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Low Levels of Follicle-Stimulating Hormone Receptor-Activation Inhibitors in Serum and Follicular Fluid from Normal Controls and Anovulatory Patients with or without Polycystic Ovary Syndrome*

IZAÄK SCHIPPER, FOCKO F. G. ROMMERTS, PAULIEN M. TEN HACKEN, AND BART C. J. M. FAUSER

Division of Reproductive Medicine, Department of Obstetrics and Gynecology (I.S., P.M.T.H., B.C.J.M.F.), and Department of Endocrinology and Reproduction (F.F.G.R.), Dijkzigt Academic Hospital and Erasmus University Medical School, 3015 GD Rotterdam, The Netherlands

ABSTRACT

In patients with normogonadotropic anovulation, either with or without polycystic ovary syndrome (PCOS), factors interfering with FSH action may be involved in arrested follicle development. The aim of this study is to assess whether factors inhibiting FSH receptor activation are elevated in serum or follicular fluid from anovulatory patients, as compared with regularly cycling women. For this purpose, a Chinese hamster ovary cell line, stably transfected with the human FSH receptor, has been applied. FSH-stimulated cAMP secretion in culture medium was measured in the presence of serum or follicular fluid. Chinese hamster ovary cells were stimulated with a fixed concentration of FSH (3 or 6 mIU/mL) to mimic FSH levels in serum or follicular fluid. Samples were added in concentrations ranging from 3–90% vol/vol to approach protein concentrations occurring in serum or follicular fluid.

In the presence of 10% vol/vol serum from regularly cycling women (n = 8), FSH-stimulated cAMP production was inhibited to 42 ± 2% (mean ± SEM of 2 experiments, each performed in duplicate) of cAMP production in the absence of serum, whereas a similar cAMP level (up to 38 ± 4% of the serum-free level) was observed at higher concentrations of serum (30–90% vol/vol). The inhibition of FSH-stimulated cAMP production in the presence of serum samples from normogonadotropic anovulatory patients, without (n = 13) or with (n = 16) PCOS, was similar to controls. Follicular fluid samples (n = 57) obtained during the follicular phase in 25 regularly cycling women and follicular fluid samples (n = 25) from 5 PCOS patients were tested in a slightly modified assay system. In the presence of 10 or 30% (vol/vol) follicular fluid, FSH-stimulated cAMP levels were decreased to 68 ± 2% and 55 ± 2% (mean ± SEM of a single experiment in triplicate) of the cAMP levels in the absence of follicular fluid, respectively. There was no correlation between the degree of cAMP inhibition and follicle size, steroid content (androstenedione or estradiol concentrations), or menstrual cycle phase. Furthermore, no differences in inhibition were found, comparing PCOS follicles with size- and steroid content-matched follicles obtained during the normal follicular phase.

It is concluded that inhibition of FSH receptor activation by proteins present in serum or follicular fluid is constant (60 and 40%, respectively) and independent from the developmental stage of the follicle, either during the normal follicular phase or in patients with normogonadotropic anovulation. Inhibition of FSH receptor activation may be of limited significance for normal and arrested follicle development. (*J Clin Endocrinol Metab* **82:** 1325–1331, 1997)

A LARGE proportion of women presenting with anovulation and infertility exhibit serum FSH concentrations within the normal range. The majority of these patients can be diagnosed as suffering from polycystic ovary syndrome (PCOS), on the basis of the sonographic appearance of the ovaries and endocrine serum parameters (1, 2). A common finding in PCOS is normal early follicle development, whereas selection of the dominant follicle is absent (3, 4). The underlying mechanism of follicle maturation arrest is unclear. The possibility of abnormal circulating FSH could be ruled out by demonstrating normal bioactivity, as assessed by an *in vitro* rat granulosa cell aromatase bioassay (1, 5). Because normal follicular growth, selection, and ovulation can be induced in some PCOS patients by administration of exogenous gonadotropins (6) and granulosa cells of these patients show normal (7) or even elevated (8) FSH-induced estradiol (E_2) production *in vitro*, it may be postulated that locally active factors, rather than defective granulosa cells, are involved in arrested follicle growth in PCOS patients.

There are many indications that local regulation of FSH action may play a role in normal and disturbed follicular development (9–12). Growth factors, such as insulin-like growth factors (IGF) (13) or activin (14), act as potentiators of FSH action *in vitro*. Ongoing growth of the dominant follicle, despite a decrease in FSH serum levels in the late follicular phase (LFP) (15), may be attributed to the enhancement of FSH action by these growth factors, acting in a para- or autocrine fashion. On the other hand, growth factors may also exert inhibitory actions, as has been described for epidermal growth factor (16, 17). Although stimulatory or inhibitory effects of growth factors are mediated by their specific receptors and pathways, inhibition of FSH action may also be caused by specific FSH receptor inhibitors (12). Sev-

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Address all correspondence and requests for reprints to: Dr. B. J. C. M. Fauser, Division of Reproductive Medicine, Department of Obstetrics and Gynecology, Dijkzigt Academic Hospital, Dr Molewaterplein 40, 3015 GD Rotterdam, The Netherlands.

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eral studies report the presence of specific FSH receptor-binding inhibitors of unknown origin in human serum (18, 19) and follicular fluid (20–23). Part of these inhibitors may be of immunological nature (24) and could lead to premature ovarian failure. It may be postulated that partial inhibition of FSH receptor activation causes arrested follicular growth and absent dominant follicle selection in PCOS patients.

The aim of this study was to assess the level of inhibitors of FSH receptor activation in serum and follicular fluid of normogonadotropic anovulatory patients, with or without PCOS, as compared with normal controls. For this purpose, a Chinese hamster ovary (CHO)-cell line, transfected with the human FSH receptor (25), has been used. Inhibition of FSH-stimulated cAMP production has been studied at physiological concentrations of FSH and at a range of concentrations of serum or follicular fluid.

Materials and Methods

Hormones and reagents

Recombinant human FSH (recFSH: Org 32489, bioactivity 8413 IU/mg, as assessed by *in vivo* bioassay relative to reference preparation IS 70/45; immunoactivity 12,000 IU/mg) was a generous gift from NV Organon (Oss, The Netherlands). Isobutyl-methyl-xanthine (IBMX) was purchased from Sigma Chemie (Bornhem, Belgium).

Cell cultures

A CHO cell line, stably transfected with recFSH receptor complementary DNA, was generously provided by NV Organon). CHO cells were cultured as described previously (25). In brief, cells (10^4 cells/cm^2) were plated on 48-well culture dishes in DMEM/Ham-F12 (1:1) (GIBCO Europe BV, Breda, The Netherlands) with the addition of 100 U/mL penicillin and 100 μg/mL streptomycin and 10% vol/vol FCS (Sebak GmbH, Adenbach, Germany). After 2 days, the CHO cells reached confluence and were used for the FSH receptor activation assay.

Serum samples

Serum samples were collected from 29 anovulatory patients, attending the Dijkzigt Hospital outpatient clinic for infertility evaluation. Inclusion criteria were: infertility, oligo- or amenorrhea, and serum FSH levels within normal limits (1–10 IU/L). The involvement of human subjects in these investigations was approved by the Ethics Review Committee of the Dijkzigt Academic Hospital and Erasmus University Medical School, and informed consent was obtained from all subjects participating. Patient samples were divided into 2 subgroups representing normogonadotropic anovulation, either without (n = 13) or with PCOS (n = 16). Patients were diagnosed as having PCOS on the basis of rigid criteria, including elevated serum LH levels (>8 IU/L) and elevated serum androgens [androstenedione (AD) more than 15.0 nmol/L and/or testosterone (T) more than 3.0 nmol/L and/or a free androgen index (FAI: [T × 100]/steroid hormone-binding globulin ratio) more than 5] (1) and polycystic ovaries, as assessed by transvaginal sonography (26). Patients diagnosed as anovulatory (without PCOS) had normal LH and androgen concentrations and normal ovaries on ultrasound. In addition, FSH levels in all serum samples were measured by immunoradiometric assay (IRMA-FSH; Medgenix, Fleurus, Belgium). Serum samples selected for this study had IRMA-FSH levels between 3 and 6 IU/L, to fulfill the FSH receptor activation assay requirements (see below).

Normal ovulatory women in the early follicular phase (EFP) of the menstrual cycle (8–10 days before the LH surge) served as controls (n = 8). These volunteers were recruited by advertisement and were paid for their participation (15). Mean cycle length was 28 ± 2 SD days. Again, control samples exhibiting IRMA-FSH levels between 3 and 6 IU/L were chosen. See Table 1 for population and endocrine characteristics of all subjects studied.

TABLE 1. Population and endocrine characteristics (mean ± SD) of study subjects from which serum was obtained

	Controls (n = 8)	Normogonadotropic anovulation (n = 13)	PCOS (n = 16)
Age (yr)	25.5 ± 4.2	30.4 ± 6.4	26.9 ± 5.3
BMI (kg/m^2)	23.2 ± 2.4	25.4 ± 5.1	26.0 ± 3.4
IRMA-FSH (IU/L)	5.4 ± 0.2	4.3 ± 1.2	4.8 ± 0.8
IRMA-LH (IU/L)	3.5 ± 1.3	4.0 ± 1.9	10.5 ± 1.2
Testosterone (nmol/L)	1.8 ± 0.5	1.3 ± 0.5	3.1 ± 1.2
Androstenedione (nmol/L)	10.5 ± 3.2	7.9 ± 2.8	23.9 ± 9.8
FAI	3.1 ± 1.0	2.6 ± 1.3	10.3 ± 5.7

BMI, Body mass index (weight/square length); IRMA-FSH, immunoreactive FSH; IRMA-LH, immunoreactive LH; FAI, free androgen index, calculated as (testosterone × 100)/steroid hormone binding globulin.

Follicular fluid samples

Follicle fluid was obtained from 22 women undergoing laparotomy for reversal of tubal sterilization or adhesiolysis and from 3 women undergoing laparoscopic tubal ligation. Informed consent was obtained from all subjects participating. All subjects were regularly cycling women with a mean cycle length of 27 ± 2 SD days and of normal weight (mean body mass index: 24 ± 2 SD kg/m^2). Mean age was 33 ± 4 SD yr, and all patients had a history of proven fertility. The day of the menstrual cycle, at the time of the follicle puncture, was assessed from the day of onset of the last menstrual period. Follicle fluid was obtained from individual follicles (between 1 and 8 per patient; Fig. 1) and assayed for E_2 and AD as described previously (27). Follicular fluid samples were classified into 5 different categories. The classification was on the basis of 3 criteria: 1) the menstrual cycle phase in which patients underwent surgery (EFP: cycle day 1–8 or LFP: cycle day 9–15); 2) follicle size (nondominant: <10 mm or dominant: ≥10 mm) (15, 27); and 3) AD/E_2 ratio (healthy: <4, or atretic: ≥4) (27, 28).

In addition, follicular fluid was obtained from five anovulatory, infertile patients diagnosed as PCOS (diagnostic criteria the same as for PCOS serum study). In two patients, two to three follicles were punctured during laparoscopy. In one patient, six follicles were punctured during elective surgery for fallopian tube correction. The remaining two patients were stimulated with human menopausal gonadotropins for infertility treatment. Because of multiple follicle development (more than three follicles larger than 16 mm), stimulation was canceled. In these patients, follicular fluid samples (five and nine, respectively) were obtained through sonographically guided transvaginal punction. See

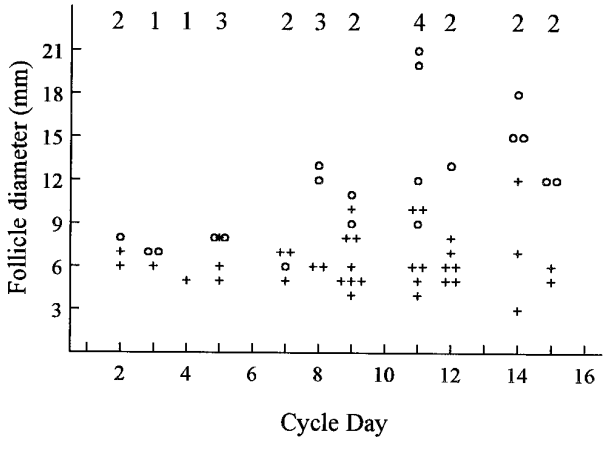

FIG. 1. Follicle diameter and menstrual cycle day in 57 follicles, obtained from 25 normally cycling women. ○, Healthy follicles (AD/E_2 ratio < 4); +, atretic follicles (AD/E_2 ratio ≥ 4). Numbers on top indicate number of subjects from whom samples were obtained on a given cycle day.

Table 2 for the classification and endocrine characteristics of the follicular fluid samples used.

FSH-receptor activation assay

Validation of this assay has been described previously (25). In brief, cultured CHO cells were incubated in 48-well culture dishes. The culture medium was replaced by FCS-free DMEM/Ham-F12 with 0.1 mmol/L IBMX and various concentrations of human serum or follicular fluid (3–90% vol/vol), and cells were stimulated with recFSH. After 4 h of incubation, medium was removed and stored at −20 C until analysis for cAMP.

For the measurement of the inhibitory effect in the serum samples, a fixed concentration of 6 mIU/mL FSH was used. To obtain this concentration of FSH in each dilution of the serum samples, endogenous FSH serum levels (range: 3–6 IU/L) were corrected by the addition of recFSH.

A pool of hypogonadotropic serum, obtained from high-dosed (50 μg ethinyl-E_2 daily) combined oral contraceptive pill users, served as a control in each assay. The FSH level of this serum pool was less than 0.5 IU/L, as assessed by IRMA.

Each serum sample was tested in duplicate in two independent FSH receptor activation assays. Results are expressed as the percentage of the cAMP production, relative to the cAMP response in serum free conditions, which was set at 100% at the given stimulatory dose of recFSH (6 mIU/mL).

Because of small volumes available, the CHO assay was adapted, to allow analysis of follicular fluid samples. For this purpose, CHO cells were cultured in 96-multiwell dishes, at a cell density of 10^4 cells/cm^2, and incubated in a vol of 50 μL/well. The sensitivity for recFSH and the degree of stimulation of the cAMP production in this assay, applying small volumes, was similar to the assay performed in 48-wells (data not shown).

The small volumes of individual follicular fluid samples allowed for testing at only two concentrations (10 and 30% vol/vol) in triplicate. The inhibitory effect of follicular fluid was assessed by stimulating CHO cells with 3 mIU/mL recFSH, instead of 6 mIU/mL. This FSH concentration was taken, to approximate the endogenous FSH levels in the follicular fluid samples (4, 8, 29, 30). Because volumes of the tested follicular fluid samples were small (in some cases only 30 μL), it was not possible to measure the FSH content.

In all experiments, DNA content of the wells was measured by a fluorometric method, as described previously (31). The intraassay coefficient of variation for DNA content/well was less than 12%. In all FSH receptor activation assays, the cAMP levels were normalized on the basis of the DNA content of the wells.

RIAs

cAMP in the media was assayed as described previously (32). In brief, after acetylation, the samples were incubated overnight with cAMP antibody (purchased from Prof. Dr. J. Stoof, Free University, Amsterdam, The Netherlands). The assay was validated for the use of culture media and corrected for addition of serum in the samples. All samples were assayed in duplicate. Sensitivity of the assay was 0.125 pmol/mL. Inter- and intraassay coefficients of variation were 20% and 8%, respectively.

E_2 and AD in follicular fluid were measured in a 1:100 dilution of the samples, as described previously (27). E_2 concentrations were estimated using an RIA kit (Diagnostic Products Corporation, Los Angeles, CA); AD concentrations were estimated using antisera and diethylether-hexane extraction, as described previously (33).

Data analysis

Experimental data are presented as the mean ± SEM if they are normally distributed and as median and range if distributed otherwise. The inhibition of FSH-stimulated cAMP production is expressed as the percentage of the cAMP response in absence of serum or follicular fluid, which is set as 100%. Results were evaluated using one-way ANOVA, comparing responses as percentages of the serum free response. Correlation of follicle characteristics with experimental results were analyzed using Spearman's rank order test. *P* values given are two-sided, with 0.05 taken as the limit for statistical significance.

Results

Effect of hypogonadotropic human serum

The dose-dependent stimulation of adenylate cyclase in CHO cells is shown in Fig. 2. Basal levels of cAMP were 0.9–2.1 pmol/μg DNA, and maximally stimulated levels were 350–420× higher. The sensitivity, defined as the dose of recFSH eliciting a response of more than twice the standard deviation of the basal cAMP production, was 0.4 mIU/mL. The half-maximal stimulation of cAMP production (ED_{50} value) was obtained at a dose of 24.9 mIU/mL FSH.

TABLE 2. Follicles (n = 83) obtained from 25 normally cycling subjects and 5 PCOS patients, divided into categories on the basis of cycle day, size, and AD/E_2 ratio

Category[a]		Number	Cycle day	Size (mm)	A/E_2 ratio
Normal cycle:	I (EFP; <10 mm; atretic)	10	5 (2–7)	6 (5–8)	42 (4–238)
	II (EFP; <10 mm; healthy)	6	4 (2–7)	7 (5–8)	0.7 (0.3–2)
	III (LFP; <10 mm, atretic)	23	11 (8–15)	6 (3–8)	30 (4–256)
	IV (LFP; ≥10 mm; atretic)	4	11 (9–14)	10 (9–12)	127 (62–173)
	V (LFP; ≥10 mm, healthy)	14	11 (8–15)	13 (9–25)	0.2 (0.04–3.3)
PCOS:	A (<10 mm; atretic)	7		5 (5–8)	9.5 (5.0–46.9)
	B (<10 mm; healthy)	4		7 (3–8)	2.4 (0.1–3.2)
	C (≥10 mm; atretic)	4		17 (16–18)	7.4 (4.3–12.8)
	D (≥10 mm; healthy)	10		16 (9–20)	0.1 (0.1–0.7)

[a] EFP, early follicular phase; LFP, late follicular phase; healthy, AD/E_2 ratio < 4; atretic, AD/E_2 ratio ≥ 4. Values given are median and range.

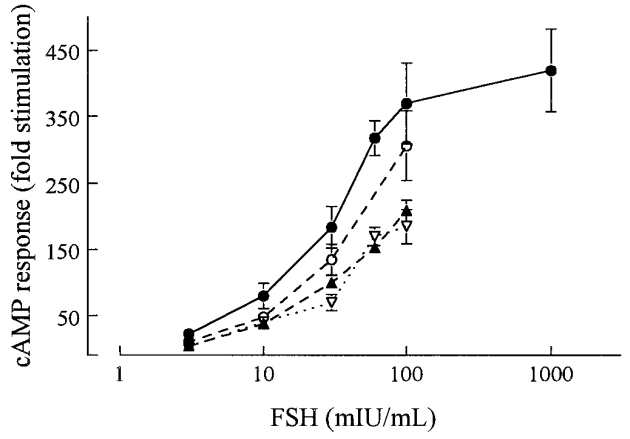

FIG. 2. cAMP response, expressed as the stimulation factor times basal production (mean ± SEM of three assays in triplicate) in CHO cells, stimulated with increasing concentrations of recFSH in the presence of 0.1 mmol/L IBMX and increasing concentrations of human hypogonadotropic serum (●, serum free; ○, 10%; ▲, 30%; ▽, 90% vol/vol).

The intraassay coefficient of variation, at different stimulatory concentrations of FSH, was less than 16%. To study the effects of serum at different doses of recFSH, CHO cells were incubated in the presence of different concentrations of human hypogonadotropic serum (10, 30, and 90% vol/vol) and stimulated with increasing doses of recFSH (3–100 mIU/mL). Basal cAMP production was not affected by the addition of hypogonadotropic serum, whereas in the presence of 90% vol/vol hypogonadotropic serum, stimulated cAMP production decreased to 49–68% of responses under serum-free conditions (Fig. 2).

When cAMP response at any given concentration of recFSH is expressed relative to cAMP production under serum-free conditions, addition of hypogonadotropic serum at concentrations more than 10% vol/vol inhibits cAMP production, independent from the concentration of serum (Fig. 3). At a high FSH concentration (30 mIU/mL), a competition between FSH and serum components is apparent over the whole serum concentration range, whereas at low FSH concentrations (3 or 6 mIU/mL), this competition occurs only at serum concentrations less than 3% vol/vol. At serum concentrations more than 10% vol/vol, a saturation of the inhibitory effect is observed. Despite the slight inhibition by serum factors, CHO cells remain sensitive to stimulation by FSH. Even in the presence of 90% vol/vol of hypogonadotropic serum, addition of 3 mIU/mL recFSH results in a 5- to 8-fold stimulation of the cAMP production (data not shown).

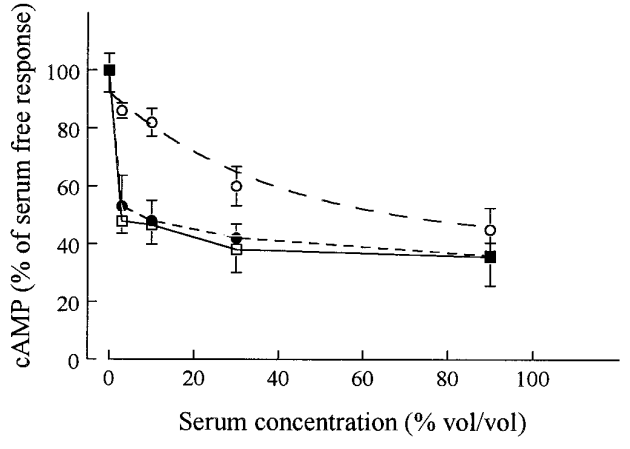

FIG. 3. Decreased cAMP production by CHO cells, stimulated with 30 mIU/mL (○); 6 mIU/mL (●), or 3 mIU/mL (□) of recFSH, incubated in the presence of increasing concentrations (3–90% vol/vol) of hypogonadotropic human serum. Results (mean ± SEM of three experiments in triplicate) are expressed as the percentage of the response under serum-free conditions (100%).

Effects of serum from normal controls, normogonadotropic anovulatory patients, and PCOS patients

Serum samples obtained from normally cycling individuals or anovulatory patients, with or without PCOS, inhibited cAMP production up to 39 ± 4%, 35 ± 2%, and 34 ± 3% of the response under serum free conditions, respectively, when tested at 90% vol/vol. Within each group, a significant further increase in inhibition was absent ($P > 0.38$) when serum concentrations were increased from 10 to 90% vol/vol. Furthermore, no statistically significant differences were found between patient groups and normal controls ($P > 0.08$), with regard to the inhibition of FSH-stimulated cAMP production at each concentration of serum (Fig. 4).

Effect of follicular fluid from normal controls and PCOS patients

Adapted bioassay conditions were used to measure the effects of small amounts of follicular fluid (see *Materials and Methods* section). The intraassay coefficient of variation of the adapted assay was greater (<21%), compared with the 48-well assay (<16%). FSH-stimulated cAMP production was clearly inhibited in the presence of 10 or 30% vol/vol of follicular fluid, although in several samples, a concentration-dependent decrease in cAMP was not observed. The mean

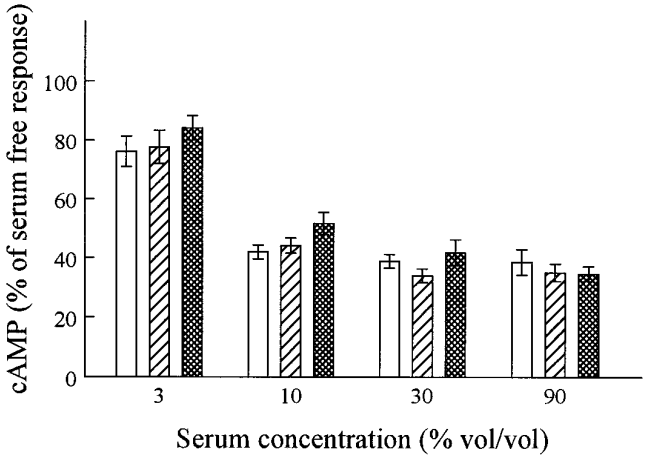

FIG. 4. cAMP production expressed as percentage of the production under serum-free conditions (mean ± SEM of two experiments in duplicate) by CHO cells, stimulated with a fixed dose of FSH (6 mIU/mL) in the presence of increasing concentrations of serum (3–90% vol/vol) from regularly cycling women (n = 8, *open bars*); normogonadotropic anovulatory patients without PCOS (n = 13, *hatched bars*); and PCOS patients (n = 16, *solid bars*).

relative cAMP production was lower at a concentration of 30% vol/vol, as compared with 10% vol/vol (55 ± 2% and 68 ± 2%, respectively; $P < 0.001$). No differences in inhibition of the cAMP response were found when comparing various classes of follicular fluid samples from normally developing follicles (Fig. 5, *upper panel*). The inhibitory effect of follicular fluid did not correlate with follicle size, with the AD/E_2 ratio of the follicular fluid, nor with the menstrual cycle phase in which the samples were obtained (data not shown). Similar results were obtained with follicular fluid from PCOS patients, as compared with follicular fluid from regularly cycling women (Fig. 5, *lower panel*).

Discussion

Inhibition of FSH action at the level of granulosa cells in the ovaries may underlie anovulation in women presenting with normal circulating FSH concentrations. The aim of this study was to compare levels of FSH receptor inhibitory activity in serum and follicular fluid from regularly cycling women and patients with normogonadotropic anovulation. The inhibitory activity was assessed by measuring the FSH-induced adenylate cyclase activity in cultured CHO cells stably transfected with the human FSH receptor (25). A similar system has been applied recently to assess bioactive FSH concentrations in serum throughout the menstrual cycle (34). In the present study, CHO cells were stimulated with physiological concentrations of FSH in the presence of untreated serum or follicular fluid. A wide range of concentrations of serum or follicular fluid was tested, from 3–90% vol/vol. High concentrations were applied, to approach protein concentrations that occur under physiological conditions.

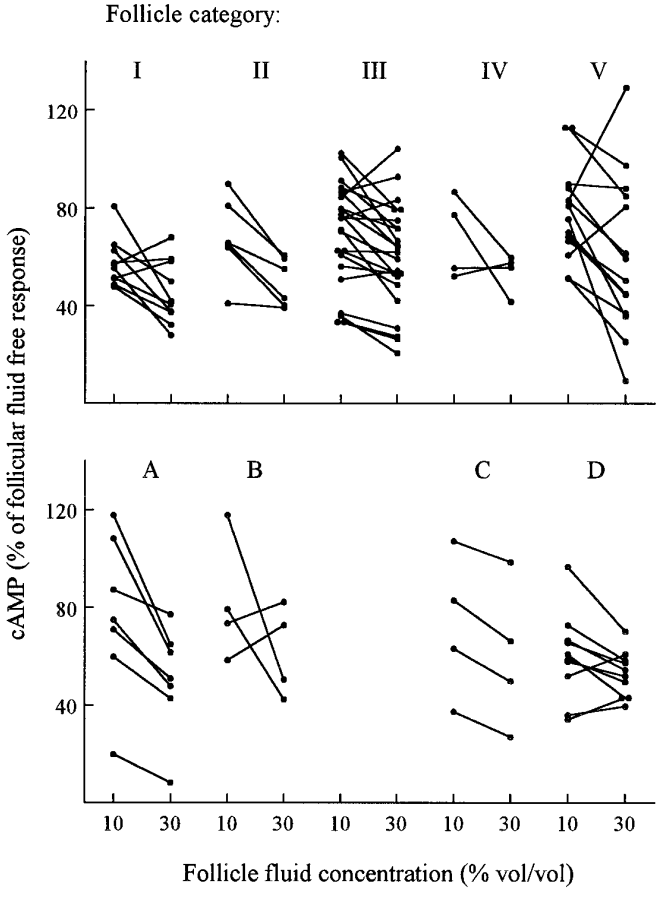

FIG. 5. cAMP response in CHO cells stimulated with 3 mU/mL recFSH in the presence of follicular fluid from regularly cycling women (*upper panel*) and PCOS patients (*lower panel*). The response is expressed as a percentage of the response in the absence of follicular fluid. *Dots* indicate responses of individual samples (tested in duplicate), with a *solid line* connecting the responses of individual samples at 10 and 30% vol/vol follicular fluid concentration. Follicle category in *upper panel:* I, EFP, <10 mm, atretic; II, EFP, <10 mm, healthy; III, LFP, <10 mm; IV, LFP, ≥10 mm, atretic; and V, LFP, healthy. Follicle category in *lower panel*: A, <10 mm, atretic; B, <10 mm, healthy; C, ≥10 mm, atretic; and D, ≥10 mm, healthy. See also Table 2.

Primary cultures of rat granulosa (35) or Sertoli cells (36) have been used as *in vitro* bioassays for FSH. These bioassays are hampered by a strong inhibition of the FSH response caused by unspecified factors in serum. An inhibition of more than 80% of the induced E_2 response can be observed in the presence of only 4% serum (25, 35). In contrast, currently used CHO cells show only a limited inhibition of FSH-induced cAMP production by serum. Even in the presence of 90% vol/vol human serum, the cAMP response is inhibited up to 60% only. Although this inhibition might be caused by metabolic changes in the cells, this seems less likely because prolonged incubation of the cells in serum affected neither cell viability nor basal cAMP production (25). In addition, the cells can be stimulated to produce large amounts of cAMP despite preincubation in 90% serum (25). It is of interest that in the presence of serum concentrations more than 10% vol/vol, the percentage inhibition of FSH-dependent receptor activation (at a fixed FSH concentration) is independent of the concentration of serum. This fixed magnitude of inhibition over a wide range of serum concentrations (10–90% vol/vol) suggests a saturating effect of the inhibitory components in serum. On the other hand, the CHO cells still respond to elevation of the FSH levels, irrespective of serum concentrations. This suggests that a competition exists between inhibiting factors and FSH for the FSH receptor. Although the mechanism of inhibition is unclear, this may be attributed to a nonspecific protein matrix effect, because bovine and equine sera show similar inhibition curves (25). Comparing sera from regularly cycling women with normogonadotropic anovulatory patients (with or without PCOS) showed no differences in inhibitory activity. This argues against the presence of increased levels of inhibitors of FSH receptor activation in serum from these patients.

Because factors inhibiting FSH receptor activation may be present exclusively in the follicular compartment, follicular fluid obtained from individual follicles throughout the follicular phase of the menstrual cycle and from PCOS patients also were tested. Follicular fluid samples were classified according to the stage of the menstrual cycle in which they were obtained, as well as by size and steroid content (expressed as the AD/E_2 ratio). Different classes of normally developing follicles did not reveal differences with regard to inhibition of FSH receptor activation. Neither did follicular fluid from PCOS patients, compared

with matched control follicles, show any difference in inhibition of FSH receptor activation. Results obtained with follicular fluid displayed a larger degree of variation, compared with results obtained from the addition of serum samples. Part of this variation may be caused by the higher intraassay coefficient of variation of the assay applying small volumes. Another explanation may be found in differences in the endogenous FSH levels of the individual follicular fluid samples. In the present study, FSH content in follicular fluid could not be assessed, because of the small volumes available. It was therefore not possible to normalize for potential differences in FSH levels. In an attempt to limit the variation in FSH levels in the follicular fluid assays, a small amount of FSH (3 mIU/mL) was added to each follicular fluid dilution. The choice for this FSH concentration was on the basis of data from the literature (4, 8, 29) that report levels of intrafollicular immunoreactive FSH ranging between 0.3 and 6 IU/L. This uncertainty in the final FSH concentration could explain the reversal of inhibition observed in some samples at increased concentration. However, despite these uncertainties, the observed responses did not correlate with any follicle characteristic, such as size or steroid content, or with menstrual cycle phase or PCOS diagnosis. Although inhibitory effects of subfractions in serum or follicular fluid have not been tested separately, the total net effect of potentially different combinations is similar in both controls and anovulatory patients. Therefore, it seems unlikely that FSH receptor inhibition plays a significant role in the (patho)physiology of normal or disturbed follicle development.

Specific FSH receptor-binding inhibitors have been described in partly purified fractions of human serum and follicular fluid (19, 23, 37). However, not all FSH-binding inhibitory activity results in inhibition of FSH, because part of these binding inhibitors have FSH agonist activity (22). Results of the present study show that the biological activity of FSH in the context of serum proteins is approximately 50% of the activity in the absence of serum. The mechanism underlying this reduction in bioactivity is not known, but it may be postulated that FSH is partly bound to plasma proteins with low affinity, thereby reducing the amount available for receptor activation. On the other hand, serum proteins also may bind reversibly to the receptor, thereby reducing the number of receptors available for interaction with FSH. One other aspect to be considered is that, in the present study, only adenylate cyclase activity has been taken as the endpoint of FSH receptor activation. It is known that FSH receptor activation results in formation of intracellular messengers other than cAMP, like calcium ions (38, 39). Although it is not yet clear whether this Ca^{2+} pathway functions independently from cAMP (39, 40), it could be postulated that specific FSH receptor inhibitors have a preference for one pathway, leaving the other less inhibited, which could thus result in expression of different cellular functions. Possible inhibition of different pathways is illustrated by findings that immunoglobulins in serum from women with premature ovarian failure block FSH-induced DNA synthesis in granulosa cells *in vitro* (24), although they do not affect FSH-induced steroidogenesis (41).

Our conclusion from these *in vitro* studies that FSH receptor inhibition is a constant, and therefore may be of only limited significance for regulation of follicle development, is being supported by recent findings from clinical studies. Growth and selection of the dominant follicle is induced by increasing levels of FSH during the luteofollicular transition in the menstrual cycle (42). Although normogonadotropic anovulatory patients display bioactive and immunoreactive FSH levels comparable with follicular phase levels during the normal menstrual cycle (1, 5), these patients lack the intercycle rise. Normal monofollicular development and ovulation can be induced in normogonadotropic anovulatory patients by the administration of exogenous FSH, applying a decremental-dose regimen (6, 43). Although the administration of gonadotropins in these patients suggests an increased FSH-threshold level that needs to be surpassed to ensure follicle development, it seems that normal follicle development can be achieved in normogonadotropic anovulatory patients without increasing FSH to supraphysiological concentrations (5). These findings dispute the presence of an increased FSH-threshold level in normogonadotropic anovulatory patients, which could be caused by locally acting factors blocking FSH action. It could well be that the absence of the transient increase in FSH levels in anovulatory patients is an important factor in the etiology of arrest of follicle development. It may be of interest that, in particular, bioactive FSH levels increase during the luteofollicular transition, as has been demonstrated recently (34). Although the bioactive-to-immunoactive FSH ratio remains constant during the EFP in regular cycling women (5, 34) and is not different in anovulatory patients (5), the change in FSH levels and the bioactive-to-immunoreactive ratio in the late luteal phase may be of significance for follicle recruitment and selection. Furthermore, under normal conditions, ongoing growth of the dominant follicle occurs despite decreasing levels of FSH in the LFP (15). This suggests an enhancement of FSH action, allowing the dominant follicle to continue its development, whereas FSH levels drop below the threshold for the remaining, less mature follicles. This enhancement of FSH action may be the result of locally active growth factors, such as IGF-I and -II, and the IGF-binding proteins (13). These factors act through their own specific receptors and therefore exert their effect through intracellular pathways and not directly through the FSH-receptor. The potential significance of IGF-II, as an intraovarian factor involved in the development of the dominant follicle in the human, has been stressed recently (44).

In conclusion, analysis of inhibition of FSH receptor activation, by serum or follicular fluid samples from both regularly cycling women and PCOS patients, indicates that increased levels of FSH receptor inhibitors do not play a significant role in the regulation of normal and abnormal follicle development. This is in concert with findings that normogonadotropic anovulatory patients do not exhibit an increased FSH-threshold level *per se*. Both the dynamics in FSH levels in the EFP and locally active growth factors, acting

through their own receptors, may be of greater significance for follicle growth and selection.

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References

1. **Fauser BCJM, Pache TD, Lamberts SW, Hop WC, de Jong FH, Dahl KD.** 1991 Serum bioactive and immunoreactive luteinizing hormone and follicle-stimulating hormone levels in women with cycle abnormalities, with or without polycystic ovarian disease. J Clin Endocrinol Metab. 73:811–817.
2. **Franks S.** 1995 Polycystic ovary syndrome. N Engl J Med. 333:853–861.
3. **Pache TD, Hop WC, de Jong FH, et al.** 1992 17 β-Oestradiol, androstenedione and inhibin levels in fluid from individual follicles of normal and polycystic ovaries, and in ovaries from androgen treated female to male transsexuals. Clin Endocrinol (Oxf). 36:565–571.
4. **Fauser BCJM.** 1994 Observations in favor of normal early follicular development and disturbed dominant follicle selection in polycystic ovary syndrome. Gynecol Endocrinol. 8:75–82.
5. **Van Dessel HJHM, Schoot BC, Schipper I, Dahl KD, Fauser BCJM.** 1996 Circulating immunoactive and bioactive follicle stimulating hormone concentrations in anovulatory infertile women and during gonadotrophin induction of ovulation using a decremental dose regimen. Hum Reprod. 11:478–485.
6. **Schoot DC, Hop WC, Pache TD, de Jong FH, Fauser BCJM.** 1993 Growth of the dominant follicle is similar to normal in patients with gonadotropin-stimulated polycystic ovary syndrome exhibiting monofollicular development during a decremental dose regimen. Acta Endocrinol (Copenh) 129:126–129.
7. **Erickson GF, Hsueh AJW, Quigley ME, Rebar RW, Yen SSC.** 1979 Functional studies of aromatase activity in human granulosa cells from normal and polycystic ovaries. J Clin Endocrinol Metab. 49:514–519.
8. **Mason HD, Willis DS, Beard RW, Winston RML, Margara R, Franks S.** 1994 Estradiol production by granulosa cells of normal and polycystic ovaries (PCO): relationship to menstrual cycle history and to concentrations of gonadotropins and sex steroids in follicular fluid. J Clin Endocrinol Metab. 79:1355–1360.
9. **Hsueh AJW, Adashi EY, Jones PBC, Welsh J, T.H.** 1984 Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocr Rev. 5:76–127.
10. **Tonetta SA, DiZerega GS.** 1989 Intragonadal regulation of follicular maturation. Endocr Rev. 10:205–229.
11. **Greenwald GS, Roy SK.** 1994 Follicular development and its control. In: Knobil E, Neill JD, eds. The physiology of reproduction. 2nd ed. New York: Raven Press; 629–724.
12. **Fauser BCJM.** 1996 Interference with follicle stimulating hormone regulation of human ovarian function. Mol Hum Reprod. 2:327–334.
13. **Giudice LC.** 1992 Insulin-like growth factors and ovarian follicular development. Endocr Rev. 13:641–669.
14. **Miró F, Hillier SG.** 1992 Relative effects of activin and inhibin on steroid hormone synthesis in primate granulosa cells. J Clin Endocrinol Metab. 75:1556–1561.
15. **Van Santbrink EJP, Hop WC, Van Dessel HJHM, de Jong FH, Fauser BCJM.** 1995 Decremental follicle-stimulating hormone and dominant follicle development during the normal menstrual cycle. Fertil Steril. 64:37–43.
16. **Steinkampf MP, Mendelson CR, Simpson ER.** 1988 Effects of epidermal growth factor and insulin-like growth factor I on the levels of mRNA encoding aromatase cytochrome P-450 of human ovarian granulosa cells. Mol Cell Endocrinol. 59:93–99.
17. **Mason HD, Margara R, Winston RML, Beard RW, Reed MJ, Franks S.** 1990 Inhibition of oestradiol production by epidermal growth factor in human granulosa cells of normal and polycystic ovaries. Clin Endocrinol (Oxf). 33:511–517.
18. **Reichert Jr LE, Sanzo MA, Darga NS.** 1979 Studies on a low molecular weight follicle-stimulating hormone binding inhibitor from human serum. J Clin Endocrinol Metab. 49:866–872.
19. **Sanzo MA, Reichert Jr LE.** 1982 Gonadotropin receptor binding regulators in serum: characterization and separation of follitropin binding inhibitor and lutropin binding stimulator. J Biol Chem. 257:6033–6040.
20. **Fletcher PW, Dias JA, Sanzo A, Reichert Jr LE.** 1982 Inhibition of FSH action on granulosa cells by low molecular weight components of follicular fluid. Mol Cell Endocrinol. 25:303–315.
21. **Sluss PM, Fletcher PW, Reichert Jr LE.** 1983 Inhibition of ^{125}I-human follicle-stimulating hormone binding to receptor by low molecular weight fraction of bovine follicular fluid: inhibitor concentration is related to biochemical parameters of follicular development. Biol Reprod. 29:1105–1113.
22. **Lee DW, Butler WJ, Horvath PM, Shelden RM, Reichert Jr LE.** 1991 Human follicular fluid contains a follicle-stimulating hormone (FSH) binding inhibitor which has FSH agonist activity, is immunologically similar to FSH, but can be distinguished from FSH. J Clin Endocrinol Metab. 72:1102–1107.
23. **Lee DW, Grasso P, Dattatreyamurty B, Deziel MR, Reichert Jr LE.** 1993 Purification of a high molecular weight follicle-stimulating hormone receptor-binding inhibitor from human follicular fluid. J Clin Endocrinol Metab. 77:163–168.
24. **van Weissenbruch MM, Hoek A, van Vliet Bleeker I, Schoemaker J, Drexhage H.** 1991 Evidence for existence of immunoglobulins that block ovarian granulosa cell growth *in vitro*. A putative role in resistant ovary syndrome? J Clin Endocrinol Metab. 73:360–367.
25. **Schipper I, Fauser BCJM, Ten Hacken PM, Rommerts FFG.** 1996 Application of a CHO cell line, transfected with the human FSH receptor for the measurement of specific FSH receptor activation inhibitors in human serum. J Endocrinol. 150:505–514.
26. **Pache TD, Hop WCJ, Wladimiroff JW, Schipper J, Fauser BCJM.** 1992 How to discriminate between normal and polycystic ovaries. A transvaginal ultrasound study. Radiology. 183:421–423.
27. **Van Dessel HJHM, Schipper I, Pache TD, van Geldorp H, de Jong FH, Fauser BCJM.** 1996 Normal human follicle development: and evaluation of correlations with oestradiol, androstenedione and progesterone levels in individual follicles. Clin Endocrinol (Oxf) 44:191–198.
28. **McNatty KP, Moore Smith D, Makris A, Osathanondh R, Ryan KJ.** 1979 The microenvironment of the human antral follicle: interrelationships among steroid levels in antral fluid, the population of granulosa cells, and the status of the oocyte *in vivo* and *in vitro*. J Clin Endocrinol Metab. 49:851–860.
29. **Erickson GF, Magoffin DA, Garzo VG, Cheung AP, Chang RJ.** 1992 Granulosa cells of polycystic ovaries: are they normal or abnormal? Hum Reprod. 7:293–299.
30. **McNatty KP.** 1979 Cyclic changes in antral fluid hormone concentrations in humans. J Clin Endocrinol Metab. 7:577–600.
31. **Downs TR, Wilfinger WW.** 1983 Fluorometric quantification of DNA in cells and tissue. Anal Biochem. 131:538–547.
32. **Harper JF, Brooker G.** 1975 Femtomole sensitive radioimmunoassay for cyclic AMP and cycli GMP after 2′0′-acetylation by acetic anhydride in aqueous solution. J Cycl Nucl Res. 1:207–218.
33. **Frohlich M, Brand EC, van Hall EV.** 1976 Serum levels of unconjugated aeticholanolone, androstenedione, testosterone, dehydroepiandrosterone, aldosterone, progesterone, and oestrogens during the normal menstrual cycle. Acta Endocrinol (Copenh) 81:548–562.
34. **Christin-Maitre S, Taylor AE, Khoury RH, et al.** 1996 Homologous *in vitro* bioassay for follicle-stimulating hormone (FSH) reveals increased FSH biological signal during the mid- to late luteal phase of the human menstrual cycle. J Clin Endocrinol Metab. 81:2080–2088.
35. **Jia X, Hsueh AJW.** 1986 Granulosa cell aromatase bioassay for follicle-stimulating hormone: validation and application of the method. Endocrinology. 119:1570–1577.
36. **Padmanabhan V, Chappel SC, Beitins IZ.** 1987 An improved *in vitro* bioassay for follicle-stimulating hormone (FSH): suitable for measurement of FSH in unextracted human serum. Endocrinology. 121:1089–1098.
37. **Lee DW, Shelden RM, Reichert Jr LE.** 1990 Identification of low and high molecular weight follicle-stimulating hormone receptor-binding inhibitors in human follicular fluid. Fertil Steril. 53:830–835.
38. **Leung PCK, Steele GL.** 1992 Intracellular signalling in the gonads. Endocr Rev. 13:476–498.
39. **Sharma OP, Flores JA, Leong DA, Veldhuis JD.** 1994 Cellular basis for follicle-stimulating hormone-stimulated calcium signalling in single rat Sertoli cells: possible dissociation from effects of adenosine 3′,5′-monophosphate. Endocrinology. 134:1915–1923.
40. **Gorczynska E, Spaliviero J, Handelsman DJ.** 1994 The relationship between 3′,5′-cyclic adenosine monophosphate and calcium in mediating follicle-stimulating hormone signal transduction in Sertoli cells. Endocrinology. 134:293–300.
41. **Anasti JN, Flack MR, Froehlich J, Nelson LM.** 1995 The use of human recombinant gonadotropin receptors to search for immunoglobulin G-mediated premature ovarian failure. J Clin Endocrinol Metab. 80:824–828.
42. **Fauser BCJM, van Heusden AM.** 1997 Manipulation of human ovarian function; physiological concepts and clinical consequences. Endocr Rev. 18:71–106.
43. **Van Santbrink EJP, Donderwinkel PFJ, Van Dessel HJHM, Fauser BCJM.** 1995 Gonadotrophin induction of ovulation using a step-down dose regimen: single-centre clinical experience in 82 patients. Hum Reprod. 10:1048–1053.
44. **Van Dessel HJHM, Chandrasekher Y, Yap OWS, et al.** 1996 Serum and follicular fluid levels of insulin-like growth factor I (IGF-I), IGF-II, and IGF-Binding protein-1 and -3 during the normal menstrual cycle. J Clin Endocrinol Metab. 81:1224–1231.