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Ability of an Extract of Pig Corpus Luteum to Inhibit Binding of ^{125}I-Labeled Human Chorionic Gonadotropin to Porcine Granulosa Cells (39810)

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Introduction. Yang *et al.* have observed that there is an inhibitor of LH binding in the 30,000*g* supernatant of Parlow "pseudopregnant" and pregnant rat ovaries and have designated it as LH RBI (LH receptor binding inhibitor) (1). They demonstrated that it was probably a low-molecular-weight polypeptide of about 3800 daltons. Our studies were undertaken to see whether this inhibitor was present specifically in corpus luteum tissue and in a species other than the rat. Inhibition of binding of iodinated human chorionic gonadotropin (hCG) to porcine granulosa cells was examined since hCG and LH share the same binding site on the granulosa cell (2). Iodinated hCG is biologically active under conditions of our *in vitro* experiments and serves as a suitable tracer molecule (3). The effect of storage in the frozen state on the recovery of the inhibitor was also examined.

Materials and methods. Porcine ovaries were obtained from a local meat-packing plant within 20 min of slaughter and were kept on ice until arrival at the laboratory about 1 hr later. Ovaries containing corpora lutea were separated from other ovaries and frozen at −35° in batches of 20 to 50 ovaries. Ovaries containing corpora hemorrhagica and corpora albicantia, as identified according to the criteria of Akins and Morrisett (4), were not studied. Lungs and hearts were collected within 5 min of slaughter and frozen. The tissues were stored at −35° for varying lengths of time and homogenates were made immediately after thawing out the tissue.

All preparations of tissue after thawing were carried out at 4° unless otherwise specified. All tissue homogenates were tested for their ability to inhibit binding of iodinated hCG to granulosa cells within 1 week of homogenization. The tissues (whole ovaries, corpora lutea, nonluteal part of the ovaries, hearts, or lungs) were weighed in lots of about 40 g and homogenized for 1 min in a Waring Blendor in 6 vol of distilled water. The homogenized tissue was then filtered through three layers of cheesecloth, and the filtrate was centrifuged at 1000*g* for 15 min; the supernatant was centrifuged for 15 min at 30,000*g* in a Sorval Model OTD ultracentrifuge. In some instances the 30,000*g* supernatant was centrifuged at 105,000*g* for 30 min. Aliquots of the 1000*g* supernatant, as well as of the 30,000 and 105,000*g* supernatants, were frozen at −35° and later assayed for protein by the Lowry technique (6) and for their ability to inhibit binding of iodinated hCG, as outlined below.

Highly purified hCG obtained from Dr. Robert Canfield (CR 117; 14,000 IU/mg) was iodinated in aliquots of 10 μg with the chloramine-T technique (7) except that the ratio of hormone to chloramine-T was 1:1. The iodination, with 1 mCi of ^{125}I (Amersham), was allowed to take place for 1 min on ice and the reaction stopped with the addition of 10 μg of sodium metabisulfite. The mixture was poured on a 15 × 0.5-cm column of Bio-Gel P-60 and 1-ml fractions were collected. The peak fraction, which had a specific activity of 20–30 μCi/μg of hCG, was saved and used for these studies. This tracer was used (see below) within 2 weeks of iodination.

Granulosa cells were harvested for the binding assay from large (5- to 12-mm diameter) porcine follicles (3, 5, 8), washed three times with Eagles medium, and resuspended in incubation buffer consisting of 100 m*M* Tris plus 0.1% beef serum albumin (BSA), pH 6.5 (solution A). Aliquots of 0.5 ml of packed cells were resuspended in 20 ml of buffer to give approximately 1 × 10^6

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cells/0.1 ml. Cells in 0.06% trypan blue were counted in a hemocytometer. The cell suspension was frozen at −35° until use 1–8 weeks later.

For the binding incubation 100 μl aliquots of the cell suspension were added to siliconized 12 × 75-mm glass tubes. An amount of cells capable of binding ^{125}I-labeled hCG to yield 10,000 cpm was used in each incubation. Prior to aliquoting, the cell suspension was thawed out, homogenized with five strokes of a glass homogenizer, and mixed on a Vortex mixer before addition to the test tubes.

Each assay consisted of the following test tubes: *control,* cell suspension plus solution A plus ^{125}I-labeled hCG; *experimental,* same, except the test solution (the 1000, 30,000, or 105,000*g* supernatant of homogenized tissue) in place of solution A; *nonspecific binding,* same as control, except solution A contained 10 μg of cold hCG. Each of these components was added in a volume of 100 μl. The iodinated hCG solution contained 5 ng of ^{125}I-labeled hCG/100 μl (~100,000 cpm). Nonspecific binding was less than 5% of total binding. In each assay, the control tubes were counted first, then the experimental tubes, in replicates of five, and finally, the nonspecific binding tubes.

The granulosa cell suspension was allowed to preequilibrate with either solution A, the unlabeled hCG solution, or the unknown test solution for 20 min at 37° in a shaking metabolic incubator; ^{125}I-labeled hCG was then added to all tubes, which were then incubated with shaking for 1 hr at 37°. The binding was terminated by the addition of 3 ml of cold wash solution B [100 m*M* Tris plus 0.1% purified pigskin gelatin (Eastman)], pH 6.5; 100 μl of celite suspension was also added to increase the size of the pellet and aid in minimizing losses during washing of the pellet. The celite suspension consisted of 1 mg of celite/1.0 ml of solution A. The tubes were centrifuged at 1000*g* for 10 min at 4°. The supernatant was decanted and the last drop was aspirated from the tubes. The tubes were washed again with an additional 3 ml of solution B followed by centrifugation and decanting as outlined above. Each tube was then counted for 10 min in a Packard auto-γ counter with a detection efficiency of about 75% for ^{125}I. Data were corrected for background counts (100–200 cpm) as well as for nonspecific binding. The corrected counts in each instance were expressed as the percentage of the counts observed in the control tubes. Each binding experiment was repeated at least twice and sometimes three times. Each tissue extract was made using a different batch of lung, heart, or ovarian material obtained from the slaughterhouse.

Results. The 30,000*g* supernatant of a homogenate of either lung, corpus luteum, or whole ovary had little or no ability to inhibit binding (Fig. 1). The 1000*g* supernatant of many of the tissues sometimes exerted a slight stimulatory effect upon binding (Table I). If the ovarian tissues were stored in the frozen state for 1.5 or more weeks, however, the ability of both the 1000 and the 30,000*g* supernatants to inhibit binding increased markedly (Fig. 1, Table I). After these and longer periods, the lung, heart, or nonluteal ovarian tissue supernatants did not inhibit binding (Fig. 1; Table I). The supernatant of a pool of granulosa cells from 3- to 6-mm follicles inhibited binding less than 10%. Similar results were obtained if each binding observation was expressed in terms of milligrams of protein of the supernatant extract. The 105,000*g* supernatant of corpus luteum, but not of lung tissues, stored for 3 or more weeks had inhibitory activity comparable to that present in the 30,000*g* supernatant (Table I).

Discussion. These findings demonstrated that after storage at −35° for 1.5 or more weeks porcine corpus luteum, but not other tissues, contained a soluble factor which could inhibit binding of iodinated hCG to porcine granulosa cells. These findings are similar to those of Yang *et al.* in the rat (1). Why the inhibitory activity appears only with prolonged storage at −35° cannot be easily explained. A similar observation was recently made by Yang and Ward in pseudopregnant rat ovaries (personal communication). It is possible that during storage an inhibitor is released from the cell membrane.

The existence of a luteal cell LH-hCG binding inhibitor in the pig may perhaps explain our previous observation that the binding of iodinated hCG to porcine corpus luteum cells was much less than to granulosa

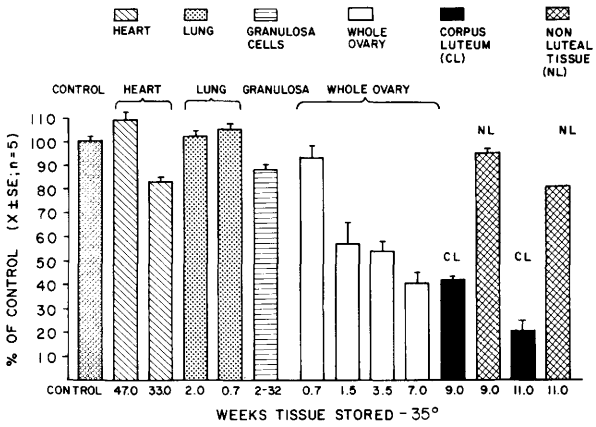

FIG. 1. Ability of 30,000*g* supernatant of various porcine tissues to inhibit binding of ^{125}-labeled hCG to porcine granulosa cells. The binding in the presence of the various supernatant fractions was expressed as a percentage of control binding in the presence of buffer alone and is given as the mean ± SE of five observations. The tissues were stored at −35° prior to homogenization and preparation of the supernatant; the duration of storage is shown at the bottom of the figure.

TABLE I. EFFECT OF 1000, 30,000, AND 105,000*g* SUPERNATANT OF VARIOUS PORCINE TISSUES UPON BINDING OF ^{125}I-LABELED hCG TO PORCINE GRANULOSA CELLS.[a]

Tissue Extracted	Storage in tissue in freezer prior to extraction (weeks)	Percentage of control binding of ^{125}I-labeled hCG[b]			
		Experiment I	Experiment II		
		1000*g*	1000*g*	30,000*g*	105,000*g*
Lung	0.7	106 ± 2.3			
	2.0	107 ± 2.0			
	3.5		133 ± 3.0	110 ± 1.7	
	6.2		110 ± 1.7		110 ± 1.7
Heart	33	103 ± 4.2			
	47	110 ± 3.0			
Whole ovary	0.7	110 ± 2.0			
	1.5	74 ± 9.6			
	3.5	67 ± 3.6			
Isolated corpus luteum	3.5		133 ± 4.0	49 ± 0.4	
			132 ± 5.2	47 ± 1.4	
	5.0		58 ± 1.4		37 ± 1.0
	5.1		90 ± 2.7	41 ± 0.4	44 ± 1.4
	9.0	50 ± 1.7			
	11.0	33 ± 4.3			
Nonluteal ovarian tissue	3.5		92 ± 1.1	83 ± 1.1	
			83 ± 2.2	73 ± 2.4	
	5.0		131 ± 5.0		76 ± 2.1
	5.1		72 ± 1.6		77 ± 1.2
	11.0	68 ± 2.8			

[a] The tissue was stored for various periods of time in the freezer at −35° and homogenized in water followed by incubation in the presence of porcine granulosa cells and iodinated hCG for 60 min at 37°. The binding at the end of the incubation period is presented as a percentage of the binding in the presence of control buffer alone.

[b] n = 5; mean ± SE.

cells harvested from large porcine follicles (9). A previous explanation of these findings was that the LH receptors had become bound to LH and that this rendered them unavailable for further binding to exogenous LH or hCG.

We have not rigorously ruled out that the luteal cell hCG binding inhibitor represents solubilized LH-hCG receptors. The observation that granulosa cell extracts do not contain inhibitor is partial evidence against this possibility. Porcine granulosa cells contain

hCG receptors (2,3) and, if the freezing released soluble receptors, it would be anticipated that it would be so for the granulosa cells and render the granulosa cell extract inhibitory of binding.

It is possible that local intraovarian diffusion of the inhibitor to granulosa cells may have a regulatory role in follicular maturation. It is also possible that the luteal inhibitor may play a role in determining the responsiveness of the corpus luteum to LH during the luteal phase. Further studies examining these two possibilities are being undertaken.

Summary. Porcine corpus luteum tissue contains an inhibitor of binding of iodinated human chorionic gonadotropin to porcine granulosa cells. The inhibitor was present in the 1000, 30,000, and 105,000*g* supernatants of the corpus luteum but not in those of nonluteal ovarian tissue, granulosa cells, lung, or heart tissue. The inhibitor appeared only after storage in the freezer for from 1.5 to 11 weeks. Freezing heart, lung, or nonluteal tissue for a comparable time period did not lead to an increase in inhibitory activity.

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