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FOLLICLE REGULATORY PROTEIN NONCOMPETITIVELY INHIBITS MICROSOMAL 3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY

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Summary—A heat- and trypsin-labile follicular fluid protein (FRP) extracted from both human and porcine follicular fluid has been shown to modulate ovarian steroidogenesis. To further investigate the effects of FRP, its effect on the kinetics of 3β-hydroxysteroid dehydrogenase activity (3β-HSD) was evaluated in cell-free microsomal preparations from human placenta. Test fractions of follicular fluid protein were preincubated with placental microsomes followed by the addition of various substrate concentrations (pregnenolone + NAD). Subsequent progesterone formation was interpreted as the velocity of the reaction. The 50% inhibitory dose (ID_{50}) of FRP for 3β-HSD for the three substrate concentrations was 300 μg/ml. Although a clear decrease in 3β-HSD activity typically occurred after pre-incubation with 730 μg/ml of FRP, a paradoxical augmentation in 3β-HSD activity was present with the lower concentrations of FRP (10–30 μg/ml) and the more concentrated microsomal preparations.

Double reciprocal plots of these reactions demonstrated a K_m for 3β-HSD of $1.8–2.1 \times 10^{-6}$ M. Analysis of all reactions was found to be consistent with a noncompetitive mode of enzyme inhibition with an apparent K_i of 120 ng/ml or $\sim 10^{-8}$ M assuming a mol. wt of 16,000 Daltons for FRP. This derived K_i for FRP is within the biological concentration of FRP in follicular fluid.

INTRODUCTION

A heat- and trypsin-labile protein extracted from both human and porcine follicular fluid has been shown to have a role in regulation of ovarian steroidogenesis [1,2]. This follicle regulatory protein (FRP) partially purified from follicular fluid by affinity chromatographic and HPLC procedures, elutes in the mol. wt range of 16,000 Daltons and has isoelectric points of pH 4.5 and 6.5 [3 and unpublished data]. FRP is secreted by granulosa cells and does not inhibit secretion of FSH by pituitary cells *in vitro* [4–6]. Therefore, FRP has biological properties different from those of inhibin [7,8]. FRP has been shown to: (1) inhibit the increase in ovarian weight and in serum estradiol levels in response to human menopausal gonadotropin (hMG) treatment in hypophysectomized, immature, DES-treated rats [9]; (2) delay follicular maturation when injected into spontaneously cycling monkeys, as evidenced by reduced serum estradiol levels, prolongation of the follicular phase, and anovulation or luteal phase dysfunction in the presence of normal serum gonadotropin levels [5]; (3) inhibit FSH induction of LH–hCG receptors in porcine granulosa cells [10]; (4) inhibit FSH-responsive adenylate cyclase activity in porcine granulosa cells [11]; and (5) inhibit *in vitro* microsomal aromatase (unpublished data) and alter production and secretion of progesterone from granulosa cells [12, 13].

Human placental microsomes contain an enzyme system which catalyze the conversion of 5-ene-3β-hydroxysteroids to 4-ene-3-ketosteroids [14–16]. This conversion which is NAD dependent, consists of two reactions: (1) oxidation of the hydroxy group at C-3 (3β-ol-dehydrogenase); and (2) a shift of the double bond from C-5 to C-4 (5-ene-isomerase) [17, 18]. The conventional abbreviation for this enzyme system, 3β-HSD, will be used here as a designation for both the dehydrogenase and isomerase steps. To further investigate the role of FRP in steroidogenesis, its effect on the kinetics of 3β-HSD activity was evaluated in cell-free microsomal preparations from placenta.

EXPERIMENTAL

Human placentas were obtained immediately after birth, dissected free of fetal membranes and homogenized in a Waring blender for 1 min in buffer A (sucrose, 0.25 M; KH_2PO_4, 0.5 M, pH 7) [19]. Preparations with 3β-HSD activity were obtained using 100 ml of buffer A to 300 *g* of tissue. The homogenate was centrifuged at 800 *g* for 30 min and that supernatant was further centrifuged at 10,000 *g* for 60 min. The resulting supernatant was centrifuged at

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120,000 *g* for 1 h. The pellet was resuspended in buffer A containing 50% glycerol and repelleted at 120,000 *g* for 1 h. The pellet was reconstituted in buffer A to yield concentrations of 15–25 mg protein/ml placental preparation. The preparation could be stored in the freezer (−50°C) for 16 weeks without apparent loss of 3β-HSD activity.

Follicular fluid fraction

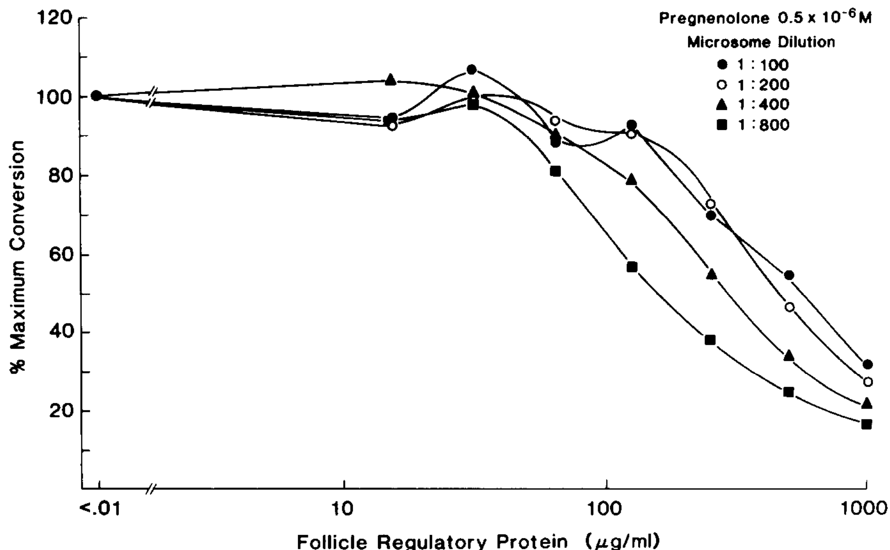
Porcine follicular fluid was thawed slowly and fractionated by dropwise addition of an equal volume of saturated ammonium sulphate during persistent agitation at 4°C [20]. After a 12 h incubation, the precipitate was recovered by centrifugation and resuspended (2:1, v/v) in 30% ammonium sulfate. An additional 12 h of mixing was followed by centrifugation at 3,000 *g* for 30 min. The resulting supernatant was dialyzed in tubing with an exclusion size of 10,000 daltons (PM-10, Amicon, Lexington, MA) against distilled water for 16 h at 4°C and then lyophilized. The resuspended retentate (500 mg of protein in 0.5 ml aliquots of buffer A without sucrose) was passed through a column (9 × 32 mm; bed vol 2 ml) containing agarose-linked matrix gel Orange A (Dyematrix, Amicon, Lexington, MA), previously equilibrated with 20 mM Tris–HCL, pH 7.5. Unbound material was eluted with 10 ml of 20 mM Tris–HCL, pH 7.5. Bound eluent fractions were dialyzed overnight against distilled water and lyophillized.

3-β Hydroxysteroid dehydrogenase

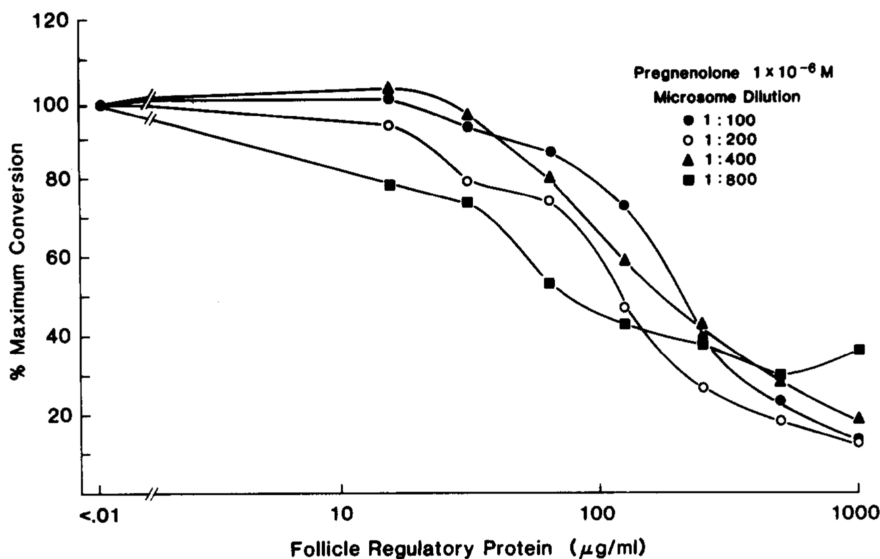
Determination of 3β-HSD activity was performed as previously described at 20°C [16, 21, 22] with minor modifications. Incubations contained 0.4 ml of the microsomal preparation in buffer A, 0.5 ml of [4-^{14}C]pregnenolone (10^{-6} M, 10 μCi/mM, New England Nuclear) and NAD (100 mM, Sigma, St Louis, Mo.) in 0.09 ml of buffer A. Test fractions of follicular fluid (0.1 ml) were preincubated with placental extracts (20°C, 20 min) followed by addition of the pregnenolone–NAD mixture. Reaction termination and quenching were performed by the addition of methanol. Aliquots (300 μl) of the reaction mixture were transferred into tubes containing non-radioactive carriers (substrate and product, 50 μg each) and immediately extracted twice with 2 vol of diethyl ether. The extracts were dryed with a steam of nitrogen. The residue was dissolved in 100 μl of methanol and separated in a Bush A paper chromatography system [24].

Chromatograms were scanned by a Packard Model 7201 radiochromatograph. From this graphic representation, R_f values for the radioactive isotopes were determined. Chromatography strips were removed from the scanner and portions corresponding to the peaks were cut out and eluted with 2 ml of methanol. A 500 μl aliquot of the eluent was placed into a scintillation vile containing 9.5 ml of scintillation fluid. Fifty μl aliquots of internal standards (5,7(*n*)-[^{3}H]pregnenolone 21 Ci/mM, and 1,2(*n*)-[^{3}H]progesterone, 50 Ci/mM, Amersham, Arlington Heights, Il, 200 μl each in 2.5 ml of methanol) were added to the 2 ml reaction volume and developed through the Bush A paper chromatography system to provide a correction factor for recovery quantitation.

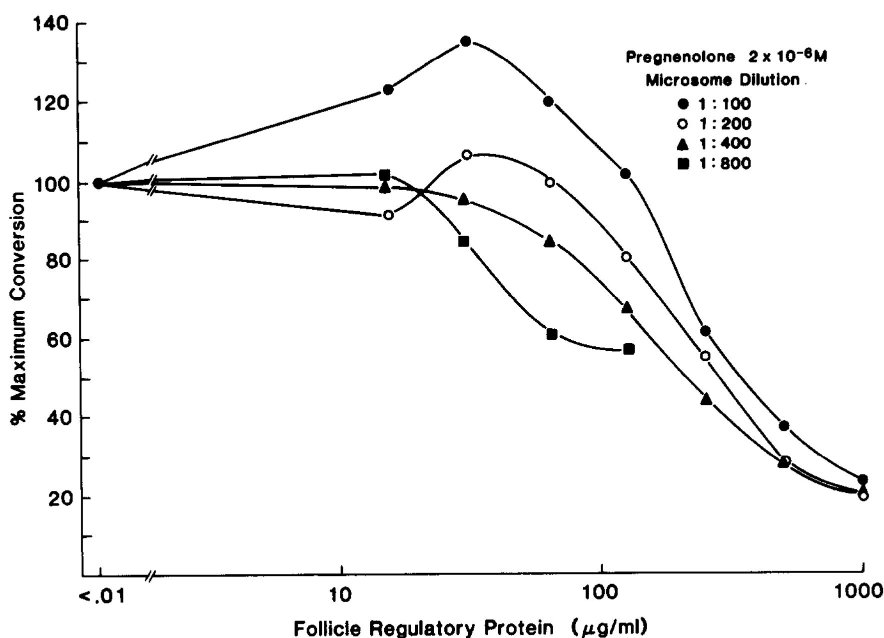
The reaction rate was calculated by determining the percentage of substrate converted to product. Areas of the chromatogram corresponding to substrate and product (e.g. pregnenolone and progesterone) accounted for 98% of the radioactivity. After confirming that the formation of product was linear over 3 min all further reactions were quenched after 2 min. Mean progesterone formation (triplicate assays; $N = 3$ each assay) was interpreted as the velocity of the reaction for the purpose of graphic



(a)



(b)



(c)

Fig. 1. The effect of a partially purified fraction of porcine follicular fluid (follicle regulatory protein) on the conversion of pregnenolone (a: 0.5×10^{-6} M; b: 1.0×10^{-6} M; c: 2.0×10^{-6} M) to progesterone by 4 different dilutions of a placental microsome prep is shown. The 50% inhibitory dose of follicle regulatory protein was 100–300 μg/ml for the respective substrate concentrations.

presentation of the data. Protein determinations were carried out by the procedure of Lowry[25].

Statistical analysis

Enzyme kinetic constants (K_m, K_i) were determined by modified Lineweaver–Burk and Dixon plots using linear regression [26]. Statistical analysis was performed using analysis of variance. All experiments were repeated at least 3 times with comparable results.

RESULTS

Determination of initial reaction rates was performed over a 20 min interval with a 1:400 dilution of the microsomal preparation and 2×10^{-6} M pregnenolone. The conversion of pregnenolone to progesterone was linear for at least 180 s. Accordingly, all further studies were performed using a 2 min incubation of pregnenolone with the microsomes and cofactor. To determine the proper microsomal concentration for assessment of kinetic parameters, a wide range of microsomal dilutions (1:40–1:3,200)

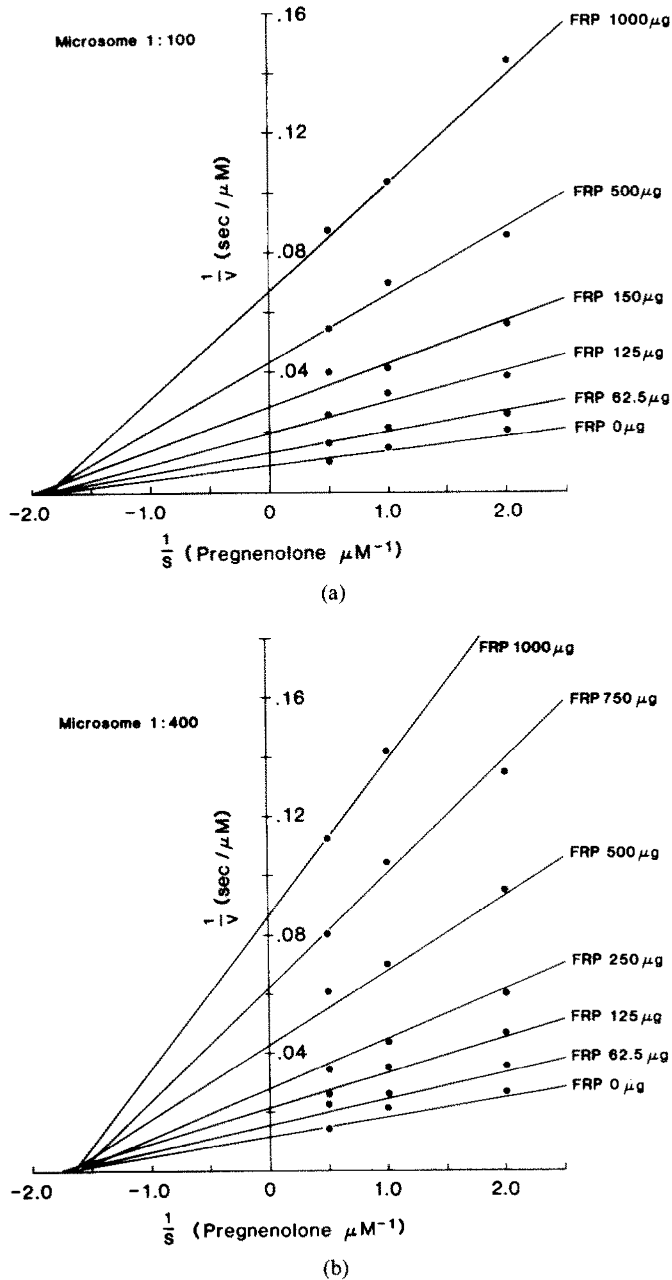


Fig. 2. Double reciprocal plots of the data shown in Fig. 1 are depicted for the 1:100 (a) and 1:400 (b) concentration of placental microsomes and the various concentrations (0–1000 μg/ml) of a partially purified fraction of porcine follicular fluid referred to as follicle regulatory protein (FRP). Kinetic constants calculated from these plots indicated a K_m for the 3β-hydroxysteroid dehydrogenase reaction of $1.8\text{–}2.1 \times 10^{-6}$ M.

were incubated with 10^{-6} M pregnenolone for 2 min. Since the amount of progesterone formed reached a plateau at 1:800, microsomal concentrations of 1:100, 1:200, 1:400 and 1:800 were chosen for the determination of kinetic parameters. The effects of preincubating microsomes with FRP on 3β-HSD activity were determined using a 1:400 microsomal dilution and 500 μg/ml of FRP. Microsomes and FRP were incubated over a 0–60 min interval followed by a 2 min incubation with substrate. Since an inflection point was reached at 20 min, all further assays employing FRP were performed with a 20 min preincubation of microsomes with or without (control) FRP prior to the 2 min incubation with pregnenolone and cofactor.

Conversion of pregnenolone to progesterone after a 2 min incubation was $20.8 \pm 1.3\%$ with a 12% interassay coefficient of variation. Kinetic parameters were determined using 1:100, 1:200, 1:400 and 1:800 dilutions of microsomes, varying concentrations of

FRP (0, 15, 30, 62, 125, 250, 500 and 1000 μg/ml) and substrate (pregnenolone: 0.5×10^{-6} M: Fig. 1a; 1×10^{-6} M: Fig. 1b; 2×10^{-6} M: Fig. 1c). The 50% inhibitory dose (ID_{50}) of FRP for 3β-HSD for the three substrate concentrations was 100–300 μg/ml. Although a clear decrease in the activity of 3β-HSD typically occurred after preincubation with 730 μg/ml of FRP, a paradoxical augmentation in 3β-HSD activity was present with the lower concentrations of FRP (10–30 μg/ml) and the more concentrated microsomal preparations [1:100] (Fig. 1c).

Double reciprocal plots of these reactions demonstrated a K_m of $1.8–2.1 \times 10^{-6}$ M for 3β-HSD (Fig. 2) which is consistent with previous observations [20, 27, 28]. To further elucidate the kinetic effects of FRP on 3β-HSD activity, the data from Fig. 1 were analyzed by Dixon plots (not shown). Analysis of all reactions was found to be consistent with a non-competitive or mixed mode of enzyme inhibition. The apparent K_i for FRP calculated from the graphs is 120 ng/ml or 10^{-8} M assuming a mol. wt of 16,000 Daltons for FRP.

DISCUSSION

These observations extend our previous reports that FRP alters production of progesterone in intact human [13], porcine [12] and rat (unpublished data) granulosa cells as well as microsomes from human granulosa cells [13]. Ledwitz-Rigby [27] using whole follicular fluid from small (1–2 dia) porcine follicles, demonstrated a reduction in secretion of progesterone after FSH stimulation in porcine granulosa cells from medium and large follicles. Shemesh[28] reported the inhibition of progesterone secretion from bovine granulosa cells by bovine follicular fluid which was partially overcome by the addition of FSH. This interaction of FRP and FSH on 3β-HSD activity was also observed with granulosa cells from rats. Schreiber *et al.* (unpublished data) examined the interactions of an identically prepared FRP preparation and oFSH on 20 a-dihydroprogesterone production in cultures of rat granulosa cells. With low doses of oFSH (6.25–37.5 ng/ml), FRP inhibited oFSH stimulation of 20 a-dihydroprogesterone production. This inhibition by FRP was overcome when oFSH was increased to 50 ng/ml.

Steroidogenic enzymes in granulosa cells appear to have FSH dependent differential sensitivities to FRP *in vitro*. FRP in concentrations of 50–200 μg/ml inhibits aromatase activity in granulosa cells [2], while stimulating 3β-HSD activity. When the concentration of FRP is increased to 500 μg/ml, both aromatase and 3β-HSD activities are inhibited. When granulosa cells are exposed to FSH alone, they produce more estrogen and less progesterone. However, when granulosa cells are exposed to FSH in the presence of FRP, more progesterone and less estrogen is produced. These observations suggest that FRP alters the dose–response relationship between FSH and 3β-HSD activity in granulosa cells. Thus, FRP may play a role in modulating the relative secretion of progesterone and estrogen by granulosa cells in response to FSH stimulation.

Recently, we found that FRP inhibits aromatase activity in a placental microsome preparation similar to the one used here [11]. The kinetic constants also suggested noncompetitive inhibition with an apparent K_i of approx. 10^{-7} M. The effect of FRP on aromatase activity in placental microsomes was associated with a decrease in the apparent V_{max} without a detectable change in the apparent K_m of the enzyme. Thus, the possibility that FRP treatment decreases enzyme activity by decreasing the affinity of the enzyme for its substrate is unlikely. Here, the apparent K_i for FRP mediated inhibition of 3β-HSD was 10^{-8} M, at least an order of magnitude more sensitive than the apparent K_i for aromatase inhibition. This derived K_i for FRP is within the biological concentration of FRP in follicular fluid, assuming that the mol. wt of FRP is 16,000 and the FRP concentration used in the reagent employed in this study was 1%. Battin [13] and Schreiber (unpublished data) using porcine and rat granulosa cells also found that 3β-HSD was more sensitive to FRP inhibition than was aromatase activity.

Thus, the developing granulosa cell, through secretion of proteins, may be capable of autoregulating steroidogenesis in its own and other follicles. Moreover, a differential sensitivity of important steroidogenic enzymes to FRP may relate to alterations in steroid secretion. Recently, Ledwitz-Rigby *et al.* (unpublished data) demonstrated that the androgenic mileu of ovarian follicles alters the response of granulosa cell progesterone production to FRP during follicular maturation. These observations suggest that, in addition to the intrafollicular steroidal mileau, a variety of non-steroidal compounds, perhaps secretory products of the granulosa cells, contribute to the paracrine–autocrine regulation of follicular steroidogenesis.

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