Relaxin protein and gene expression in ovarian follicles of immature pigs

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ABSTRACT

Relaxin production by the ovarian follicle of gonadotropin-primed, prepubertal gilts is well documented. As far as we are aware, a source of relaxin in pig follicles, independent of gonadotropins, has not yet been reported. Therefore, the objective of this study was to determine whether relaxin is produced in porcine follicles in the absence of exogenous or cyclic gonadotropins. In immature pigs, immunoreactive relaxin was detected in fluids from small (1–3 mm), medium (4–5 mm) and large (>6 mm) follicles and localized to the theca interna of large follicles. Relaxin levels in follicular fluid significantly increased with follicle size ($P<0.05$). Relaxin mRNA was detected in

whole small- and medium-sized follicles. In large follicles, the relaxin gene was expressed in thecal layers, but not granulosa cells. The abundance of relaxin transcript did not change with follicle size. In summary, relaxin protein and mRNA were detected in porcine follicles from immature animals, indicating that relaxin is produced in the porcine follicle in the absence of exogenous or cyclic gonadotropins. Relaxin's *in vitro* growth effects on porcine granulosa and theca cells support this follicular relaxin as a growth modulator during porcine follicular development.

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INTRODUCTION

The corpus luteum (CL) is well established as the primary source of ovarian relaxin in the pig (Ali *et al.* 1986, Fields & Fields 1985, Bagnell *et al.* 1990a). The first studies to support a follicular source of relaxin were reported in 1980, when relaxin was found in media of follicle wall cultures and follicular fluid from pregnant and non-pregnant pigs (Bryant-Greenwood *et al.* 1980). Evidence that this represented a non-luteal source of relaxin came from the studies of Matsumoto & Chamley (1980) who showed immunoreactive relaxin in ovarian extracts and follicular fluid of immature pigs. The specific cellular source of relaxin in the pig follicle was identified as the theca cell (TC) in larger, more developed follicles from gonadotropin-primed pigs (Bagnell *et al.* 1987, 1990b). *In vitro* relaxin production was also detected by thecal explants from gonadotropin-primed pigs (Evans *et al.* 1983), and by preovulatory porcine granulosa cells (GC) after treatment with luteinizing hormone (LH) (Loeken *et al.* 1983). Thus, while there is evidence that exogenous gonadotropins stimulate relaxin production by porcine follicular tissue, whether

follicular relaxin is produced in the absence of exogenous gonadotropins has not been reported.

The growth-promoting effects of low levels of relaxin *in vitro* on porcine GC and TC from follicles of all sizes (Zhang & Bagnell 1993, 1994) points to a local source of relaxin in mediating follicle growth *in vivo*. Although the presence of relaxin in follicular fluid has been attributed to diffusion from nearby CL (Bryant-Greenwood *et al.* 1980), the source of follicular relaxin in the absence of CL (Matsumoto & Chamley 1980) has never been identified. Therefore, the objective of the present study was to investigate whether relaxin is produced by ovarian follicles from immature pigs. We chose to use immature pigs to avoid the possibility of diffusion of relaxin from nearby CL and eliminate the impact of cyclic gonadotropins on follicular relaxin production. In small, medium and large follicles from immature pigs, relaxin protein was measured in follicular fluid using a homologous radioimmunoassay, and sought by immunohistochemistry using specific porcine relaxin antisera. In addition, semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used to characterize relaxin gene expression in different sized porcine follicles.

MATERIALS AND METHODS

Materials

Purified, monoacylated porcine relaxin was provided by Dr G D Bryant-Greenwood (University of Hawaii, Honolulu, HI, USA). Relaxin antisera (R6) for the relaxin radioimmunoassay was a gift from Dr B G Steinetz (New York University Medical Center, New York, USA). Antisera to purified porcine relaxin (Gg) used for immunostaining was raised in a rabbit by the method of Vaitukaitis *et al.* (1971) and provided by Dr G D Bryant-Greenwood, (University of Hawaii). The Vectastain ABC kit and normal rabbit serum were obtained from Vector Laboratories, Inc. (Burlingame, CA, USA) for immunostaining experiments. The Gene-Amp PCR Reagent Kit, including AmpliTaq polymerase, was purchased from The Perkin-Elmer Corp. (Norwalk, CT, USA). The Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and RNasin RNase inhibitor were obtained from Promega Corp. (Madison, WI, USA), and random primers were purchased from Life Technologies, Inc. (Grand Island, NY, USA). PCR primers for porcine relaxin were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX, USA). PCR primers and competimers (alternate pair) for 18 s ribosomal RNA (18 s rRNA) were obtained from Ambion, Inc. (Austin, TX, USA).

Collection of follicular fluid and tissue

Ovaries from immature gilts (73–82 kg) were collected at a local slaughterhouse. Only follicles considered morphologically healthy according to the criteria of Moor *et al.* (1978) were used for collection of follicular fluid and tissue. Fluid was collected by needle aspiration from small (1–3 mm), medium (4–5 mm) and large (>6 mm) follicles. Follicular fluid was gathered from more than ten different collections taken over a period of 6 months. At each collection, fluid from follicles of the same size class were pooled, centrifuged at 500 *g* for 10 min to remove cellular debris and stored at –80 °C. For each follicle size, the number of pooled follicular fluid samples analyzed for relaxin content was: small, *n*=19; medium, *n*=17; and large, *n*=9.

For gene expression studies, follicular tissue was isolated and frozen on-site at a local slaughterhouse to decrease the chance of RNA degradation during subsequent travel and follicle isolation. Groups of small (1–3 mm) and medium follicles (4–5 mm) were dissected from the ovaries and frozen. Large follicles (6 mm and >7 mm) were excised from the ovary and cut in half. In order to determine the

cellular source of relaxin in these follicles, TC and GC were separated. After GC were gently scraped from follicle linings and collected into a petri dish containing M199, the remaining follicle wall, containing theca interna, theca externa and connective tissue, was frozen in liquid nitrogen. GCs in M199 were pelleted by centrifugation and frozen in liquid nitrogen. Between three and four pools of tissue from each follicle size were collected for isolation of total RNA.

Radioimmunoassay for relaxin

Relaxin levels in follicular fluid from developing follicles were determined using a homologous porcine relaxin radioimmunoassay (Afele *et al.* 1979) with modifications. Monoacylated porcine relaxin was iodinated using chloramine T (Greenwood *et al.* 1982). Anti-porcine relaxin antibody (R6; 50 µl 1:30 000) was incubated with 300 µl follicular fluid for 24 h at 4 °C, and ¹²⁵I-relaxin (12 000 c.p.m.; 50 µl) was added for an additional 24 h at 4 °C. Separation of bound and free hormone was carried out by conventional double-antibody procedure. Assay sensitivity was 66 pg/ml and specificity of this assay has been described previously (Bryant-Greenwood & Greenwood 1979). Inter and intra-assay coefficients of variation were 8.6 ± 1.6 and 6.2 ± 0.5%, respectively.

Tissue preparation and immunohistochemistry

In order to distinguish different sized follicles at the histologic level, ovaries were cut into small pieces containing either small and medium or large follicles, prior to fixation. Tissues were fixed in Bouin's solution for 24 h at room temperature, dehydrated in increasing concentrations of alcohol and embedded in paraffin. Prior to immunostaining, the tissues were sectioned at 7 µm, mounted on glass slides with gelatin, deparaffinized, and rehydrated in decreasing concentrations of alcohol. At least four different animals were studied for each follicle size.

Relaxin was localized in tissue sections as described by Hsu *et al.* (1981) using the Vectastain ABC kit (Vector Laboratories, Inc.). All steps were performed at room temperature in a humidified chamber. Briefly, sections were pretreated with 0.3% hydrogen peroxide for 10 min to remove endogenous peroxidase activity. Non-specific binding sites for immunoglobulin G (IgG) were saturated with 3.0% normal goat serum (NGS) for 20 min. Tissues were then incubated for 1 h with rabbit antiporcine relaxin serum or normal rabbit serum (NRS) diluted 1:500 in phosphate-buffered

saline (0.015 M; PBS) with 1.0% NGS. The appropriate dilution of relaxin antisera for immunostaining was previously determined (Bagnell *et al.* 1987). After 2×3 min washes in PBS, tissue sections were incubated with biotinylated goat anti-rabbit IgG for 30 min, and washed 2×3 min in PBS. Sections were then covered with the ABC reagent for 45 min. After washing, peroxidase activity was visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride in 0.01% hydrogen peroxide/0.1 M Tris-HCl buffer (pH 7.2) for 2–8 min. The sections then were rinsed in water, counterstained with hematoxylin, and mounted using Permount. Sections of CL obtained from non-pregnant sows at day 15 of the estrous cycle, known to contain immunoreactive relaxin (Ali *et al.* 1986), were used as a positive control, and to control for interexperiment reproducibility. Method specificity was tested by substituting NRS for the rabbit antiporcine relaxin serum. Antiserum specificity was demonstrated by preabsorbing the relaxin antiserum with purified porcine relaxin (100 ng/ml) at 4 °C for 48 h prior to use.

Total RNA isolation

Total RNA was isolated from follicular tissue as previously described by Cathala *et al.* (1983) with modifications (Ryan *et al.* 1996). Tissues were homogenized in GT buffer (4 M guanidine thiocyanate; 50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 0.5% sodium N-lauroyl sarcosine, 8% 2-mercaptoethanol; 10 ml/g tissue) and homogenates were precipitated with 5 volumes 4 M LiCl. After centrifugation, the precipitate was resuspended in 2.5 ml TES buffer (50 mM Tris-HCl, pH 7.5; 5 mM EDTA; 0.5% SDS) and digested in proteinase K (Life Technologies). The remaining solution was then extracted with phenol:chloroform:isoamyl (25:24:1) and chloroform:isoamyl (24:1). Total RNA was ethanol precipitated, rinsed with ice-cold 80% ethanol, dried and resuspended in diethylpyrocarbonate-treated water. Quantity and purity of the RNA was assessed spectrophotometrically at 260 and 280 nm.

Reverse transcriptase-polymerase chain reaction

Although Northern analysis has been used to detect follicular relaxin gene expression in gonadotropin-primed prepubertal pigs, preliminary experiments indicated that this approach was not sensitive enough to detect the low copy relaxin transcript expected in follicles from immature pigs. Therefore, relaxin mRNA expression in follicular tissue was

monitored using RT-PCR as described by Knox *et al.* (1994) with modifications, and quantified relative to the amount of 18 s rRNA. DNA contamination was eliminated as reported by Huang *et al.* (1996) with modifications. Briefly, 1 µg total RNA was incubated at 37 °C for 30 min in $1 \times$ MMLV-RT reaction buffer; 1.5 µg random primers; 125 µM dATP, dCTP, dGTP and dTTP; 1 U DNase (RNase-free); and 20 U RNasin (RNase inhibitor). Subsequently, the DNase was inactivated for 7 min at 75 °C, and 200 U MMLV-RT were added for a 45 min reaction at 42 °C. A blank was included with each set of DNase-treated, RT samples, in which sterile water was substituted for RNA in the DNA elimination and RT reactions.

Primer sequences used to amplify porcine relaxin cDNAs were synthesized as described by Knox *et al.* (1994) and are specific for the 5'-(TACAGCA GCTGCAGTATCTA-OH) and 3'-(TGTCACCTGA GAATACATGTG-OH) untranslated regions of the relaxin gene. Relaxin and 18 s rRNA alternate primers:competimers (Ambion, Inc.) generated fragments of 663 and 324 base pairs respectively. Five- and one-microliter aliquots of the RT reaction were used to amplify relaxin and 18 s rRNA fragments respectively, with the GeneAmp DNA Reagent Kit in the following 50 µl reaction: $1 \times$ PCR buffer II; 2 mM MgCl₂; 50 µM each of dATP, dCTP, dGTP and dTTP; 10 pmol 5'- and 3'- relaxin primers or 18 s rRNA primers:competimers (8:2; 4 µl); and 1.25 U AmpliTaq polymerase. Amplification of relaxin and 18 s rRNA cDNAs was performed for 30 and 15 cycles respectively, each cycle consisting of 1 min denaturation at 95 °C, 45 s annealing at 56 °C and 1 min extension at 72 °C in the Ericomp DeltaCycler I System (Ericomp, Inc., San Diego, CA, USA). Each set of PCR included the appropriate volume of the RT blank described above. A pool of follicle RNA from pigs 60 and 72 h after equine chorionic gonadotropin (eCG)-treatment, which contains relaxin mRNA (Bagnell *et al.* 1990b), and liver from neonatal pigs, were used as positive and negative controls for relaxin transcript respectively. The post-eCG follicle pool was included in each set of RT-PCR to account for variability between each set of reactions. To verify that relaxin and 18 s rRNA cDNAs were amplified in the exponential range, an RT reaction of the post-eCG follicle pool was serially diluted (up to 1:32) and each dilution was used as template for relaxin and 18 s rRNA PCR.

Southern analysis

PCR products were fractionated on 5% polyacrylamide-Tris-borate-EDTA gels at 100 V in Tris (90 mM)-borate (90 mM)-EDTA (2 mM) buffer ($1 \times$ TBE). Gels were stained with ethidium

bromide (0.5 µg/ml) for 15 min, destained in distilled, deionized water for 15 min and photographed. Gels containing relaxin PCR fragments were prepared for Southern analysis by denaturing in 0.25 M NaOH for 5 min and neutralizing in 1 × TBE for 10 min prior to transfer. Amplified relaxin cDNAs were electrophoretically transferred to BrightStar-Plus, positively-charged, nylon membranes (Ambion, Inc.) in 0.5 × TBE with 400 mA for 1 h. Nucleic acids were fixed with 0.4 M NaOH for 10 min and crosslinked to the membrane with UV light (Stratalinker, Stratagene, Inc., La Jolla, CA, USA).

Relaxin antisense riboprobe was transcribed in a large scale reaction using the Gemini System (Promega Corp.). The riboprobe was labeled with biotin by irradiating 10 µl probe (50 ng/µl) with long wave UV light (366 nm) in the presence of psoralen-biotin for 45 min in the well of a microtiter plate on ice, according to the manufacturer's directions (Ambion, Inc.). Excess psoralen-biotin was removed from the labeled probe with two butanol extractions. The biotin-labeled relaxin probe was then used in Southern analysis of PCR products to detect relaxin fragments. The membranes were prehybridized at 42 °C for 15 min in hybridization buffer (10 ml) containing 50% formamide; 6 × SSPE (1 × SSPE=150 mM NaCl; 11.5 mM NaH₂PO₄; 1 mM EDTA); 5 × Denhardt's; 0.5% SDS and 50 µg/ml salmon testes DNA. Subsequently, the membranes were hybridized with biotinylated relaxin riboprobe in hybridization buffer (10 ng/ml; 5 ml) overnight at 42 °C. Membranes were then washed in the following: 2 × SSC, 0.5% SDS (1 × SSC=150 mM sodium chloride; 15 mM sodium citrate) for 5 min at 22 °C; 2 × SSC, 0.1% SDS for 20 min at 22 °C; 0.1 × SSC, 0.5% SDS for 20 min at 37 °C; 0.1 × SSC, 0.5% SDS for 20 min at 65 °C; and 0.1 × SSC for 20 min at 22 °C.

Relaxin antisense probe hybridized to relaxin PCR products was detected using the BrightStar BioDetect Nonisotopic Detection Kit (Ambion, Inc.). All steps of the detection procedure were performed at room temperature with rocking. The membranes were washed 2 × 5 min in wash buffer; 2 × 5 min in blocking buffer; and 30 min in blocking buffer. Subsequently, the membranes were incubated with a streptavidin-alkaline phosphatase conjugate for 30 min, and washed 10 min in blocking buffer; 3 × 5 min in wash buffer; 2 × 2 min in assay buffer; with a final incubation in the CDP-Star chemiluminescent reagent for 5 min. On the following day, the membranes were exposed to X-ray film (Hyperfilm-ECL; Amersham Corp., Arlington Heights, IL, USA) for 2–15 min. X-ray film images (relaxin PCR products) and Polaroid

negatives (18 s PCR products) were scanned into the computer using Adobe Photoshop 4.0 (Adobe Systems, Inc., San Jose, CA, USA). The area of pixel unit intensity (optical density units=OD_{units}) for each band was determined with SigmaGel 1.0 (Jandel Scientific Software, San Rafael, CA, USA). The positive control, post-eCG follicle PCR products from the appropriate set of RT-PCR were run on each gel. To correct for variability between the Southern blotting and non-radioactive detection procedures, optical densities generated from the relaxin and 18 s PCR products of each sample were divided by the densitometric value of the relaxin and 18 s fragments from the follicle pool on the same blot or gel. Subsequently, optical densities of the relaxin cDNAs were corrected to the optical density of the 18 s rRNA cDNAs of the same sample.

Statistics

The correlation between relaxin concentrations in follicular fluid and follicle size was determined using linear regression analysis. To calculate the mean concentration of relaxin in follicular fluid, each sample below the detection limit of the assay was assigned a value of 33 pg/ml, the midpoint of the detection limit of the assay (66 pg/ml). The midpoint of the assay detection limit was chosen since a value of zero would under-estimate mean relaxin concentrations, whereas 66 pg/ml would over-estimate mean relaxin levels. For statistical analysis of the gene expression results, optical densities from at least three pools of tissue from each follicle size were compared. Analysis of Variance and Duncan's Multiple Range Test were used to analyze radioimmunoassay and RT-PCR data. A $P < 0.05$ was accepted as significant.

RESULTS

Relaxin protein in the follicular fluid of developing follicles

Immunoreactive relaxin was detected in fluid from follicles of all sizes. Figure 1 illustrates the range and variation in relaxin concentrations of follicular fluid from different sized follicles. There was a positive correlation between increasing follicle size and relaxin concentration ($y = -332.9 + 241.9x$; $r = 0.694$; $P < 0.01$). Some of the values were below the detection limit of the assay. For example, in small follicles, relaxin was detected in 6 of 19 samples and ranged from 371 to 843 pg/ml. In medium follicles, relaxin concentrations in 11 of 17

FIGURE 1. Relaxin content of fluids from porcine follicles of increasing size. Fluids from pools of small (1–3 mm; $n=19$), medium (4–5 mm; $n=17$) and large (>6 mm; $n=9$) follicles of prepubertal pigs were collected by needle aspiration. Relaxin was measured using a homologous radioimmunoassay.

samples above the detection limit of the assay, ranged between 292 and 1369 pg/ml. In large follicles, eight of nine samples had detectable relaxin levels, which ranged from 1114 to 1989 pg/ml. Mean relaxin levels in follicular fluid significantly increased with follicle size ($P<0.05$), and averaged 185 ± 67 , 650 ± 139 , and 1465 ± 223 pg/ml for small, medium and large follicles respectively.

Localization of relaxin in developing follicles by immunostaining

Immunoreactive relaxin was localized in the theca interna layer, but not the granulosa layer, of large porcine follicles (Fig. 2a). When normal rabbit serum was substituted for the relaxin antisera, relaxin immunostaining was not evident in the theca of the large follicle (Fig. 2b). Preabsorption of relaxin antisera with purified relaxin also abolished specific relaxin immunostaining (Fig. 2c). There was no evidence for immunoreactive relaxin in the TC of small- or medium-sized follicles (data not shown). In addition, immunoreactive relaxin was not present in morphologically atretic follicles of any size (data not shown).

Relaxin mRNA in porcine follicles of different sizes

A linear relationship was observed between relaxin and 18s rRNA PCR product yields and the dilutions of positive control RT reaction used as

template in the PCR (data not shown). Relaxin gene expression was detected in whole small (1–3 mm) and medium (4–5 mm) follicles (Fig. 3a). In follicle walls containing thecal layers, relaxin mRNA was expressed in larger follicles (6 mm and >7 mm; Fig. 3b and c). However, relaxin transcript was not detected in GC from 6 mm and >7 mm follicle groups (Fig. 3b and c). In addition, relaxin mRNA was not found in liver from neonatal pigs, used as a negative control for relaxin transcript (Fig. 3b). While relaxin transcript was found in small, medium and large follicles, there was no difference in the relative abundance of relaxin mRNA between follicle sizes.

DISCUSSION

This study is the first to demonstrate that relaxin protein and gene are expressed in porcine follicles, independent of cyclic or exogenous gonadotropins. In ovaries of immature animals, relaxin protein was detected in follicular fluid by RIA and localized in the TC of the follicle wall by immunohistochemistry. The presence of relaxin mRNA in small-, medium- and large-sized follicles, detected by RT-PCR, supports the concept that these follicles are a source of relaxin protein. The data reported here, together with the *in vitro* growth-promoting effects of relaxin on GC and TC from developing porcine follicles (Zhang & Bagnell 1993, 1994), support the hypothesis of relaxin as a modulator of

follicular growth. Relaxin produced by the pig follicle may act in an autocrine/paracrine manner to stimulate GC and TC growth associated with follicular development.

The data presented here support the original reports indicating the presence of relaxin in fluid from large follicles of non-pregnant (Bryant-Greenwood *et al.* 1980) and immature pigs (Matsumoto & Chamley 1980). In addition, the present study extends these findings in two ways. By focusing on follicles recovered from ovaries without CL, we ruled out the CL as a source of relaxin in follicular fluid analyzed in our study. We also demonstrated the presence of relaxin mRNA not only in large follicles, but also in small- and medium-sized pig follicles. Furthermore, the concentration of relaxin in follicular fluid significantly increased with follicle size. However, there was considerable variation in relaxin content within each follicle-size class. A similar variation in relaxin levels is also reported in follicular fluid of women (Wathes *et al.* 1986) and horses (Ryan *et al.* 1997). The explanation for the high variation of relaxin in follicular fluid is unknown. However, diffusion of relaxin from larger follicles to small and medium follicles is a possibility and could be a source of variation. Another explanation may be that individual follicles differ in their rates of relaxin transcription, translation and/or secretion, leading to variable levels of relaxin protein and message in different follicles.

The fact that we found relaxin protein and gene expression in porcine follicles, independent of cyclic or exogenous gonadotropin stimulation, demonstrates that elevated gonadotropins are not a prerequisite for follicular relaxin production. This finding is important since several studies demonstrate that relaxin is produced in the follicle in response to exogenous gonadotropin administration in a number of species. When prepubertal pigs were given gonadotropins to induce follicular growth and ovulation, relaxin was detected in the follicular fluid (Bagnell 1991), and relaxin gene and protein were localized in TCs (Bagnell *et al.* 1987, 1990b).

FIGURE 2. Immunolocalization of relaxin in the wall of a large porcine follicle. Immunoreactive relaxin was sought in tissue sections using a specific relaxin antiserum (a; 1:500) and the Vectastain ABC kit. Immunoreactive relaxin localized to the theca interna (ti) layer of the follicle wall. Nearby sections were incubated with either normal rabbit serum (b; 1:500); or relaxin antisera preabsorbed with 100 ng/ml relaxin (c). Note the reduction in relaxin immunoreaction product in the theca (b, c) when compared with (a). g: GCs; Bar=50 μ m

FIGURE 3. Relaxin (RLX) gene expression in different sized porcine follicles. Total RNA was isolated from small (1–3 mm) and medium (4–5 mm) whole follicles, and GC and TC from large follicles (6 mm and >7 mm). Relaxin transcript was detected by RT-PCR and Southern analysis and relatively quantified as described in the Materials and Methods. (a) small ($n=3$) and medium ($n=4$) whole follicles; (b) TC ($n=3$) and GC ($n=3$) from 6 mm follicles and the negative control liver sample (L); (c) TC ($n=4$) and GC ($n=3$) from follicles >7 mm. +: positive control.

Relaxin secretion from thecal explants of pigs given exogenous gonadotropins is also stimulated by LH (Evans *et al.* 1983). In addition, follicular relaxin is found in women stimulated with gonadotropins (Yki-Jarvinen *et al.* 1984, Wathes *et al.* 1986, Haning *et al.* 1996). In women, baboons and monkeys, plasma relaxin also increases after human chorionic gonadotropin (hCG) administration (Quagliarello *et al.* 1980, Castracane *et al.* 1983, Ottobre *et al.* 1984). Although cyclic or exogenous gonadotropins were absent in the present study, we cannot rule out the possibility that follicular relaxin

production is influenced by the low circulating levels of gonadotropins reported in prepubertal gilts (Colenbrander *et al.* 1982, Diekman *et al.* 1983, Grier *et al.* 1986).

Evidence for the cellular source of relaxin in follicles from immature animals points to the theca, since relaxin transcript was detected in TC of larger-sized follicles. While relaxin production has been reported in porcine and human GC (Gagliardi *et al.* 1992, Loeken *et al.* 1983), this was likely due to the luteinization of these GC in culture. Relaxin is also produced by differentiating porcine GC during formation of the CL (Bagnell *et al.* 1989, 1993b, Denning-Kendall *et al.* 1989). However, RT-PCR analysis of TC and GC from larger follicles in this study indicates that GC do not express relaxin transcript, and therefore, theca are the sole source of follicular relaxin in the prepubertal pig. This relaxin gene expression in TC is consistent with our immunostaining results which localize immunoreactive relaxin to the TC of large follicles. Collectively, the data presented here extend the reports of thecal relaxin production in gonadotropin-primed pigs (Bagnell *et al.* 1987, 1990b) by showing that exogenous gonadotropins are not required for follicular relaxin expression. Our inability to immunolocalize relaxin in small- and medium-sized follicles may be related to the sensitivity of the immunohistochemical detection system used. This is supported by the fact that relaxin transcript was detected in follicles of all class sizes by RT-PCR. The presence of relaxin mRNA in small and medium follicles indicates that these less mature follicles are capable of producing relaxin.

Of interest in the present study is that the relative abundance of relaxin mRNA did not differ between small, medium and large follicles, while relaxin protein in follicular fluid was significantly correlated with follicle size. The reason for the increase in follicular fluid relaxin without a change in relaxin transcript is unknown. One possibility is that the semiquantitative RT-PCR method used in the present study was insensitive to increases in relaxin mRNA with follicle size. However, this approach accurately detects relative differences in mRNA abundance when both the relaxin and 18s cDNAs are amplified within the linear range, which we verified in the validation of our specific RT-PCR protocol. Another possibility is that relaxin in the follicle is translationally regulated, therefore, differences in relaxin protein would be expected without changes in relaxin mRNA. Lastly, it is possible that relaxin gene transcription and hormone secretion in the follicle are independent events. For example, when relaxin secretion and gene expression in

individual large LC (LLC) were studied using the reverse hemolytic plaque assay coupled with *in situ* hybridization, a direct correlation between relaxin secretion and relaxin mRNA was not demonstrated in all cells (Bagnell *et al.* 1993a). While a proportion of LLC that secreted relaxin contained relaxin mRNA, other relaxin-secreting LLC did not express relaxin transcript (Bagnell *et al.* 1993a). Thus, it is possible that rates of relaxin transcription are different from those of relaxin secretion in LLC, as well as in the follicle. A similar phenomenon has been reported in other protein-secreting cells (Tsang *et al.* 1990, Scarbrough *et al.* 1991, Larson & Wise 1994) and a temporal dissociation between hormone secretion and transcription has been suggested (Tsang *et al.* 1990, Scarbrough *et al.* 1991).

In summary, the presence of relaxin protein and mRNA in follicles of increasing size in the immature porcine ovary supports the concept of a follicular source of relaxin. This follicular relaxin may contribute to GC and TC growth associated with follicular development in prepubertal as well as cyclic pigs. Further studies are necessary to identify factors regulating follicular relaxin production, and relaxin's mechanism of action and physiologic roles in the pig ovary.

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