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Herbimycin, a Tyrosine Kinase Inhibitor with Src Selectivity, Reduces Progesterone and Estradiol Secretion by Human Granulosa Cells

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The purpose of the present study was to investigate whether the tyrosine kinase inhibitor herbimycin with some selectivity to block Src would alter the stimulatory effects of follicle-stimulating hormone (FSH) and cyclic adenosine monophosphate (cAMP) on estradiol secretion by human granulosa cells. Granulosa cells were taken from ovaries of premenopausal women undergoing oophorectomy for reasons unrelated to ovarian pathology. Granulosa cells from follicles ranging from 5–20 mm in diameter were subjected to culture. Granulosa cells were cultured with human FSH (2 ng/mL) or cAMP (0–1 m*M*) and testosterone (1 µ*M*) in the presence and absence of herbimycin (0–2 µ*M*). Media were collected at 24, 48, and 72 h. Accumulation of cAMP, progesterone, and estradiol in the media was determined by radioimmunoassay. Herbimycin dose dependently inhibited the ability of FSH to induce increases in progesterone and estradiol secretion. Although herbimycin increased ($p < 0.0001$) the accumulation of cAMP in response to FSH, this was evident only at the high concentrations of herbimycin (2 µ*M*). To determine whether herbimycin would inhibit the ability of exogenous cAMP to induce estradiol and progesterone secretion, granulosa cells were incubated with 0–1 m*M* cAMP in the presence and absence of various doses of herbimycin. Herbimycin inhibited cAMP-induced estradiol and progesterone secretion in granulosa cells. The results from seven experiments indicate that herbimycin inhibits FSH stimulation of estradiol and progesterone secretion and that this inhibition may be, in part, at post-cAMP site(s).

Key Words: Granulosa cell; ovary; Src; FSH; cAMP; human.

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Introduction

Evidence from both in vivo and in vitro studies indicates that granulosa cells of preovulatory follicles are the primary site of ovarian aromatase activity *(1)*. Androgen aromatization to estrogen is regulated by follicle-stimulating hormone (FSH), which in human granulosa cells stimulates aromatase activity and requires RNA and protein synthesis for expression of this action *(2)*. Estrogen biosynthesis is an FSH-stimulated reaction that occurs within the granulosa cells after the diffusion of thecal origin androgens into the granulosa cell and access of the aromatase enzyme. Cyclic adenosine monophosphate (cAMP) has also been shown to stimulate aromatase activity in ovarian tissue *(3)*. In the granulosa cell under the regulation of FSH, the availability of cAMP to stimulate aromatase activity is largely dependent on the presence of phosphodiesterase, which metabolizes cAMP *(4)*.

Src tyrosine kinase, a non-receptor-related tyrosine kinase, has been implicated in the regulation of luteinizing hormone (LH)- and FSH-induced steroidogenesis in rodent gonadal cells by regulating the activity of phosphodiesterase *(5–9)*. In those studies, genetic and pharamacologic approaches were used to inhibit or stimulate Src tyrosine kinase. A dominant negative Src increased the sensitivity of the MA-10 Leydig cell line to LH as determined by increased secretion of cAMP, progesterone, and androstenedione *(8)*, via inhibiting phosphodiesterase. Conversely, overexpression of Src using a temperature-sensitive mutant led to a decrease in LH-stimulated cAMP and steroidogenesis and increased phosphodiesterase activity in Leydig cell lines *(5)*. By contrast, in the rat granulosa cell, inhibition of Src using pharmacologic agents, such as herbimycin, which is a tyrosine kinase inhibitor with specificity toward Src tyrosine kinase *(10,11)*, reduced estrogen secretion in response to LH but not FSH *(7)*. Because of potential species differences in the regulation of ovarian steroidogenesis, and because of several ovarian disorders in which gonadotropins fail to stimulate ovarian function, it was of interest to determine whether Src tyrosine kinase might be involved

Table 1
Medical Data on Study Patients

Patient	Age (yr)	Weight (lb)	Follicular phase <10 mm	Follicular phase >10 mm	Luteal phase <10 mm	Luteal phase >10 mm	Acyclic <10 mm	Acyclic >10 mm	Surgical indications
1	39	170	√	√					Uterine fibroids
2	35	144			√				Invasive cervical cancer
3	46	150	√						Uterine fibroids
4	34	224					√	√	Right ovarian leiomyosarcoma
5	40	126	√	√					Uterine fibroids[a]
6	41	264	√	√					Uterine fibroids[a]
7	41	261			√				Uterine fibroids, D.U.B.

[a] Provera was given to the patient.

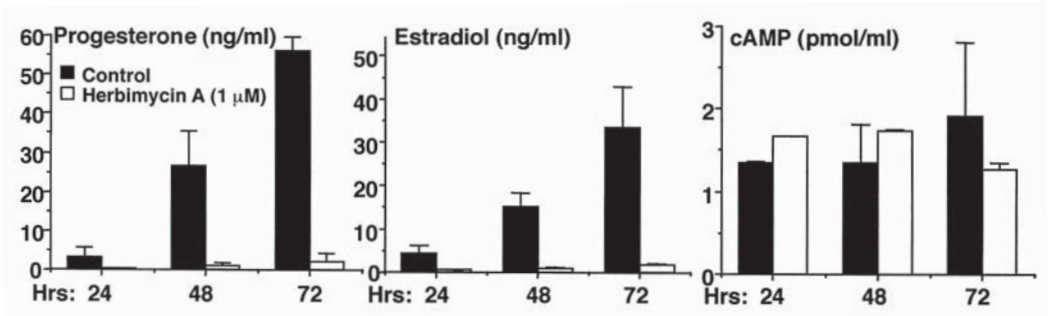

Fig. 1. Effects of herbimycin (1.0 μ*M*) on accumulation of progesterone, estradiol, and cAMP by human granulosa cells during follicular phase of menstrual cycle. Granulosa cells (20,000 cells/0.5 mL) from 5- to 10-mm-diameter follicles were cultured for 72 h in the presence of 2 ng of FSH/mL. Media were collected for radioimmunoassay (RIA) at 24, 48, and 72 h by removal of 20 μL. All cultures included 1 μ*M* testosterone as precursor for estradiol. Data were analyzed by analyzed by two-way ANOVA. Data were collected from triplicate determinations using two patients (patients 1 and 3 from Table 1).

in human granulosa cell function. Thus, the present study determined the in vitro effects of herbimycin on FSH-stimulated granulosa cell secretion of progesterone, estradiol, and cAMP in humans.

Results

Medical Data on Patients

The medical data on the patients including age, weight, surgical indications, and reproductive medications are presented in Table 1. Values were similar among the various groups with respect to age and weight of the patients.

Effects of Herbimycin on FSH-Stimulated Progesterone, Estradiol, and cAMP Using Granulosa Cells from Small Follicles (<10 mm diameter)

In granulosa cells taken from two patients studied during the follicular phase of the menstrual cycle, FSH increased progesterone and estradiol in a stepwise manner over the 72 h of culture (Fig. 1, $p < 0.03$, time effect for progesterone and estradiol by two-way analysis of variance [ANOVA]). Progesterone, estradiol, and cAMP were not detectable in the media in the absence of FSH (<0.625 ng/mL for progesterone, <0.125 ng/mL for estradiol, <0.05 pmol/mL; data not shown). Herbimycin significantly inhibited the accumulation of progesterone and estradiol by 24 h of culture, and both remained low throughout the remainder of the culture period ($p < 0.0002$ and 0.004 for progesterone and estradiol, respectively; treatment effect, two-way ANOVA). In the presence of FSH, cAMP was approx 1 pmol/mL throughout the 72 h of culture. cAMP was unaffected by herbimycin during the 72-h culture period ($p > 0.8$; treatment effect, two-way ANOVA).

Similar to results from the follicular phase in a patient studied during the luteal phase, FSH increased accumulation of progesterone and estradiol in the media over the 72 h of culture ($p < 0.0001$; time effect, two-way ANOVA). Herbimycin dose dependently inhibited the accumulation of progesterone and estradiol in FSH-treated granulosa cells from follicles <10 mm in diameter (Fig. 2; $p < 0.0004$ and 0.0001 for progesterone and estradiol, respectively; dose effect, two-way ANOVA). As observed in the follicular phase at a dose of 1 μ*M* herbimycin or less, cAMP was unaffected; however, cAMP concentrations increased in response to herbimycin at a dose of 2 μ*M* herbimycin ($p < 0.0001$; dose effect, two-way ANOVA). Further analysis of the cAMP concentrations in response to 2 μ*M* herbimycin revealed a significant decline over the 72-h culture period ($p < 0.0004$; one-way ANOVA).

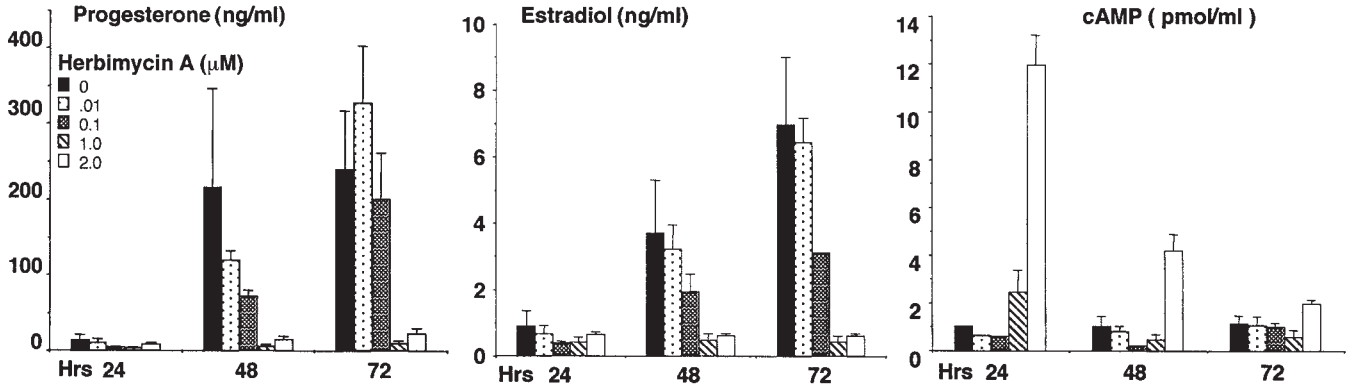

Fig. 2. Effects of herbimycin (1.0 μ*M*) on accumulation of progesterone, estradiol, and cAMP by human granulosa cells from 5- to 10-mm-diameter follicles during luteal phase of menstrual cycle. As described in Fig. 1, granulosa cells were cultured in the presence of FSH and data analyzed by two-way ANOVA. Data were collected from triplicate determinations using one patient (patient 3 from Table 1).

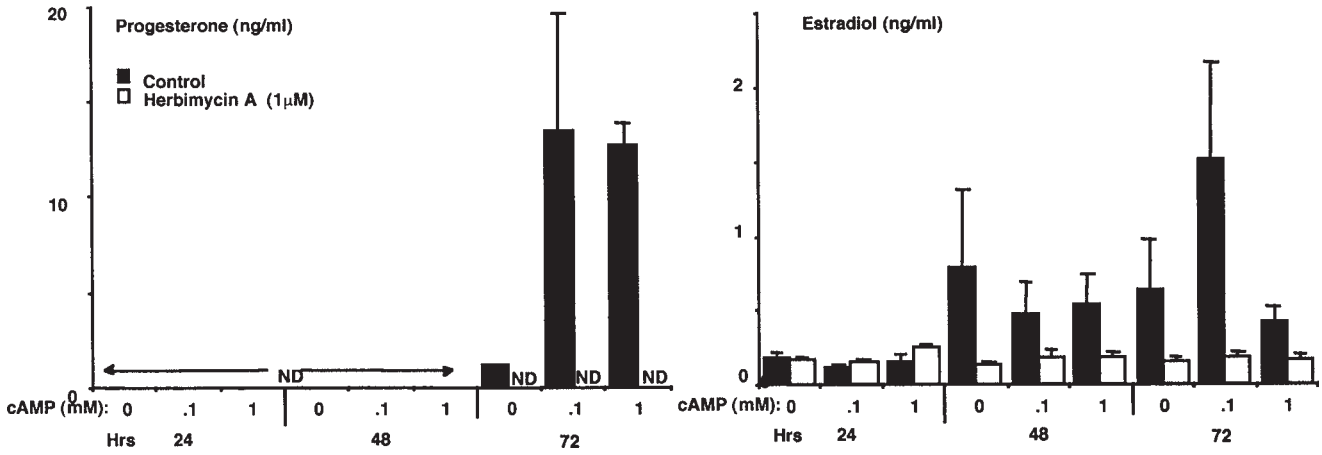

Fig. 3. Effects of herbimycin (1.0 μ*M*) on accumulation of progesterone and estradiol by human granulosa cells from 5- to 10-mm-diameter follicles taken from ovaries of an acyclic woman. Granulosa cells were cultured in the presence of cAMP (0–1 m*M*) and testosterone (1 μ*M*) and data analyzed by two-way ANOVA. Data were collected from triplicate determinations using one patient. ND, not detectable by progesterone RIA (<0.625 ng/mL) (patient 4 from Table 1).

Effects of Herbimycin on cAMP-Stimulated Progesterone and Estradiol Using Granulosa Cells from Small Follicles (<10 mm diameter)

Of the four patients studied with small follicles, granulosa cells from three patients did not respond to cAMP in vitro. The nonresponders included two patients in the follicular phase and one in the luteal phase. The only cells that responded to cAMP were from an acyclic patient. The data from the granulosa cells taken during acyclicity revealed that in the presence of testosterone, cAMP stimulated progesterone and estradiol secretion by 72 h (Fig. 3; time effect, $p < 0.0004$ for both progesterone and estradiol, two-way ANOVA). Herbimycin in vitro reduced the accumulation of progesterone and estradiol in cAMP-treated granulosa cells from the acyclic patient (Fig. 3; treatment effect, $p < 0.003$ for progesterone and estradiol, two-way ANOVA).

Effects of Herbimycin on Progesterone and Estradiol Using Granulosa Cells from Large Follicles (>10–25 mm diameter)

Only four of seven patients exhibited large follicles. One of those had nonviable granulosa cells; the remaining three patients were studied. Two of the patients were in the follicular phase and one was acyclic. Cells from each of the three patients were treated differently. Similar to granulosa cells from small follicles, in granulosa cells taken from large follicles during the follicular phase, progesterone and estradiol (but not cAMP) increased over the 72 h of culture in the presence of FSH (Fig. 4; $p < 0.05$ for progesterone and estradiol, time effect, two-way ANOVA). Herbimycin significantly inhibited FSH-stimulated progesterone and estradiol secretion (Fig. 4; treatment effect, $p < 0.0001$ by two-way ANOVA) similar to granulosa cells from small

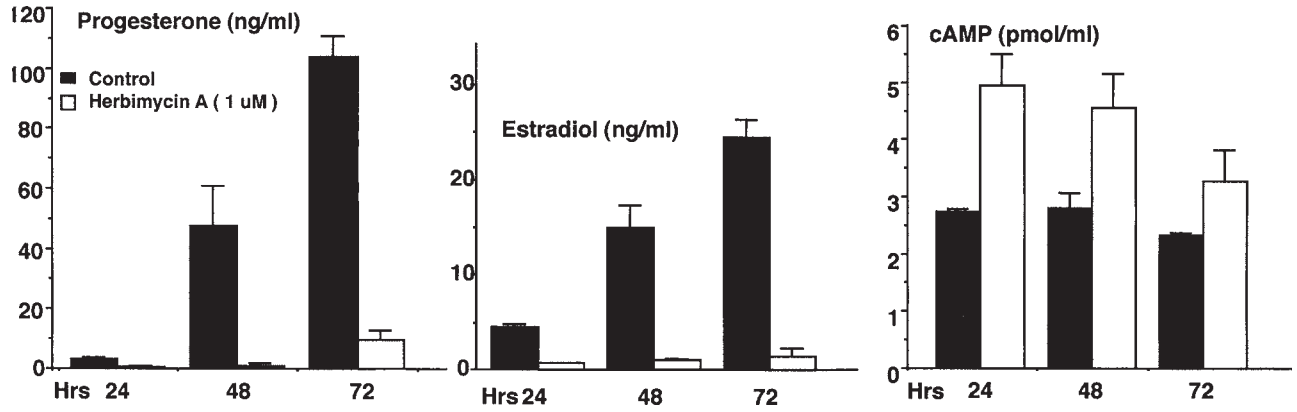

Fig. 4. Effects of herbimycin (1.0 μ*M*) on accumulation of progesterone, estradiol, and cAMP by human granulosa cells from follicles >10 mm in diameter during follicular phase of menstrual cycle. As described in Fig. 1, granulosa cells were cultured in the presence of FSH and testosterone and data analyzed by two-way ANOVA. Data were collected from triplicate determinations using one patient (patient 1 from Table 1).

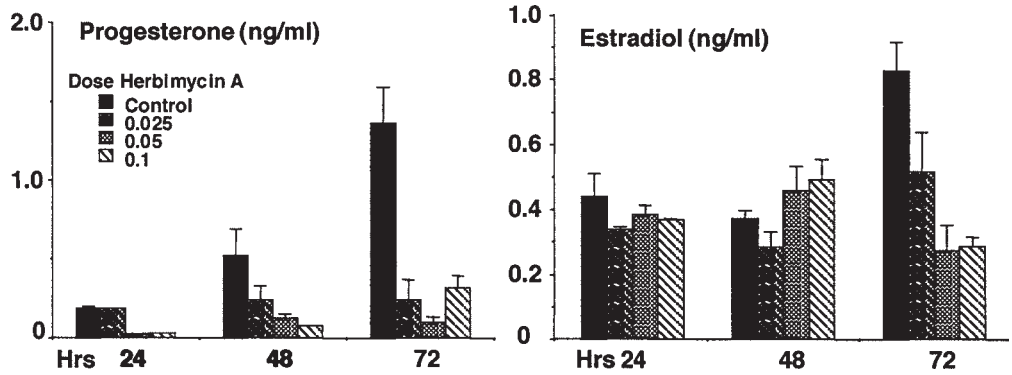

Fig. 5. Effects of herbimycin (0–0.1 μ*M*) on accumulation of progesterone and estradiol by human granulosa cells from follicles >10 mm in diameter taken during follicular phase. Granulosa cells were cultured in the presence of cAMP (0.1 m*M*) and testosterone (1 μ*M*) and data analyzed by two-way ANOVA. Data were collected from triplicate determinations using one patient (patient 6 from Table 1).

follicles. Unlike granulosa cells from large follicles, cAMP was increased by 1 μ*M* herbimycin (Fig. 4; treatment effect, $p < 0.05$ by two-way ANOVA).

The data from the other patient whose granulosa cells were taken during the follicular phase revealed that progesterone accumulation increased over the 72 h of culture (Fig. 5; $p < 0.0001$, time effect, two-way ANOVA); this was not evident for estradiol. In the presence of exogenous cAMP, herbimycin dose dependently reduced accumulation of progesterone ($p < 0.0001$) and estradiol ($p < 0.007$) in granulosa cells from large follicles (Fig. 5; treatment effect, two-way ANOVA) in a manner similar to that of small follicles (cf. with Fig. 3).

The remaining patient was acyclic and the granulosa cells were cultured with cAMP (Fig. 6). In the presence of cAMP, progesterone and estradiol accumulated in the media over the 72 h of culture. Herbimycin in the presence of cAMP reduced the accumulation of progesterone and estradiol when compared with cAMP alone (Fig. 6; treatment effect, $p < 0.0003$ and <0.02 for progesterone and estradiol, respectively; two-way ANOVA).

Discussion

The present studies revealed that in vitro herbimycin, a tyrosine kinase inhibitor with specificity toward Src tyrosine kinase, reduced the ability of FSH and cAMP to stimulate the secretion of progesterone and estradiol. The studies point to a potential new signal transduction pathway, the nonreceptor Src tyrosine kinase, for investigations related to alterations in gonadotropin responsiveness in humans. Our finding that herbimycin inhibited granulosa cell steroidogenesis is consistent with a single observation in a previous study from our laboratory in which herbimycin inhibited LH-stimulated estradiol secretion by rat granulosa cells *(7)*. The present study extends those observations by elucidating that FSH-stimulated progesterone and estradiol is inhibited and that potential post-cAMP site(s) of action of herbimycin may exist. Although only seven patients were used in the present study, the results consistently indicated that herbimycin reduced gonadotropin-stimulated estradiol without a major effect on cAMP levels.

By contrast, previous studies from our laboratory using rat thecal cells and mouse Leydig cell lines (MA10 and TM3),

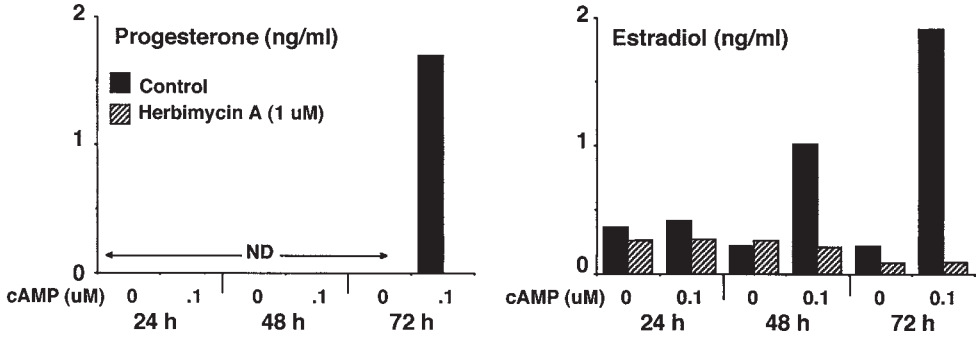

Fig. 6. Effects of herbimycin (1.0 μ*M*) on accumulation of progesterone and estradiol by human granulosa cells from follicles >10 mm in diameter taken from ovaries of an acyclic woman. Granulosa cells were cultured in the presence of cAMP (0 and 0.1 m*M*) and testosterone (1 μ*M*) and data analyzed by two-way ANOVA. Data were collected from duplicate determinations using one patient. ND, not detectable by progesterone RIA (<0.625 ng/mL) (patient 4 from Table 1).

embryologic homologs of thecal cells in the male, have shown that loss of Src activity, with the expression of a dominant negative Src kinase or by pharmacologic blockade by herbimycin, increases the responsiveness of the cells to LH stimulation as measured by cAMP and progesterone secretion *(5,7,8)*. This increase in cAMP and progesterone was in part attributed to a decrease in phosphodiesterase activity, which would reduce degradation of cAMP and lead to increased accumulation of cAMP in the cells and media. The accumulation cAMP after inhibition of Src would then amplify the gonadotropin action, leading to an increase in steroidogenesis. In contrast to the results from the dominant negative MA10 Leydig cells, in the present study herbimycin at high concentrations (2 μ*M*) increased cAMP accumulation in the media, and this was still accompanied by reduced progesterone and estradiol secretion. Thus, it appears that herbimycin may also be acting at other sites to inhibit gonadotropin/cAMP-stimulated steroidogenesis in granulosa cells. The observations of increased concentrations of cAMP in media of granulosa cells treated with high doses of herbimycin in vitro support a previous report that herbimycin increased cAMP in rat thecal interstitial cells *(5)*. However, in rat thecal cells, steroidogenesis was stimulated. The reasons for differences between thecal and granulosa cell steroidogenesis in response to herbimycin is unknown at present.

Src activity in the human granulosa has not been measured at present largely because of the large number of granulosa cells required for those studies. Such studies would require the immunoprecipitation of Src, the addition of an Src (specific) substrate, which is subsequently phosphorylated by immunoprecipated Src in the presence of P^{32}-γ-ATP (immune-complex kinase assays). It is not possible to carry out these studies at present because hundreds of thousands of granulosa cells from normal ovaries would be required. A study using thecal cells from porcine ovaries has identified Src by Western blot and measured its activity *(9)*. Other methods for identifying and measuring Src activity would include tyrosine phosphorylation of immunoprecipitated Src and changes in total protein tyrosine phosphorylation indicating that tyrosine kinase activity is changed in granulosa cells. Identification of Src in human granulosa cells is the subject of ongoing investigation and seems worthy based on the herbimycin data in the present study. Possibly, human luteinized granulosa cells collected from patients undergoing in vitro fertilization may be a source of sufficient cell numbers for such initial studies.

In vitro herbimycin inhibited granulosa cell steroidogenesis in cells taken from the follicular and luteal phases, and in cells taken from acyclic women. In addition, cells from small (<10-mm-diameter) and large (>10-mm-diameter) follicles, responded similarly to herbimycin with decreased progesterone and estradiol accumulation. Thus, at this preliminary stage of investigation, it appears that phase of the cycle and size of the follicle does not affect the ability of herbimycin to reduce the secretion of progesterone and estradiol.

Herbimycin inhibits tyrosine phopshorylation of Src and targets the degradation of the protein kinase *(12,13)*. Additional studies using genetic approaches such as dominant negative and transient transfections were not feasible owing to the limited number of granulosa cells obtained from the ovaries. Previous studies using the various genetic approaches with rat, mouse, and pig ovarian cells have supported the pharmacologic studies using herbimycin *(5,8,9)*. Thus, in the human, further studies are required to be clearly certain that the effects of herbimycin are entirely owing to the inhibition of Src.

The present study presents evidence that herbimycin consistently inhibited FSH- and cAMP-stimulated secretion of estradiol and progesterone without significant alteration in cAMP. In addition, the results indicate that herbimycin may act at post-cAMP site(s) in the inhibition of granulosa cell steroidogenesis. In conclusion, it appears that Src tyrosine kinase may be involved in human granulosa cell steroidogenesis.

Materials and Methods

Patients

Patients undergoing total abdominal hysterectomy with either unilateral or bilateral salpingo-oophorectomy were used in this study. The ovaries used were considered normal. Reproductive diseases of the patients in which ovaries were removed included fibroids, cervical carcinoma, and ovarian leiomyosarcoma (in one ovary only and that ovary was not used). Medical data on the patients are provided in Table 1. Stage of cycle was determined from endometrial biopsy as presented in medical records of each patient. Acyclicity was indicated by a period of three consecutive months without menses.

This study was approved by the Institutional Human Studies Committee of the University of Kansas Medical Center. It was given exemption status because of the discarded nature of the tissue.

Collection of Granulosa Cells

Granulosa cells were collected as previously described *(14)*. Briefly, they were collected in the surgical room within 15 min of removal of the ovaries from the abdominal cavity. Follicular diameter was determined using a millimeter rule by measuring the diameter of each follicle on the ovarian surface. Granulosa cells were collected by follicular aspiration using a 21-gauge needle attached to a 1-cc syringe. Follicular aspirates containing blood were not used. Straw-colored follicular aspirates were pooled into two groups according to follicular diameter: 5–10 mm (small) and >10–25 mm (large). Granulosa cells were pooled from three to five follicles per patient in the small-follicle group; in the large-follicle group, either granulosa cells from one follicle per patient were utilized or two follicles per patient were pooled. Aspirates were placed in sterile, capped, plastic 12 × 75 mm culture tubes and transported at room temperature to the cell culture laboratory.

Culture of Granulosa Cells

Follicular aspirates were washed twice with 2 mL of culture medium (Medium 199 containing Hank's salts, 25 m*M* *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid buffer, 2 m*M* L-glutamine, 50 µg/mL of streptomycin, 0.1% [wt/vol] bovine serum albumin, and 1.0% fetal bovine serum) as previously described *(14)* and then centrifuged at 1000*g* for 5 min. The supernatant fluid was discarded, and the cells were resuspended in a known volume of culture medium. Cell viability and counting were determined by adding 2% trypan blue to an aliquot of the cells. Cells were diluted with culture medium so that ~20,000 viable cells/well were added in 0.5 mL of medium in individual wells of a 24-well culture plate. In most cases, duplicate or triplicate treatments as well as control vehicle were added to each well in 10-µL aliquots. Treatments included 2.0 ng of human recombinant FSH/mL (~12,000 IU/mg), 0–1 m*M* cAMP, 1 µ*M* testosterone, and 0–2 µ*M* herbimycin. The doses of cAMP, FSH, and testosterone were chosen based on effectively stimulating doses previously reported *(14–16)*. In addition, the dose of herbimycin was based on a previous study using rat granulosa cells *(7)*. Cells were incubated for 72 h at 37°C in a humidified incubator, with 95% air and 5% CO_2. Media were collected for RIA after 24, 48, and 72 h.

Radioimmunoassay

Progesterone, estradiol *(17)*, and cAMP *(18)* from unextracted media were analyzed as previously described.

Statistical Analyses

The hormone and cAMP data were analyzed by one- or two-way ANOVA as appropriate. Differences were considered significant if $p \leq 0.05$.

Acknowledgments

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