III (n = 11). Similar trends were observed for expressed concentrations of FS-315 plus FS-288 mRNAs.

To evaluate FS gene expression and follicular size, we summarized the incorporated radioactivity for small (< 5.0 mm), medium (> 5.0–7.0 mm) and large follicles (> 7.0 mm) within each group (Fig. 3). Expression of FS-315 and FS-315 plus FS-288 mRNAs was affected only marginally by follicular size within each group. In group II there was a small but significant increase as follicles increased from
FOLLISTATIN IN PORCINE FOLLICLES

FIG. 3. Amounts of DNA amplified from mRNAs encoding for FS-315 and FS-315 plus FS-288 were plotted against follicles of small, medium, and large sizes for each group. Amounts of DNA amplified from porcine β-actin RNA were used to normalize the results. Means differ (p < 0.05) within each size of amplified DNA that do not share a common superscript. Numbers of follicles evaluated: for group I, 13 (≤ 5 mm); for group II, 14 (5-7 mm), and 14 (> 7 mm); for group III, 2 (≤ 5 mm), 2 (5-7 mm), and 7 (> 7 mm).

≤ 5 mm to > 5-7 mm. In contrast, the concentration of FS-315 mRNA was 1.6 times higher (p < 0.05) in small follicles of group I females than in follicles of similar size in group II females. Likewise, concentration of FS-315 mRNA in large follicles of group II females was twice (p < 0.05) that observed in large follicles of group III females.

Expression of Different Forms of FS mRNA (RNase Protection Assay)

A schematic diagram of the assay and the expected protected fragments for FS-315 and FS-288 mRNAs are illustrated in Figure 4A. Consistent with the RT-PCR data, RNase protection assays also demonstrated that the expression levels for both FS-315 and FS-288 decreased as animals approached ovulation (Figs. 4B and 5). No significant changes in the ratio of FS-315 mRNA to FS-288 mRNA were detected among the three different follicular stages (groups) but this ratio was quite variable. As indicated in Figure 4A, two fragments (273 and 45 nucleotides) were protected when the antisense pFS-9 riboprobe hybridized with FS-288 mRNA, but the 45 nucleotide fragment was too small to be detected with the gel conditions used here. In contrast, no fragments were protected from RNase digestion when the sense pFS-9 riboprobe was used (data not shown), indicating that the protected fragment for the antisense pFS-9 riboprobe was specific for the FS mRNAs.

Follicular Fluid FS Concentrations

FS concentrations in follicular fluid decreased (p < 0.01) as females approached ovulation (Fig. 6). As expected from our scheme for classification of females, estradiol concentrations increased (p < 0.01) as follicles developed from the early (group I) to the mid stage (group II) and then decreased (p < 0.01) after the onset of estrus (group III).
Expression of Inhibin/Activin (I/A) Subunits (RNase Protection Assay)

RNase protection assays with the antisense riboprobes for the I/A subunits indicated that expression of α and βA subunits increased significantly (p < 0.05) as follicles developed from early (group I) to mid (group II) follicular stages (Fig. 7), and then declined (p < 0.01) in follicles nearing ovulation (group III). No significant change was detected for the βB mRNA between follicles of the early and mid stages. Similar to the other two I/A subunits, expression of βB subunit decreased (p < 0.01) as follicles approached ovulation.

DISCUSSION

Expression of the FS gene, observed by either quantitative RT-PCR or RNase protection assays, decreased in porcine ovarian follicles during advancing stages of the follicular phase of the estrous cycle. This contrasts with earlier reports on bovine [14] and porcine follicles [31] in which FS mRNA concentrations increased with increasing follicular size. Differences in procedures for collection of follicles and stage of follicular development probably account for these differences. The previous report with porcine follicles [31] evaluated FS gene expression in follicles collected as pools of similar follicular size from prepubertal females whereas, in the present study, cluster analysis provided more homogenous groups at later, defined stages of the follicular phase. In general, we conclude that in antral porcine follicles, FS gene expression increases with follicular size during pubertal development ([31]; unpublished results) and presumably during the luteal phase of the estrous cycle; but during the follicular phase, size becomes secondary and stage of the follicular phase becomes increasingly more relevant.

Similar to the mRNA species observed in rats, cattle, and sheep, two bands of about 2.5 kb and 1.5 kb were detected from porcine follicles by Northern hybridization (data not shown), and the concentrations of these two mRNA species were relatively higher in the early- and mid-stage follicles than in follicles from the late stage. Again, this indicates that the FS gene is more highly expressed in follicles of the early follicular phase than in those of later stages. By using RT-PCR with the primers designed specifically for the FS mRNAs, we found that expression of the FS gene within an animal was less dependent on follicle diameter. Differences in procedures for collection of follicles and stage of follicular development probably account for these differences. The previous report with porcine follicles [31] evaluated FS gene expression in follicles collected as pools of similar follicular size from prepubertal females whereas, in the present study, cluster analysis provided more homogenous groups at later, defined stages of the follicular phase. In general, we conclude that in antral porcine follicles, FS gene expression increases with follicular size during pubertal development ([31]; unpublished results) and presumably during the luteal phase of the estrous cycle; but during the follicular phase, size becomes secondary and stage of the follicular phase becomes increasingly more relevant.

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FS concentrations in the follicular fluid also decreased during the follicular phase of the estrous cycle and in general were consistent with changes observed in gene expression. FS in follicular fluid exists in multiple forms be-
cause of proteolytic cleavage of FS-315 to FS-303 and because of various forms of glycosylation of FS-315, FS-303, and FS-288 [34]. These truncated forms of FS bind to heparan sulfate proteoglycans on cells [35]; thus, concentrations within follicular fluid probably differ somewhat from those within the granulosa cell layer. During the early stages of the follicular phase, follicular fluid FS concentrations were higher than those observed later in larger, estrogen-active follicles from females during the mid stage of the follicular phase. The concentration of FS decreased more dramatically in follicular fluid than did that of its mRNA in follicular tissue. This may reflect differences in the two separate populations of females used in these studies or, more likely, differences in tissue compartmentalization. FS binds activin with an affinity similar to that of the I/A subunits. No significant differences were observed for the I/A subunits. No significant differences were observed for the I/A subunits. No significant differences were observed for the I/A subunits. No significant differences were observed for the I/A subunits.

Cluster analysis provided a technique for grouping animals that relies on information from multiple variables associated with follicular development. The objective of cluster analysis is to look for groups in which all subjects in a group are relatively “similar” to each other but relatively “different” from all subjects in other groups. With this multivariate analytical technique, we used morphological and biochemical characteristics of the follicles from each female. This technique provided more homogenous groups than attainable by assigning females on the basis of days from their previous estrus.

In summary, we demonstrated with RT-PCR and RNase protection techniques that the FS gene is more highly expressed in follicles during early than during late stages of the follicular phase. This pattern of expression was related more to the animal’s stage of the follicular phase than to follicle size itself, and this pattern differed from that observed for the I/A subunits. No significant differences were detected in the ratio of mRNA for FS-315 to that for FS-288 among follicles at the three different stages of the follicular phase. In accordance with changes in gene expression, concentrations of FS in follicular fluid decreased during the final stages of follicular development. FS binds activin with high affinity, but how these two proteins interact in vivo to modulate antral follicular development is not understood. Increased concentrations of FS within developing follicles should protect these follicles from the inhibition of steroidogenesis by activin that has been observed in cultured, highly differentiated, granulosa cells [38, 41–43].

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