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In Situ Amplification of the Cytochrome P-450 Cholesterol Side-Chain Cleavage Enzyme mRNA in Single Porcine Granulosa Cells by IGF-1 and FSH Acting Alone or in Concert

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To investigate the cellular mechanisms and cell–cell heterogeneity of the actions of insulin-like growth factor-1 (IGF-1) and follicle-stimulating hormone (FSH) exerted alone and in combination on ovarian cholesterol side-chain cleavage gene expression (P450scc mRNA) in (pig) granulosa cells, we implemented semiquantitative *in situ* molecular hybridization at the single target-cell level. To this end, a 1-kb cDNA specific to the catalytic region of porcine p450scc gene was subcloned into pGEM-3 and directionally transcribed in vitro in the presence of ^{35}S-dUTP to yield radiolabeled antisense (and sense, negative control) cRNA hybridization probes. Swine granulosa cells harvested nonenzymatically from immature (1–5 mm) Graafian follicles were anchored on eight-chamber multiwell slides and treated with control solvent, human recombinant IGF-1 (10 n*M*), ovine FSH (10 n*M*), or both hormones, for 48 h to stimulate progestin biosynthesis maximally. After appropriate cellular permeabilization, cRNA hybridization, and solvent washes, granulosa cells were exposed to Kodak NTB-2 emulsion for 6 wk. Semiquantitative automated image analysis software (NIH IMAGE 1.5) was used to evaluate the number of silver grains deposited/20,000 square pixels. Specificity controls included labeled sense riboprobe, pretreatment with RNase, and 100-fold molar excess unlabeled cRNA. Grain counts and their distributions were examined by ANOVA and the Wilcoxon nonparametric test. The mean number of silver grains deposited per granulosa cell increased over control (reflecting specific P450scc mRNA expression) in granulosa cells pretreated with IGF-1, FSH, or IGF-1 + FSH ($p < 0.05$ by ANOVA). The rank order of abundance of expression of P450scc mRNA (grains/ovarian cell) was (IGF-1 + FSH) > FSH > IGF-1 > control treatment. Distributional analysis showed that each treatment introduced skewed distributions toward granulosa cells expressing more P450scc per cell than controls ($p < 0.01$). The median grain count of granulosa cells treated with FSH was significantly increased over that of IGF-1 treatment ($p < 0.05$). Treatment with both IGF-1 and FSH further shifted the grain count distribution per cell to favor granulosa cells expressing more P450scc mRNA compared to IGF-1 or FSH treatment alone ($p < 0.05$). Accordingly, a demonstrable mechanism of IGF-1 and FSH's regulation of specific P450scc gene expression at the single granulosa cell level is amplification in the number of target ovarian cells expressing this enzymatically rate-determining gene transcript. Interestingly, the induction of P450scc mRNA is not sufficient to explain fully the synergistic increases in progesterone accumulation driven by combined treatment with IGF-1 and FSH, thus suggesting that other steroidogenic control points are also targets of IGF-1/FSH action.

Key Words: P450scc; granulosa cells; ovary; porcine.

Introduction

In steroidogenic cells of the gonad, cholesterol metabolism and steroid-hormone biosynthesis are regulated by relevant systemically delivered as well as locally produced hormonal effectors. In the ovary, the gonadotropin follicle-stimulating hormone (FSH), presumably acting via cAMP, is a primary regulator of granulosa cell cytodifferentiation and steroidogenesis *(1)*. In addition, intrafollicular paracrine and autocrine regulators, such as insulin-like growth fac-

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tor-1 (IGF-1), acting via tyrosine kinase signaling pathways act and are produced locally within the Graafian follicle, and potently stimulate the cytodifferentiation of and sex-steroid hormone production by granulosa cells *(2)*. Indeed, an array of positive and negative regulators likely act in concert to control steroid biosynthesis by granulosa cells in the developing Graafian follicle.

FSH and IGF-1 synergize remarkably to augment progestin production in rat and porcine granulosa *(2–6)*. One critical locus of FSH and IGF-1's regulation is the cytochrome P-450 cholesterol side-chain cleavage enzyme (P450scc), the rate-limiting enzymatic step in steroid hormone biosynthesis. This inner mitochondrial enzyme complex hydroxylates and cleaves sterol substrate (cholesterol) to generate pregnenolone, which is converted to other biologically potent progestins *(7)*. Therefore, in addition to regulated sterol substrate uptake by the cell and delivery into the mitochondria, the P450scc enzyme itself is a primary candidate for hormonal control of progestin biosynthesis in granulosa cells. Although either FSH or IGF-1 alone can increase P450scc mRNA *(5,6)* and protein synthesis in ovarian cell populations *(8,9)*, little is known concerning the mechanisms of synergy between these two key regulators of P450scc gene expression whether in cell populations or especially in single cells. Indeed, at the level of enzymatic protein expression, marked cell-to-cell heterogeneity exists within apparently homogeneous granulosa cell populations *(10,11)*. Thus, cell population responses may reflect preferential gene activation either in the same or diverse cell groups.

The goal of the present study was to examine the mechanism of synergism between FSH and IGF-1 at single granulosa cell level. Serum-free primary porcine granulosa cell cultures were used, since this defined in vitro system is hormonally responsive to several distinct effectors that regulate ovarian steroid biosynthesis *(3–6,8,9)*. Interactions of IGF-1 and FSH were studied using *in situ* molecular hybridization to examine net transcription of the P450scc gene in individual granulosa cells. We hypothesized that FSH and IGF-1 would interact synergistically by amplifying the amount of specific P450scc expressed per granulosa cell and/or by increasing the number of individual granulosa cells expressing detectable P450scc mRNA.

Results

In Situ *Hybridization*

In situ hybridization with the specific porcine antisense P450scc riboprobe yielded multiple silver grains over primary porcine granulosa cells. This is illustrated in Fig. 1A,B. The number of silver grains over cells hybridized with the sense P45Oscc probe (even when treated with IGF-1 and FSH; Fig. 1C) was greatly diminished, which confirms specificity of antisense hybridization. Silver grains were also greatly decreased in frequency or abolished when cells granulosa were pretreated with an excess of unlabeled antisense probe or RNase (data not shown), further confirming hybridization specificity.

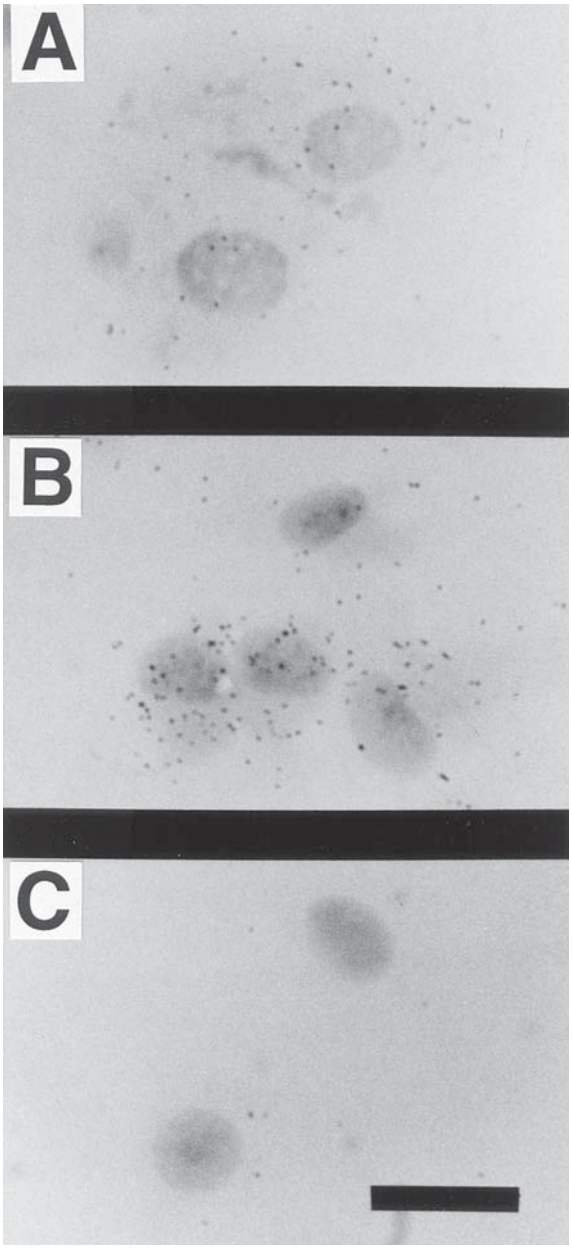

Fig. 1. *In situ* molecular hybridization of P450scc mRNA in primary porcine granulosa cells. Illustrative photomicrographs of: Panel **A** control primary porcine granulosa cells hybridized with a specific antisense cRNA to the P450scc gene; panel **B** granulosa cells treated with IGF-1 and FSH, and hybridized with a specific antisense P450scc cRNA; and panel **C** porcine granulosa cells pretreated with IGF-1 and FSH, but hybridized with a specific sense cRNA to the P450scc gene. Bar = 20 μm. Quantitative data are given in Figs. 2 and 3.

The mean number of silver grains deposited per cell was higher ($p < 0.05$ by ANOVA) in all three individual treatment (FSH, IGF-1, or both) groups over control (Fig. 2). In addition, FSH treatment alone increased the mean number

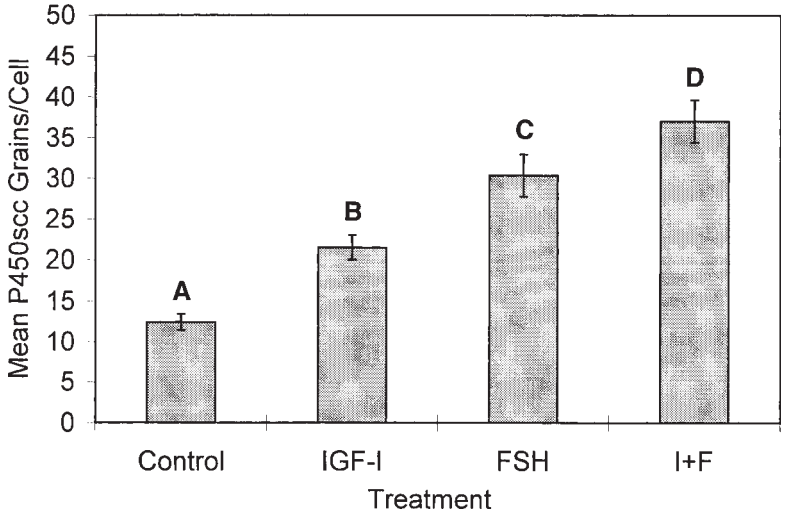

Fig. 2. Mean P450scc grains/cell in cultured porcine granulosa cells following FSH and/or IGF-1 treatment. Cells were treated for 48 h with or without (control) 10 n*M* of IGF-1 (I) and/or FSH (F) in serum-free MEMedia. Cells were hybridized within an antisense porcine P450scc riboprobe. Grain counts were determined from 20 cells/treatment in 8 independent studies. Bars = means ± SE. Bars with different superscripts are different ($p < 0.05$) by ANOVA..

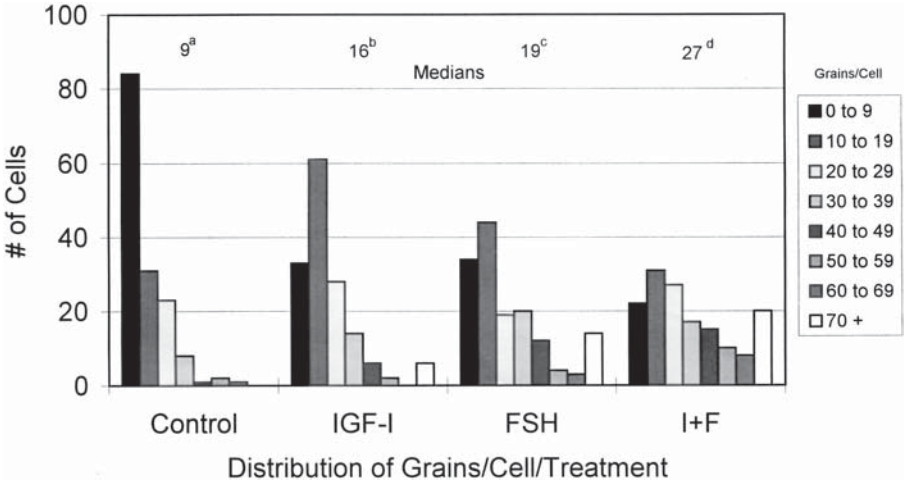

Fig. 3. Impact of FSH and/or IGF-1 on the distribution of in situ hybridized P450scc grains/cell in porcine granulosa cells in $N = 8$ independent experiments. Cells were treated with 10 n*M* of FSH or IGF-1 alone or in combination (I + F) for 48 h. The distributions of cells expressing a given number of P450scc grains were compared using the Wilcoxon rank sum tests to test *a priori* hypotheses. Median values are given above each histogram. Bars = number of cells within a pooled grain group. Treatments with different superscripts are different ($p < 0.05$).

of silver grains significantly more than IGF-1 treatment alone ($p < 0.05$). Combined treatment with FSH and IGF-1 elicited an additional increase ($p < 0.05$) over that of FSH and IGF-1 alone.

The distributions of the numbers of grains per cell for each treatment were found to be nonnormal ($p < 0.05$). These distributions for eight experiments are shown in Fig. 3. Each of the three treatment groups showed skewed distributions towards cells expressing significantly more grains (thus, more P450scc mRNA) per granulosa cell than the control group ($p < 0.01$). In addition, FSH-treated cells had distributions more skewed toward cells expressing a higher number of grains than IGF-1-treated cells ($p < 0.05$). The IGF-1 + FSH treatment yielded more cells expressing more grains over the cells than either IGF-1 ($p < 0.01$) or FSH ($p < 0.05$) alone. The median numbers were 9, 16, 19, and 27 grains per granulosa cell for the control, IGF-1, FSH, and IGF-1 + FSH treatment groups, respectively. These data indicate that increased accumulation of P450scc mRNA in porcine granulosa cells after combined treatment with IGF-1 and FSH is owing in part to augmented expression of this gene by individual granulosa cells.

Progesterone

Progesterone concentrations in the spent media (normalized as a percentage of control) increased ($p < 0.05$)

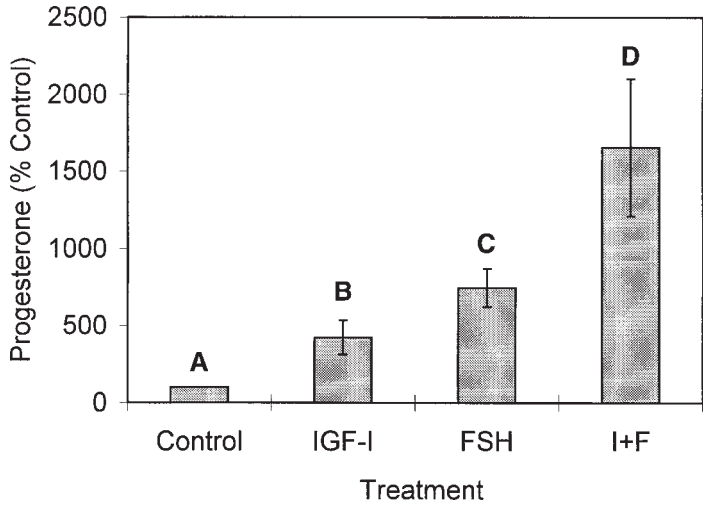

Fig. 4. Progesterone production (percentage of basal) by primary porcine granulosa cells studied in eight-chamber poly-L-lysine-coated microscope slides. Individual chambers were treated with 10 n*M* each of FSH and IGF-1 alone, and in combination (I + F) for 48 h. Progesterone accumulation was measured in the media by RIA. Bars = means ± SE. Bars with different letter superscripts are different by ANOVA ($N = 10$ experiments, which include two studies in which grain counts were not available: Materials and Methods). Absolute basal rates of progesterone accumulation averaged 2.95 ± 0.88 ng/mL medium/48 h.

significantly over controls in all three treatment groups (Fig. 4). Absolute basal progesterone accumulation in these studies averaged 2.95 ± 0.88 ng/mL medium/48 h. Progesterone concentrations rose 4.2-, 7.4-, and 17-fold above controls in response to IGF-1, FSH, and IGF-1 + FSH treatment, respectively. Thus, in the multichambered slide culture system, we confirm that combination treatment with IGF-1 and FSH is synergistic with respect to progesterone production by (porcine) granulosa cells.

Discussion

The present studies using computer-assisted grain counting for semiquantitative *in situ* molecular hybridization of a specific homologous P450scc riboprobe in immature porcine granulosa cells demonstrate that IGF-1 and FSH individually and jointly stimulate the increased accumulation of steady-state levels of specific mRNA encoding the cytochrome P450scc enzyme at the single ovarian target-cell level. Our single granulosa cell approach in serum-free monolayer cultures further indicates that the accumulation of P450scc mRNA after treatment with IGF-1 and FSH is the mechanistic result of a shift in the granulosa cell population distribution of gene expression, such that more P450scc mRNA is expressed per target granulosa cell. Interestingly, median P450scc mRNA accumulation per granulosa cell was 9, 16, 19, and 27 grains/cell for control, IGF-1, FSH, and (IGF-1 + FSH), thus with corresponding treatment ratios of 1, 1.8, 2.1, and 3.0, whereas progesterone synthesis increased, respectively by 1-, 4.2-, 7.4-, and 17-fold for the same treatments. Thus, whereas combined IGF-1 + FSH treatment augmented specific P450scc mRNA accumulation per granulosa cell significantly more than treatment with either IGF-1 or FSH alone, the (subadditive) increase was insufficient to account for the biologically synergistic enhancement of progesterone production by granulosa cells treated with combined IGF-1 and FSH. Therefore, we infer that yet additional sites and mechanisms of IGF-1 and FSH actions(s) likely participate posttranscriptionally and posttranslationally in regulating overall progestin biosynthesis in granulosa cells.

The biochemical actions of IGF-1 and FSH in inducing specific P450scc mRNA accumulation at the level of single immature swine granulosa cells are consistent with our previous observations *(3–6,8,9)*, including at the level of Northern blot analysis (e.g., *5,6*). These data also are in accord with others *(12)* in populations of swine granulosa cells studied in monolayer culture, as well as with inferences made earlier in populations of rat and human granulosa cells *(1,2)*. To our knowledge, similar data are not yet available for single theca cells derived from the same follicle. At the single granulosa cell level, we observe that the gonadotropin, FSH, and IGF-1, each significantly augments the amount of specific porcine P450scc mRNA expressed per granulosa cell, and that this action occurs with considerable cell–cell heterogeneity. This finding at the mRNA level is consistent with cytochemistry studies showing cell–cell heterogeneity in cytochrome P-450 protein expression, as well as that of other enzymes, in granulosa cells of rat preovulatory follicles *(10)*, and in ovine granulosa cells *(11)*. Independently, FSH has been shown to exert diverse effects on cytosolic Ca^{2+} signaling in individual (porcine) granulosa cells *(13)*, further supporting the notion of significant functional cell-to-cell heterogeneity of granulosa cells. Analogous observations regarding cellular diversity are not yet available to our knowledge at

the levels of cAMP or tyrosine kinase second-messenger signaling in individual FSH or IGF-1-responding granulosa cells. In addition, we note that further subclassification of follicles into atretic or nonatretic states, and individual cells as apoptotic or nonapoptotic, may also explicate some of the cell-to-cell nonuniformity of gene expression. Moreover, the replicative and/or differentiative phase of the individual granulosa cell might modulate its level of gene expression.

Monniaux and coworkers reported in ovine granulosa cells that IGF-1 primarily stimulates proliferation of granulosa cells from small follicles, but acts in synergy with FSH in larger follicles to increase progesterone secretion and *in situ* expression of the P450scc proteins *(11)*. Such facilitative interactions between IGF-1 and FSH on steroidogenesis (observed here and earlier *[3,4]* in pig granulosa cells) might be explained by way of dual-signal pathways. FSH potently activates a cAMP-PKA signaling pathway *(1)*, whereas IGF-1 stimulates a tyrosine kinase effector system presumably *(2)*. FSH treatment alone augments progesterone production by (swine) granulosa cells with a more rapid time-course than IGF-1 under identical experimental conditions *(5,6)*. Such observations, in conjunction with our findings of enhanced, but nonsynergistic P450scc mRNA accumulation in response to combined FSH and IGF-1 treatment, support the hypothesis that IGF-1 and FSH act through nonidentical cellular pathways to stimulate granulosa cell progestin synthesis. Rather, synergistic amplification of progesterone biosynthesis induced by these two first messengers may reflect differential coamplification of other sterol-regulating genes, and/or other components or the P450scc complex. In the latter regard, immunoprecipitation studies *(3)* earlier disclosed increases in cytochrome P450scc (enzyme) protein levels following treatment with IGF-1 and FSH alone and in combination (additive, but not synergistic), which are qualitatively similar to those identified here for P450scc mRNA *in situ*. The same immunoisolation experiments indicated that adrenodoxin, another key component of the cholesterol side-chain cleavage apparatus, also is markedly induced by combined IGF-1 and FSH stimulation. Thus, additional actions of FSH and/or IGF-1 on adrenodoxin and/or its reductase could account for facilitative interactions between IGF-1 and FSH. Alternatively, or concurrently, posttranslational enzyme modification and/or coamplification of other sterol-modulating genes may contribute to synergistic or additive effects of IGF-1 and FSH on progesterone production.

In mammals, the primary source of cholesterol substrate utilized for ovarian steroid hormone biosynthesis is blood-borne lipoprotein sterol. In pigs and humans, low-density lipoprotein (LDL) is the major cholesterol carrier, and the uptake of LDL by the ovary is mediated through the LDL receptor *(14)*. Moreover, the number of LDL receptors in porcine granulosa cells is increased significantly by IGF-1 and FSH acting alone *(15,17)*. Combined treatment with both trophic hormones evokes a pronounced synergistic effect *(3)*. Corresponding expression of the LDL receptor gene in porcine granulosa cells is also stimulated in vitro by FSH and IGF-2, with the IGF-2 probably acting through a type I IGF receptor *(18)*.

Certain other sterol-modulating loci and their genes are significantly regulated in (pig) granulosa cells by IGF-1 and FSH, namely the hydroxymethylglutaryl coenzyme A (HMG CoA) reductase enzyme necessary for the *de novo* synthesis of cholesterol *(19)*, and sterol carrier protein 2 (SCP-2), which may be involved in cholesterol transport within the cell *(20)*. In the rat, the enzyme mediating the conversion of pregnenolone to progesterone, 3-β-hydroxysteroid dehydrogenase, also is regulated by FSH *(31)*. Most recently, the steroidogenic acute regulatory (StAR) protein has been sequenced and cloned, and shown to respond to acute trophic hormone stimulation *(22)*. StAR is apparently rate-limiting in the delivery of cholesterol to the inner mitochondrial membrane for P450scc action. IGF-1 and FSH stimulate StAR protein and gene expression synergistically in culture (swine) granulosa cells *(23)*. Thus, IGF-1 and FSH likely play regulatory and interactive roles at multiple key loci of sterol metabolism in the ovary.

In conclusion, in serum-free primary monolayer cultures of swine granulosa cells, FSH and IGF-1 individually and jointly stimulate increases in specific P450scc mRNA concentrations in single granulosa ovarian cells as measured by *in situ* molecular hybridization. Mechanistically, the increased accumulation of P450scc mRNA, as driven by FSH and/or IGF-1 action(s), arises by way of an increase in the number of granulosa cells individually expressing more P450scc transcripts. Distributional analysis indicates that FSH and IGF-1 alone and together produce a right-shift in the frequency histogram of the amounts of specific P450scc mRNA expressed per granulosa cell. Since the median increases in P450scc mRNA do not fully explain the synergistic amplification of progesterone biosynthesis by combined treatment with IGF-1 and FSH, our *in situ* observations and the available literature suggest that these two key interactive regulators of steroidogenesis also convergently impact the expression and/or activity of other sterol-regulating loci within ovarian cells.

Materials and Methods

Cell Harvesting, Culture, and Hormonal Treatment

Granulosa cells were harvested nonenzymatically from immature porcine follicles (1–5 mm in diameter) by fine-needle aspiration, as previously described *(3)* and plated on eight-chamber poly-L-lysine-coated tissue-culture slides (Nunc, Inc., Naperville, IL). Cells were incubated as 250,000 viable cells by trypan blue vital-dye exclusion in 300 μL for 24 h at 37°C initially in 3% fetal bovine serum (FBS)/Eagle's Minimum Essential Medium (MEM) to allow

anchorage. After 24 h, medium was replaced with serum-free MEM. At this time, granulosa cells were treated with:

1. 10 n*M* recombinant human IGF-1 (UBI, Lake Placid, NY);
2. 10 n*M* ovine FSH (NIH);
3. IGF-1 + FSH; or
4. Media only (control) for 48 h.

These IGF-1 and FSH concentrations were previously found to stimulate granulosa cell steroidogenesis maximally *(5,6)*. At the time of cell processing, aliquots of media from each chamber were collected for later measurement of progesterone by RIA. Subsequently, the cells were processed for *in situ* molecular hybridization. All experimental observations were made in duplicate. The data reported are from eight independent granulosa cell experiments reflecting cell collections from 400–500 ovaries each. Data from two experiments were lost by inadvertent light exposure of slides early in this study, but the progesterone data were available.

Gene Probes

A 1-kb cDNA probe specific to porcine P450scc *(24)* was kindly supplied by Thomas Wise, USDA HMARC, Clay Center, NE. This probe was subcloned into a pGEM-3 vector containing T7 and SP6 RNA polymerase promotors to synthesize antisense and sense cRNA probes, respectively *(12)*.

In Situ *Hybridization*

The *in situ* hybridization procedure was performed as described *(25–27)* with modifications we have found suitable for primary granulosa cell culture *(28)*. Hormonally treated cells plated on eight-chamber slides were washed with Hank's Buffered Saline Solution (HBSS), and fixed for 30 min in 4% paraformaldehyde/phosphate-buffered saline (PBS). Slides containing fixed cells were rinsed in PBS, and permeablized with 0.5 g/mL proteinase K for 30 min. The slides were then acetylated with 0.25% acetic anhydride in 0.1 *M* triethanolamine (pH 8.0) for 10 min, soaked in 2X SSC for 3 min at room temperature, and dehydrated through graded ethanol washes. The slides were dried and stored until hybridization.

P450scc antisense riboprobes were labeled with ^{35}S-dUTP using an in vitro transcription kit (Ambion, Inc., Austin, TX), and purified over Sephadex G-50 minispin columns (Worthington Biochemical Corp., Freehold, NJ). The P450scc probe underwent a bicarbonate hydrolysis step, followed by ethanol precipitation, leaving an average length of 150 nucleotides for better probe penetration into the cells (R. A. Steiner, unpublished protocol). The probes were diluted in a hybridization buffer containing 50% formamide, 10% dextran sulfate, 1X Denhardt's solution, 4X SSC, 10 m*M* dithiothreitol (DTT), 0.25 mg/mL yeast tRNA, and 0.5 mg/mL salmon sperm DNA, and applied to the cells at a dilution of 1–10 × 10^6 CPMs/500 μL. The slides were cover slipped with parafilm and hybridized at 37°C overnight in a moist chamber. Following hybridization, slides were treated with 30 μg/mL RNase A in 10 m*M* Tris (pH 8.0)/1 m*M* EDTA/0.5 *M* NaCl (TEN) for 30 min at 37°C to remove all single-stranded RNA, thus leaving only riboprobe–RNA hybrids. The slides were rinsed in TEN and subjected to consecutive 30-min stringency washes of 2X SSC/1 m*M* DTT, 1X SSC/1 m*M* DTT, 0.5X SSC/1 m*M* DTT at room temperature, followed by a high-stringency wash of 0.5X SSC/1 m*M* DTT at 42–60°C for 30 min. Hybridized slides were air-dried and exposed to Kodak NTB-2 emulsion for 6 wk, developed, counterstained in hematoxylin-eosin, and cover slipped.

Grain-counting measurements were made using a microscope fitted with a video camera, and quantitative computer imaging equipment and software. Ten cell images were randomly captured from each treatment chamber and cataloged as TIFF files using OPTIMAS 4.1 (Optimas Corp., Edmonds, WA) image analysis software. Each eight-chamber slide represented one experiment from a weekly collection of granulosa cells for a total of eight independent experiments. Each slide consisted of two chambers for each of the four treatments. Captured image files were then transferred over a mainframe UNIX computer to a MacIntosh Quadra 650 (Apple, Palo Alto, CA) computer, and grains over cells quantitated using a customized macro on the NIH IMAGE 1.5 (NIH) image analysis software. Grain counts were standardized as grains/20,000 square pixels area (approximately equal to an average granulosa cell area). In addition, measurements (usually negligible) were also taken beyond the periphery of the cells and subtracted as background in slides hybridized with the antisense riboprobe.

Specificity controls for the *in situ* hybridization experiments included:

1. Hybridization with labeled sense (rather than antisense) riboprobes.
2. Pretreatment of slides with RNase to degrade cellular RNA.
3. Incubation of cells with labeled probe and a 100-fold molar excess of unlabeled probe.

Progesterone Assay

Media for progesterone RIA were pooled from the eight-chamber slides by treatment group for each weekly granulosa cell harvest (n = 10). Steroids were extracted with hexane, dried, dissolved in 50 μL ethanol, and diluted with 200 μL of MEM. Progesterone concentrations in media were measured by RIA using antibody-coated tubes manufactured by ICN Biomedicals *(5,6)*. The progesterone RIA has a sensitivity of 0.15 ng/mL and <1% crossreactivity with other pertinent steroid hormones.

Statistical Analysis

Effects of treatments on hormonal and RNA levels were analyzed using ANOVA, and a Duncan's New Multiple-Range test *(29)*. An Anderson-Darling test was used to test for normality of the grain count distributions *(30)*. Grain count distributions were examined using a nonparametric Wilcoxon rank sum test between treatments *(31)*.

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