# ESTROGEN STIMULATION OF PROGESTERONE SYNTHESIS BY PORCINE GRANULOSA CELLS IN CULTURE

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## SUMMARY

Estrogen stimulates synthesis of progesterone by porcine granulosa cells in tissue culture. This enhancement is inhibited by cycloheximide, suggesting that protein synthesis is required for the effect. Estrogen is synergistic with hCG in increasing progesterone synthesis. Sequential treatment with hCG followed by estradiol causes maximal stimulation of progesterone production.

## INTRODUCTION

Estrogen, in addition to gonadotropins, is an important factor in maintaining corpus luteum function in vivo (1-6). We have investigated the effect of estrogen on granulosa cells, and have demonstrated for the first time that estrogenic compounds have a direct stimulatory effect on progesterone synthesis by granulosa cells in culture. Gonadotropins also stimulate progesterone production by granulosa cells in tissue culture (7,9). Therefore, we have also determined the combined effect of estrogen and gonadotropins on granulosa cell progesterone synthesis.

## MATERIALS AND METHODS

For these experiments, granulosa cells from preovulatory follicles were chosen to study the hormones controlling

progesterone synthesis. Porcine granulosa cells were harvested from 5 to 8 mm antral follicles by aspiration with a 26 gauge needle after searing an area of the ovarian capsule with a hot spatula (10). The cells were centrifuged, washed, resuspended, counted in a hemocytometer and diluted with complete media consisting of F10 medium 82%, horse serum 13.9% and fetal bovine serum 3.2% with added penicillin, streptomycin and amphotericin B. Replicate cultures were prepared with $2.5 \times 10^6$ cells in each 30 ml plastic flask and 4 flasks were used for each control and each treatment group. After 48 hours, the medium was removed and replaced with complete medium containing the test substances. These media were completely changed every 48 hours throughout the course of the experimenst. Progesterone content in the unextracted medium was determined by radioimmunoassay (11), after we had previously shown that values before and after extraction with petroleum ether were similar. At the end of each experiment, the cells were dispersed with 0.05% trypsin and 0.02% sodium versenate, re-suspended, and counted.

## EXPERIMENTS AND RESULTS

In the initial experiments, cultures were treated with either human chorionic gonadotropin (hCG) 10 IU/ml, diethylstilbestrol (DES) 10 ug/ml, or hCG 10 IU/ml and DES 10 ug/ml combined (hCG-DES). Progesterone production in control cultures ranged between 50 and 100 ng/flask/48 hrs (Figure 1). The addition of hCG in these experiments resulted in a significant increase in progesterone levels ranging from 20 to 200 percent over control values. DES consistently produced a greater increase in progesterone levels than hCG. Combined

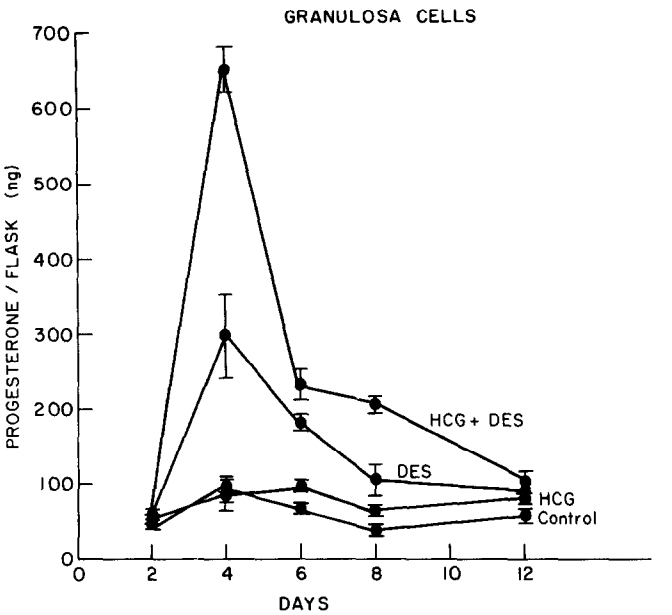


Fig. 1. Total progesterone content in 48-hour collections of media harvested on day indicated after initiation of hormone treatment with human chorionic gonadotropin (hCG) 10 IU/ml, diethylstilbestrol (DES) 10 IU/ml, or a combination of both. Control cultures received basic medium. Brackets indicate ± standard error of the mean (SEM).

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hCG-DES treatment resulted in a 6 to 8-fold increase in progesterone production over control values with maximum concentrations generally found in the samples removed on day 4 or day 6. Maximum progesterone production per 48 hrs per cell in the hCG-DES cultures was 1.28 pg/cell compared to 0.1 pg/cell in the untreated cultures.

Estradiol ($E_2$) similarly increased progesterone synthesis. With either estrogen, the increase in progesterone synthesis was not the result of an increased number of cells. In fact, DES at 10 ug/ml caused a decrease in cell number with a mean of $5.1 \pm 0.12 \times 10^5$ cells per flask compared to $1.03 \pm 0.12 \times 10^6$ cells per flask for controls at the end of the 12 day experiment.

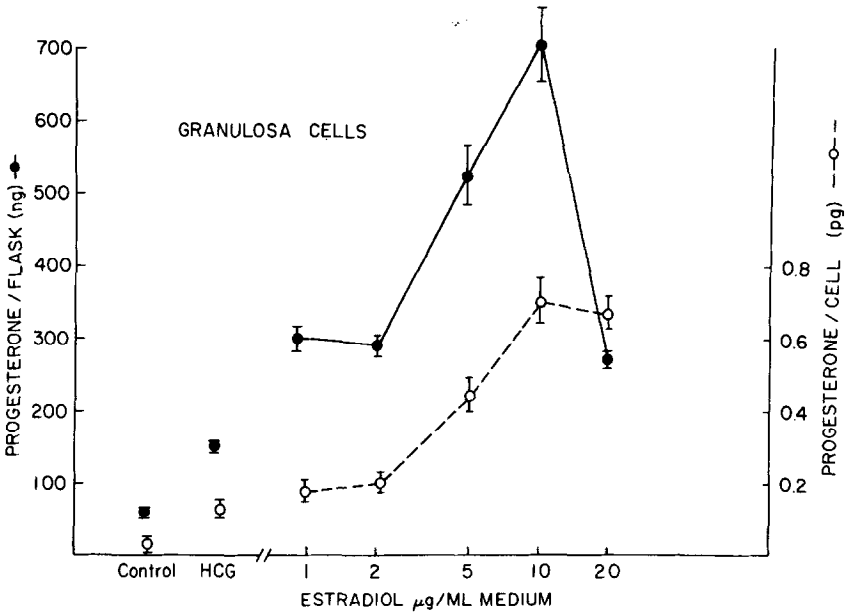


Fig. 2. Comparison of progesterone levels in 48-hour collections of media on day 6 after treatment of granulosa cells with hCG 10 IU/ml alone and in combination with graded doses of estradiol. Brackets indicate ±SEM.

Estradiol did not depress cell counts until 15 to 20 ug/ml were added to the media.

Increasing concentrations of either estrogen, when given with hCG were associated with increasing progesterone production per culture until cell counts began to decrease. When high estrogen concentrations were used and cell counts decreased, a simultaneous decrease in progesterone production per flask occurred, although progesterone synthesis per cell was relatively constant (Figure 2).

To determine the importance of the order of hormonal treatment on granulosa cell progesterone production, we gave hCG and estrogen sequentially (Figure 3). $E_2$ (10 ug/ml) alone for 2 days followed by hCG 10 IU/ml alone in the media for 12 days

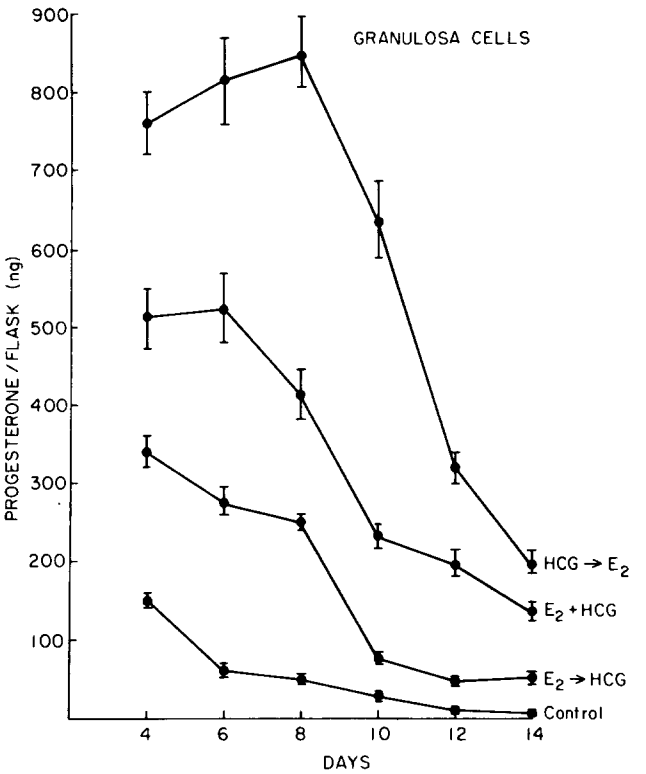


Fig. 3. Comparison of progesterone levels in 48-hour collections of media after combined and sequential treatment of granulosa cells with hCG 10 IU/ml and estradiol ($E_2$) 10 ug/ml. Combined E + hCG treatment was carried out for entire 14 days. Sequential treatment consisted of either hCG or $E_2$ for 2 days followed by the other hormone alone for 12 days. Brackets indicate ± SEM.

caused a 2-4 fold increase in progesterone over control with maximum production occurring on the 4th day after the beginning of the experiment. hCG for 2 days followed by continuous estrogen treatment increased progesterone production until 6 days after the addition of $E_2$ at which time there was a 17-fold increase over control, and a significantly ($p < .01$) greater amount of progesterone than resulted from addition of the two hormones simultaneously. Sequential addition of hCG and followed by $E_2$ therefore resulted in the greatest progesterone production

over the longest period of time.

To determine if protein synthesis was necessary for the induction of progesterone by the granulosa cells, cycloheximide in doses ranging from 0.01 ug/ml to 10 ug/ml was added to cultures treated with hCG 10 IU/ml and $E_2$ 10 ug/ml. Cycloheximide, at non-toxic concentrations between 0.05 and 0.5 ug/ml blocked the stimulation of progesterone production by E and hCG. These results suggest that protein synthesis was necessary for induction of progesterone synthesis.

Since hCG appeared to have a synergistic effect with estrogen on progesterone synthesis, we substituted another gonadotropic hormone, follicle stimulating hormone (FSH), to determine if it would synergize with estrogen in stimulating progesterone production in this system. Ovine FSH (NIHFSH-S6) 20 to 50 ug/ml did not produce additional progesterone synthesis when given with $E_2$, indicating that FSH did not duplicate the effect of hCG in synergizing with estrogen.

## DISCUSSION

In all experiments, control cultures were found to produce measureable quantities of progesterone, indicating at least some degree of the "spontaneous luteinization" previously described by Channing (7). Estrogen, when added to these cultures, markedly increased progesterone production. hCG, which has the ability to luteinize granulosa cells morphologically (10), also increased progesterone synthesis, but not to the the extent seen in the estrogen treated cultures. These experiments suggest that although further cellular changes characteristic of luteinization might have taken place, hCG did not reproduce the effect of estrogen on progesterone synthesis.

Estrogen and hCG given together increased progesterone synthesis to a greater extent than the additive effect of individual responses. When a short exposure to hCG was followed by continuous estrogen treatment, an even greater degree of progesterone synthesis over a longer time period was achieved. hCG is known to induce synthesis of adenosine 3',5'-monophosphate (cyclic AMP), a step thought necessary in mediating the action of this hormone (12). Steroid hormones, however, generally do not appear to act through this pathway (13). We suggest that hCG may induce cyclic AMP synthesis enabling the granulosa cell to synthesize progesterone and at the same time causing morphological luteinization. Estrogen, apparently by a different mechanism, stimulates and maintains progesterone synthesis in cells already acted upon by hCG.

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