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Follicle Stimulating Hormone Stimulation of ^{125}I-Human Chorionic Gonadotropin Binding in Porcine Granulosa Cell Cultures[1] (38780)

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It has been known for a long time that pituitary follicle stimulating hormone (FSH) must be administered along with pituitary luteinizing hormone (LH) in order to promote ovarian follicular growth, ovulation, and luteinization in the immature or hypophysectomized rat (1). The mechanism by which the FSH exerts this "synergistic" action with LH is not known. FSH also synergizes with LH in promoting luteinization of porcine (2) and monkey (3, 4) granulosa cells *in vitro*. Furthermore, as the follicles mature *in vivo* under the influence of LH and FSH, there is an increase in the ability of granulosa cells to bind hCG (5). This appears to reflect an increase in numbers of LH-hCG receptors per cell rather than a change in affinity for LH or hCG (6, 7). Zeleznick *et al.* (8) demonstrated that FSH administration *in vivo* in the rat could stimulate ovarian granulosa cells and luteal tissue binding of iodinated human chorionic gonadotropin (hCG) or LH *in vitro*. These findings led to the hypothesis that FSH acts by stimulating the formation of LH receptors. The present studies were aimed to prove that FSH can directly stimulate formation of LH-hCG receptors in granulosa cells in an all *in vitro* system. For this purpose granulosa cells obtained from immature small porcine follicles were grown in the presence of purified human FSH for a 2-day period and the influence of the FSH upon the ability of the cells to bind iodinated hCG measured.

Materials and Methods. Granulosa cells were harvested from 1 to 2 mm small porcine follicles according to methods detailed elsewhere (2, 5, 9). Subsequently, the cells were counted in a hemocytometer in 0.2% lissamine green and inoculated in aliquots of 1×10^7 cells/ml in a volume of 14 ml culture medium in 250 ml Falcon plastic culture flasks. Cells were grown in both serum-containing medium (2) and serum-free medium (9). Serum-containing medium (designated medium Y) contained 15% serum obtained from immature male pigs in a balance of culture medium 199 plus 25 m*M* HEPES buffer and Earles salts (purchased from Grand Island Biological Co., Grand Island, New York; GIBCO). The serum-free medium (designated medium 199D) contained 0.4% bovine serum albumin (BSA; GIBCO), 0.2% lactalbumin hydrolysate (GIBCO) in a balance of culture medium 199 plus Earles salts and 25 m*M* HEPES buffer. Both medium 199D and Y contained supplemental L-glutamine (2 m*M*) and 50 units/ml penicillin and 50 μg/ml streptomycin and 25 μg/ml fungizone as antibiotics. In some experiments, 50 μg/ml Gentamicin (Schering Corp., Port Reading, NJ) alone was used as an antibiotic.

At the end of 2, 4, and 6 days, cultures were terminated by scraping with a rubber policeman. Each culture was divided into 4–6 equal aliquots. Five replicate aliquots were incubated for 30 min with shaking at 37° in the presence of 0.1 μg/ml biologically active ^{131}I or ^{125}I labeled hCG as outlined previously (5, 9, 10). One to three of these replicate aliquots were incubated in the presence of excess unlabeled hCG (1 μg/ml) to determine nonspecific binding. Specific binding (>80% of total binding in each instance) was estimated by subtracting the binding obtained in the presence of 1.0 μg/ml unlabeled hCG from the total binding. The data presented in Tables I and II have therefore all been corrected for nonspecific binding which represented <20% of total binding in each instance. One or two aliquots of the cells were used for a Lowry protein determination (11). Cell numbers were estimated by a planimetric method (2, 3). If

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TABLE I. EFFECT OF HUMAN FSH UPON BINDING OF IODINATED hCG TO CULTURES OF PORCINE GRANULOSA CELLS.[a]

Experiment	CPM Bound/Culture ($\bar{X} \pm SE$)			FSH Effect (FSH/control)
	Medium Y	199D + 10 munit Insulin (Control)	199D + 10 munit Insulin + 0.05 μg/ml FSH	
I	1983 ± 381 (3)	962 ± 45 (3)	3933 ± 1787 (3)	4.08
II	—	1132 ± 70 (6)	1660 ± 277 (3)	1.47
III	—	1473 ± 200 (3)	2330 ± 289 (3)	1.58
IV	6584 (2)	11,369 (2)	16,281 (2)	1.43
V	—	7003 (2)	17,412 (2)	2.49

[a] Granulosa cells were grown for 2 days in either serum-containing medium Y or serum-free medium 199D plus 10 munit/ml insulin. Binding of iodinated hCG was determined by a subsequent incubation for 30 min in the presence of 0.1 μg/ml ^{125}I- or ^{131}I-hCG.

cultures were grown for more than 2 days, the medium was changed on alternate days. Binding was expressed as cpm/culture or cpm/mg protein. Within an experiment (Tables I and II) binding per culture was normalized to binding per 25% confluent culture (i.e., a culture 25% covered with cells).

In each experiment replicate cultures were grown in medium Y or medium 199D + 10 munits crystalline zinc insulin (Squibb) or medium 199D + 10 munits insulin plus 0.05 μg/ml human FSH. The FSH was obtained from Dr. W. D. Peckham of the Department of Physiology of the University of Pittsburgh School of Medicine, Pittsburgh, Pa. The human FSH preparation had a biologic potency of ~200 U/mg in terms of NIH-FSH-S1 in the Steelman Poohley human assay (12). FSH preparations prepared in a similar fashion contained less than 0.05 unit/mg of contaminating LH activity using the Parlow ovarian ascorbic acid depletion assay (13). Each experiment was repeated five times using separate batches of porcine granulosa cells. The Student's *t* test was employed to evaluate significant differences between groups.

In four experiments using granulosa cells obtained from large 6–12 mm follicles (which already have large numbers of receptors), the influence of culture conditions upon "preformed specific receptors" was estimated after 2 days in culture. Prior to culture freshly harvested cells from large follicles specifically bound 361, 200 ± 16,800 cpm/mg protein. Aliquots of the same cells bound 91,920 ± 4480 cpm/mg protein after culture for 2 days in medium 199D + 10 munit/ml insulin. In the presence of excess cold hCG nonspecific binding was <10% of the total binding before and after culture. Therefore, under these culture conditions there is a loss of about 60–70% of "preformed receptors." In the case of cells obtained from small follicles in Experiment III (Table I), initially the cells bound 8287 ± 311 cpm/mg protein and after 2 days of culture in medium 199D + 10 munits insulin, they bound 4086 ± 313 cpm/mg protein. Therefore, in the case of small follicle cells the loss in binding was less, being less than 50%. Granulosa cells cultured for 2 days in Experiment I had the following total cpm hCG bound/mg protein: medium Y: 10,965 ± 2110; medium 199D + 10 munits insulin:

TABLE II. INFLUENECE OF INSULIN UPON THE ABILITY OF FSH TO STIMULATE hCG BINDING IN PORCINE GRANULOSA CELL CULTURES.[a]

Culture medium	CPM ^{125}I-hCG Bound/ Culture ($\bar{X} \pm SE$; $n = 3$)
199D	735 ± 160
199D + 10 munit/ml insulin	1473 ± 200
199D + 0.05 μg/ml human FSH	1335 ± 113
199D + 10 munit/ml insulin + 0.05 μg/ml human FSH	2330 ± 289

[a] Granulosa cells were grown for 2 days in culture medium 199D with or without insulin or human FSH. Binding to iodinated hCG was determined by a subsequent incubation for 30 min in the presence of 0.1 μg/ml ^{125}I- or ^{131}I-hCG.

24,320 ± 1150; medium 199D + 10 munits insulin + 0.05 μg/ml human FSH: 62,160 ± 28,253. This demonstrated that normalization of cpm bound per culture or per mg protein gave roughly similar results. Therefore data in Tables I and II are expressed per culture.

Results. In five separate experiments after a 2-day culture period, addition of FSH stimulated specific hCG binding two- to fourfold above control cultures (Table I). Lengthening the culture period to 2 or 6 days did not significantly alter the binding. In the chemically defined media, by 6 days there were some signs of cellular necrosis demonstrating that some other factor essential for maintenance of cell viability was lacking. In two experiments insulin was omitted from the medium 199D; binding was also diminished 50%. However, there also was poorer cell growth and some signs of cellular necrosis. FSH also stimulated binding even in the absence of insulin but its effect was less (Table II).

Discussion. These data demonstrate that over a 2-day period FSH can consistently stimulate hCG binding two- to fourfold in porcine granulosa cells cultured in chemically defined media. This could indicate that FSH is involved in stimulating formation of LH-hCG receptors. Alternately, the FSH could alter the turnover of the LH-hCG receptors leading to a net increase in available receptor sites. These findings would support the *in vivo* findings of Zeleznick *et al.* (8) and Midgley *et al.* (14) who demonstrated that FSH injections *in vivo* stimulated hCG binding in rat ovaries. These data further demonstrate that FSH can have a direct effect *in vitro* upon a homogeneous target cell, a finding not demonstrated by others. An alternate explanation for these findings is that the FSH first stimulates estrogen secretion which in turn brings about an increase in hCG binding. This explanation is supported by the finding by Lee and Ryan (15) who demonstrated that administration of estrogen to pseudopregnant rats brought about a subsequent increase in the ability of the luteinized ovary to bind ^{125}I-hCG *in vitro*.

In vivo during follicular maturation the increase in LH-hCG binding in granulosa cells obtained from large mature follicles (6–12 mm) compared to cells from small (1–2 mm) follicles is in the order of 20- to 500-fold. Such FSH stimulation of binding was not achieved in our 2 day granulosa cell cultures. This could indicate that something other than FSH is also required for receptor induction. This is supported by the finding in the rat that LH and estrogen administration *in vivo* can augment the FSH stimulation upon ovarian binding to ^{125}I-hCG (8, 14). Further experiments examining the effects of LH and estrogen addition to the cultures are being carried out to test this hypothesis. Inability to observe a greater stimulation after 4 or 6 days compared to 2 days in cultures is most likely due to an "endocrine deficiency" in the chemically defined medium since after 4–6 days cellular necrosis was noted. We have demonstrated previously that addition of thyroxin and cortisol to medium 199D can improve granulosa cell viability and responsiveness to LH and FSH up to a period of 8 days (C. P. Channing and V. Tsai, unpublished observations). Lack of these hormones as well as perhaps estrogen and LH may be also responsible for the net loss in hCG binding observed after 2 days compared to freshly harvested unincubated cells observed here and elsewhere (Ledwitz-Rigby and Channing, manuscript in preparation; 9, 16).

FSH addition to granulosa cell cultures also improves the ability of LH to stimulate progesterone secretion in cultures of granulosa cells harvested from small porcine (2, and Channing, unpublished observations) and monkey follicles (3, 17). In light of the present findings it would be most reasonable to postulate that the FSH improves responsiveness to LH by stimulating LH-hCG receptors or altering the turnover of the receptors. The mechanism of this stimulation as well as interaction with other hormones remain a subject for future investigation.

Summary. In order to see if FSH acts directly upon the granulosa cell to stimulate hCG binding, granulosa cells harvested from small 1–2 mm porcine follicles were grown in 250 ml flasks in chemically defined media containing 0.05 μg/ml highly purified human FSH for 2, 4, and 6 days. The defined medium consisted of culture medium

199 plus 0.4% bovine serum albumin, 0.2% lactalbumin hydrolysate and 10 munit/ml insulin. The cultures were harvested by scraping with a rubber policeman and incubated with 0.1 μg/ml ^{131}I- or ^{125}I-hCG. Binding expressed as cpm/culture or per mg protein yielded similar results. In five separate experiments addition of FSH stimulated hCG binding two- to fourfold above control cultures. In a typical experiment after 2 days of culture, the specific binding of control cultures to hCG was 962 ± 45 cpm/culture ($\bar{x}$ ± SE; $n = 3$) and the binding in cultures grown in the presence of 0.05 μg/ml FSH was 3933 ± 1787 ($n = 3$; $P < 0.01$). Granulosa cells harvested from large (8–12 mm) follicles grown under similar conditions bound 29,669 ± 948 cpm/culture ($n = 4$). These data demonstrate that FSH may have a direct stimulatory role upon induction of granulosa cell LH-hCG receptors *in vitro*.

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