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Evidence for a Sertoli cell, FSH-suppressible inhibiting factor(s) of testicular steroidogenic activity

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SUMMARY By using a model of immature porcine Leydig and Sertoli cells cultured in serum free defined medium, we evidenced a paracrine control of Leydig cell steroidogenic activity by Sertoli cells via a secreted inhibiting protein(s). This protein(s), partially purified using gel filtration(M.W. 20,000-30,000) suppresses the steroidogenic responsiveness to LH/hCG by decreasing the specific LH/hCG binding (52% decrease)and hormone steroid biosynthesis (73% decrease) at a level(s) located between cAMP production and pregnenolone formation. The suppression of this inhibitor(s) by FSH, in a dose dependent manner, is one mechanism by which FSH "sensitizes" Leydig cell response to LH/hCG stimulation.

The testicular development and function are predominantly under the control of pituitary hormones, Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). Steroidogenic Leydig cell activity is regulated by LH and tubular (Sertoli) cells by FSH through specific membrane receptors (1). However, there is evidence for the involvement of FSH in the development of Leydig cells, since in immature hypophysectomized rat, FSH treatment enhances the capacity of the testis to bind LH and to secrete testosterone (2,3). By studying the activity of immature porcine Leydig cell cocultured with Sertoli cells or cultured in Sertoli cell conditioned medium, we have shown that FSH increases both LH/hCG binding and hCG-stimulated testosterone secretion via Sertoli cell secreted proteins (4,5). Since biochemical (6) and morphological (7) studies have suggested that seminiferous tubules secrete steroidogenesis stimulating factor(s), it was hypothesized that FSH could enhance Leydig cell

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steroidogenesis by increasing such factor(s) (4,8-10). However, in the present report the fractionation of conditioned medium by both unstimulated or FSH-stimulated Sertoli cells shows: (i) the secretion by Sertoli cells of an inhibiting factor(s) of Leydig cell steroidogenesis activity and (ii) that the suppression of this inhibitor(s) is one mechanism by which FSH "enhances" testicular (Leydig cell) steroidogenic responsiveness to LH/hCG stimulation.

MATERIAL AND METHODS

Human Chorionic Gonadotrophin (hCG CR 121 13,450) was a gift of Dr CANFIELD. Porcine Follicle Stimulating Hormone (pFSH) (NIH FSH P2)was obtained from NIADDK, National Pituitary Agency. Collagenase was obtained from Boehringer. Dulbecco Modifed Eagle's medium (DME) and Ham's F12 medium were obtained from GIBCO.Soybean trypsin inhibitor, Vitamin E, Transferrin, Insulin, Hepes, Desoxyribonuclease (Dnase1) were supplied by Sigma.

Leydig and Sertoli cell cultures and conditioned medium by Sertoli cells. Routinely, cells were prepared from immature porcine testes as described elsewhere(4,5). Following digestion with collagenase, cells were washed and centrifugated. The cell pellet was resuspended and two successive sedimentations of 5 and 15min. were performed. The crude interstitial (Leydig) cells were recovered from the supernatants and Sertoli cells from the sedimented tubules. Leydig cells were purified from interstitial cells using a discontinous Percoll gradient. After several washings with DME/F12 medium (1:1) the tubules were washed and treated with a 1 M glycine buffer pH7.2 (11). The tubules were dissociated by collagenase (0.5 mg/ml) and DNAse I (0.01 mg/ml) treatments (15 min, 33°C), washed and filtered through 160 mesh Nitex.

Leydig and Sertoli cells were cultured in DME/F12 (1:1) containing 1.2 mg/ml sodium bicarbonate, 15 mM hepes and 20 µg/ml gentamycine. This medium was supplemented with insulin (5 µg/ml), transferin (5 µg/ml), vitamin E (10 µg/ml). Cells were cultured at 32°C in a humidified atmosphere at 5 % CO_2, 95 % air.

Preparation of partially purified Sertoli Cell Androgenic Inibiting Factors (SCAIF). SCAIF was preparerd from Sertoli cell conditionned medium (SSCM). For the preparation of SCCM, the cells were cultured in Falcon flasks (75 cm^2 or 175 cm^2) for two weeks. The medium was collected every two days,centrifuged at 3.900g for 30 min. The conditioned medium was concentrated using Amicon Stirred cell with Diaflo membrane YM2 (MW app cutt off 1,000), dialysed using Spectrapor 3 membrane (MW app cutt off 3,500) against acetic acid 0.1M at 4°C for 24hrs Following these treatments, SCCM was dried under vacuum before resuspended in acetic acid

1M.This extract was then chromatographied on Sephadex G100 column (1.7x62cm) equilibrated with acetic acid 1M at 4°C. Leydig cells were incubated with these chromatograpy fractions for 2 days and on the third day their activity was evaluated (see below).

Leydig cell activity. Leydig cells were cultured in the absence or presence of different fractions from Sertoli cell conditioned medium. The cells were then washed and their activity was evaluated mainly by two parameters: (i) LH/hCG binding: Leydig cells were incubated for 4hrs at 32°C with [^{125}I]-hCG in the absence (total binding) or presence (non specific binding), of an excess of 500 fold hCG. The cells were then washed twice with cold phosphate buffer 0.2% BSA, solubilized in 0.5% N NaOH 0.4% deoxycholate solution, and radioactivity was measured in a γ counter; (ii) testosterone secretion: after 3 hrs incubation in the absence or presence of hCG (10^{-9}M),the testosterone content of the medium was measured by radioimmunoassay.

RESULTS

The conditioned medium by unstimulated Sertoli cells was subjected to different extraction procedures (dialysis, concentration and acid extraction) and fractionation by Sephadex G100 as indicated in Fig.1. The

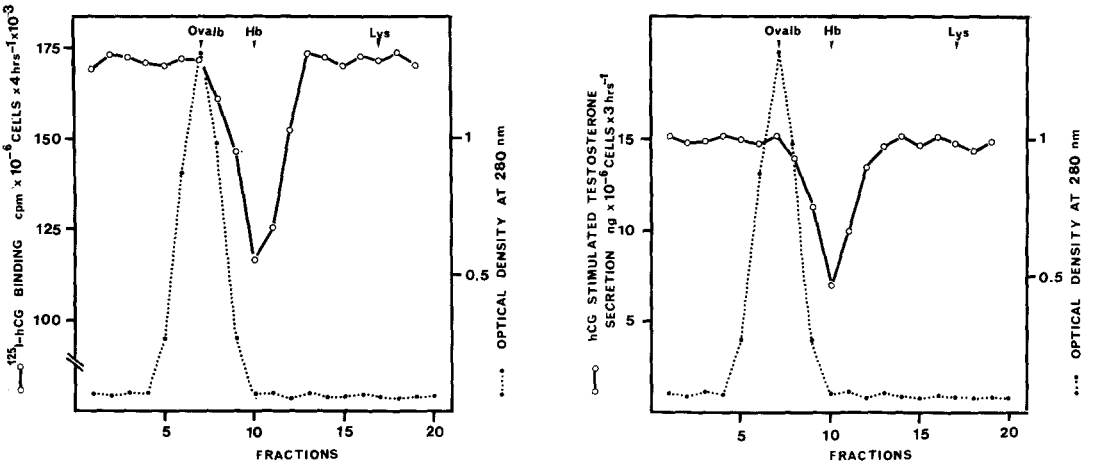

Fig 1: Chromatography of the conditioned medium by unstimulated Sertoli cells using a Sephadex G100 column.

The Sertoli cell conditioned medium (SCCM) was chromatographied on Sephadex G100 column with acetic acid 1M at 4°C. Different fractions obtained were dried and resuspended in culture medium. Leydig cells were incubated with these fractions for 2 days and on the third day their activity was evaluated using [^{125}I] -hCG binding (upper panel) and hCG-stimulated testosterone secretion (lower panel). Each point represents the mean of triplicate determinations. Dotted lines represent the optical density (OD) at 280nm. Standard proteins include Ovalbumin (Ovalb. 45,000),Hemoglobin (Hb 20,000) and Lysozyme (Lyso.12,000). Fractions 9-11 were pooled and used as SCAIF.

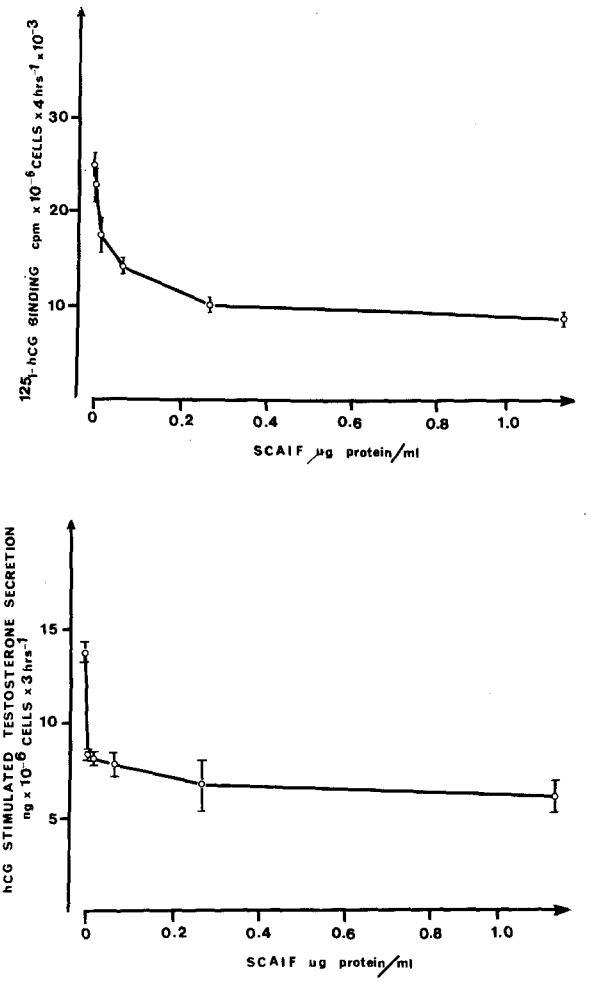

Fig 2. Leydig cell inhibition by SCAIF:dose dependency. Leydig cells were cultured for 48 hrs in the presence of indicated concentrations of SCAIF (0-1100ng/ml). Leydig cell activity was evaluated by [^{125}I]-hCG binding (upper panel) and hCG-stimulated testosterone secretion (lower panel). The results represent the mean ± SD of triplicate determinations.

gel filtration of Sertoli cell conditioned medium yielded inhibitory fractions of Leydig cell function evaluated by LH/hCG binding and hCG-stimulated testosterone production (Fig1). These inhibitory fractions which eluted between M.W. 20,000-30,0000 were pooled and called SCAIF (Sertoli Cell Androgenic Inhibiting Factors). The inhibition of Leydig cell activity was dependent on the concentration of the SCAIF (Fig.2) with maximal reduction observed at 250 and 70 ng/ml respectively for LH/hCG binding and hCG-stimulated testosterone secretion. The maximal reduction

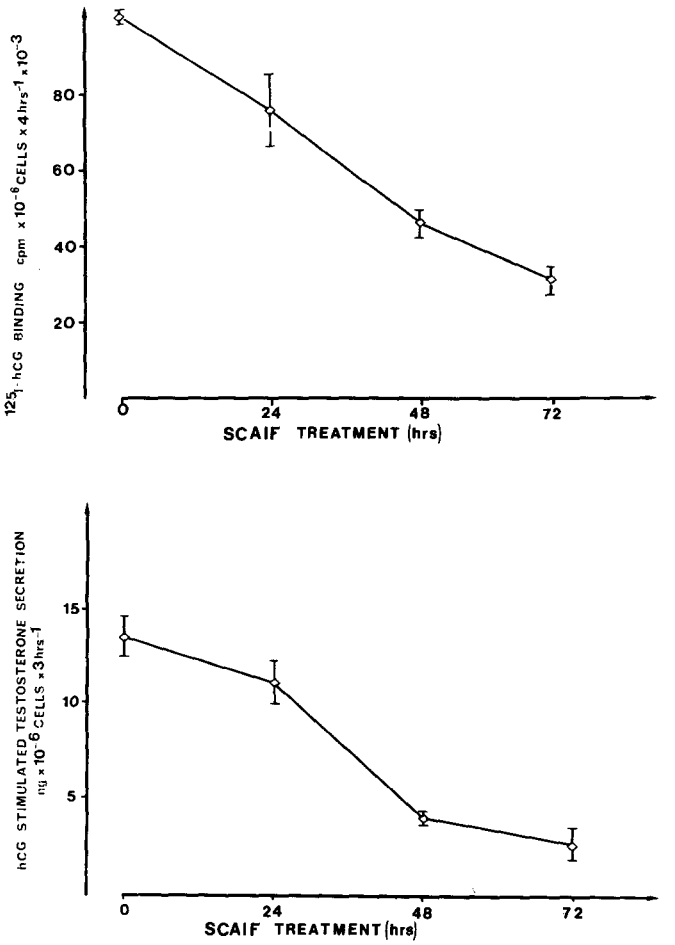

Fig 3. Leydig cell inhibition by SCAIF: time dependency. Leydig cells were cultured for the duration indicated (24-72 hrs) in the presence of SCAIF (250 ng/ml). Leydig cell activity was evaluated by [^{125}I]-hCG binding (upper panel) and hCG-stimulated testosterone production (lower panel). The results represent the mean ± SD of triplicate determinations.

of both LH/hCG binding and hCG-stimulated testosterone secretion was respectively 52% decrease and 73% decrease as compared to untreated cells. The maximal reduction of Leydig cell activity was observed following 48 hrs of SCAIF treatment (Fig.3).

Because FSH enhances Leydig cell steroidogenic activity via Sertoli cell proteins(5), the effects of FSH on SCAIF were studied; Sertoli cells were cultured with increasing concentrations of pFSH (0-100ng/ml) and Sephadex G100 fractions related to SCAIF were tested on Leydig cell

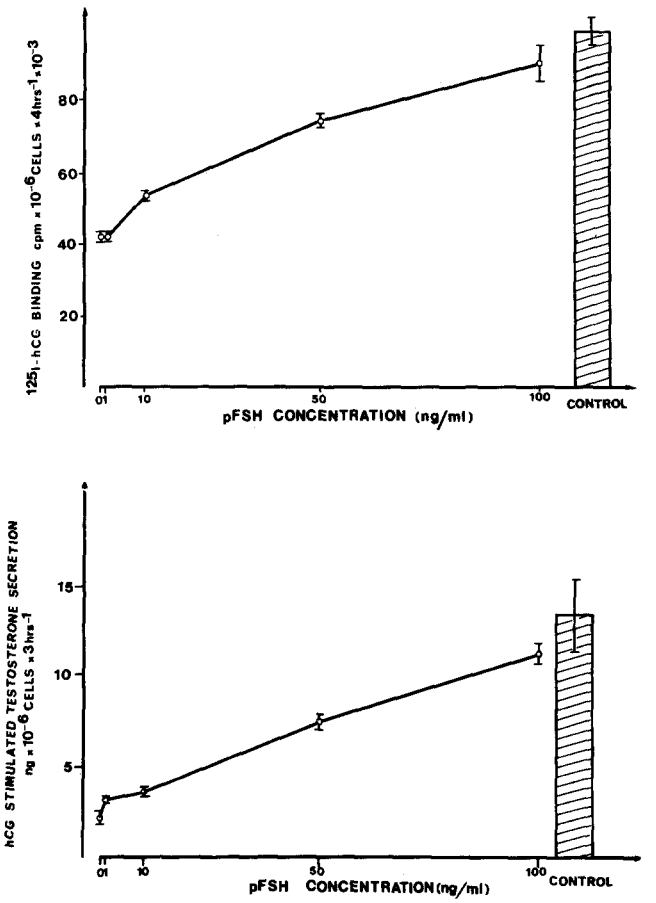

Fig. 4. Suppression of SCAIF activity following treatment of Sertoli cells by increasing concentrations of FSH (0-100 ng/ml). SCAIF activity is evaluated by inhibition of $[^{125}I]$-hCG binding (upper panel) and of hCG-stimulated testosterone production (lower panel). A control value for both $[^{125}I]$-hCG binding (upper panel) and hCG-stimulated testosterone production (lower panel) is represented by hatched columns. The results represent the mean ± SD of triplicate determinations.

activity. As shown on Fig.4, FSH suppressed, in a dose dependent manner, the effects of SCAIF on both LH/hCG binding and hCG-stimulated testosterone secretion. With 100ng/ml pFSH, SCAIF was completely suppressed since SCAIF-treated Leydig cell activity was similar to that of untreated cells (Fig.4).

The inhibitory effect of SCAIF on steroidogenesis was also observed when Leydig cells were stimulated with forskolin, an adenylate

Table 1-Effect of hCG, forskolin, and Δ5 Pregnenolone on testosterone production by Leydig cells treated or not (control) with SCAIF

	Testosterone (ng x 10^{-6} cells x 3 hrs^{-1})	
	Control	SCAIF
no treatment	0.8 ± 0.1	0.83 ± 0.1
hCG	8.8 ± 0.1	2.4 ± 0.3
Forskolin	8.5 ± 0.9	2.8 ± 0.5
Δ5 Pregnenolone	28.0 ± 0.2	24.2 ± 0.9

Leydig cells were cultured for two days with SCAIF. On the third day, the cells were washed and incubated with hCG (10^{-9}M), forskolin ($5x10^{-5}$M) or Δ5 Pregnenolone (10^{-6} M) for 3 hrs. The medium was then collected and testosterone assayed. The data represent the mean ± SD of triplicate determinations.

cyclase activator (12) instead of hCG (Table1). The effects of SCAIF on conversion of pregnenolone to testosterone were examined in cultured Leydig cells. As shown in Table 1, SCAIF was ineffective in decreasing testosterone production when Leydig cells were cultured with exogenous pregnenolone.

DISCUSSION

The present findings evidence the production by Sertoli cells of a protein(s) which greatly inhibits both LH/hCG binding and hCG- stimulated testosterone secretion (Figs1-4), but not basal secretion (Table1). The specificity of SCAIF is shown by its FSH dependency, namely its suppression by FSH in a dose dependent manner (Fig 4).

Although the exact origin of SCAIF must be questioned, the ability of FSH (Fig 4) to supress SCAIF in Sertoli cell conditioned medium, reveals that Sertoli cell are undoubtedly the most likely source of this factor(s). Cultured Sertoli cell are poorly contaminated with peritubular myoid cells (about 5-10 %), and thus, the possibility cannot be excluded that Sertoli cells may interact with peritubular myoid cells in the

production of SCAIF as suggested for certain Sertoli cell proteins (13,14).

Inhibiting factors of gonadal activity have been reported both from the testis (15) and the ovary (16). Morphological studies have reported a hypertrophy of Leydig cells following seminiferous tubule (Sertoli) cell damage by implantation of infertility compounds in the testis (17), experimental cryptorchidism and efferent duct ligation (18); these studies suggested that the seminiferous tubules secrete an inhibitor(s) of Leydig cell function, the levels of which were reduced in states of tubule damage. More recently, the existence of an inhibitor(s) of Leydig cell function from stages VIII-XI of seminiferous tubules has been reported(15). The inhibiting activity of this factor(s) was observed only after a short treatment (3-4hrs) on testosterone production but not on LH/hCG binding. SCAIF decreased Leydig cell function, after a long term treatment (48 hrs), at different levels including LH/hCG binding. Whether this inhibitor(15) and SCAIF are different remains yet unknown.The decrease of forskolin-stimulated testosterone in SCAIF-treated Leydig cells (Table1) suggests a post-cAMP effect of the inhibitor(s). Interestingly, SCAIF was with no effect on the conversion of pregnenolone into testosterone, indicating an absence of effect on steroid hormone biosynthesis enzymes. Taken together, these findings suggest that SCAIF decreases hCG-stimulated testosterone secretion mainly at two level(s): LH/hCG binding and at a step(s) located somewhere between cAMP production and pregnenolone formation.

One major difficulty in studying an inhibiting activity is to rule out a toxic activity.The observed effect of SCAIF cannot be related to a toxic byproduct of the fractionation procedures for the following reasons: (i)cell viability quantificated by trypan blue exclusion was not affected by SCAIF, (ii)Leydig cell steroidogenic activity is completely recovered after the removal of SCAIF(unpublished data); (iii) the inhibiting activity of SCAIF on testosterone production is not observed in the

presence of exogenous pregnenolone (Table1) and (iv) SCAIF is hormono (FSH) suppressible (Fig.4).

FSH is one of the major hormones involved in the differentiation of Leydig cells during sexual maturation, and particularly at the onset of puberty (3). Biochemical studies have suggested that FSH "sensitizes" the response of Leydig cells to LH stimulation (3). Our data show,for the first time, that the suppression of a Sertoli cell inhibiting factor(s) of steroidogenic activity (SCAIF) could be one mechanism by which FSH "enhances" Leydig cell response to LH stimulation. However, this mechanism of action of FSH seems not to be unique, since, while FSH suppresses SCAIF, it induces the secretion by Sertoli cells of a factor(s) which stimulates testosterone secretion (5).

In summary, the presence of an inhibiting factor(s) of testicular steroidogenic responsiveness (to LH/hCG stimulation) from Sertoli cells supports the concept of paracrine (tubular) control of Leydig cells and the suppressibility of such inhibitor by FSH is a mechanism by which FSH "sensitizes" Leydig cells to LH stimulation. This is the first time that such a mechanism has been reported.

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