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Biological characteristics of a factor suppressing follicle stimulating hormone (inhibin) from ovine testes

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Abstract. Inhibin (follicle stimulating hormone suppressing factor) isolated from ovine testes has been characterized for its biological activity using a variety of tests. The bioassay used – inhibition of the human chorionic gonadotropin induced increment in the mouse uterine weight– demonstrates that there is a significant increment in specific activity (approx. 300-fold) with the progress of purification. Eventhough the final product has not been obtained in a homogenous state it has been possible to show that (*a*) [^{125}I]-labelled inhibin is preferentially taken up and retained by the pituitary, pretreatment of rats with testosterone facilitating this uptake; (*b*) it is able to suppress specifically the levels of follicle stimulating hormone in castrated as well as immature intact rats and (*c*) treatment of immature male rats with inhibin preparation for ten days results in impairment of testicular function as judged by ^{3}H-thymidine incorporation into testicular DNA and testicular hyaluronidase activity.

Keywords. Regulation of FSH; testicular function; gonadotropin.

Introduction

Attempts are continuing to be made in several laboratories to isolate the follicle stimulating hormone (FSH) suppressing factor (inhibin) in a homogenous state from the gonad or its secretions. Although partial purification of inhibin from ovine testes has been reported earlier both by Murthy *et al.* (1979) and Sheth *et al.* (1979) the type of molecule each of these groups have isolated appear to be distinctly different. While, we have reported that the FSH release suppression activity is associated in the testis with a protein fraction whose molecular weight is around 15,000 daltons, Sheth *et al.* (1979) noted that FSH suppressing activity is exhibited by a low molecular weight peptide of approximately 1500 daltons, which they have isolated. From the ram rete testicular fluid, reports on the isolation of both types of material exist (Baker *et al.,* 1976). The low molecular weight material in addition to suppressing FSH secretion in castrated rats (Moodbidri *et al.,* 1980) appears to inhibit binding of [^{125}I]-FSH to rat testicular and ovarian membrane preparations (Vijayalakshmi *et al.,* 1980). It was therefore considered essential before continuing with the isolation procedure to understand better the biological

Abbreviations used: FSH, Follicle stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone.

characteristics of the inhibin isolated by us from the ram testes. We wish to report here some of the results of our studies using the immature rat and mouse as animal models.

Materials and methods

Isolation of ovine testicular inhibin

Inhibin was isolated from ovine testes using conventional protein purification techniques, the details of which have been provided earlier (Murthy *et al.,* 1979).

The preparations P-1 to P-5 used here refer to the active fractions obtained by ammonium sulphate fractionation followed by chromatography on Sephadex G-75, DEAE, CM-cellulose and Sephadex G-50 respectively.

Bioassay of inhibin

Inhibin activity of the various fractions were determined using the mouse uterine weight assay described earlier by Ramasarma *et al.* (1979). This assay is based on the ability of inhibin, when given in two equal doses 1 h prior to human chorionic gonadotropin injection, to suppress the hCG-stimulated increase in uterine weight of 27 day old Swiss albino female mice. The usefulness and validity of this assay in quick screening of bioactivity of inhibin preparations has earlier been discussed (Ramasarma *et al.,* 1979).

Radioimmunoassay (RIA) of rat FSH and luteinizing hormone (LH)

These hormones were assayed using the rat FSH and LH RIA kits provided by NIAMDD, Bethesda, USA. The hormones were iodinated using carrier-free [^{125}I]-Na (Amersham, UK) by the chloramine-T method. The assay done according to Moudgal *et al.* (1979), consisted of incubating serum samples with the appropriate dilution of antiserum and the label at 37°C for 24 h, the antigen antibody complex being precipitated by the addition of goat antibody to rabbit gamma globulin and continuing incubation for an additional 12 h at 37°C. The coefficient of variation for intra and interassay were 3 and 9% respectively.

Radioiodination of inhibin and uptake studies with [^{125}I]-inhibin

The iodination of inhibin preparation (P-5) was carried out using the lactoperoxidase method (Moudgal *et al.,* 1979). The iodinated protein was separated from free iodine and lactoperoxidase by chromatography on Sephadex G-50 and hydroxylapatite column. Hydroxylapatite (column chromatography grade, BioRad, USA) packed in a 2 ml glass syringe was equilibrated with 0.005M phosphate buffer, pH 6.65, containing 0.1% bovine serum albumin (BSA). The absorbed inhibin was eluted with 0.1M phosphate buffer, pH 6.65 containing 0.1% BSA.

The [^{125}I]-Labelled inhibin was injected to 23 day old immature male albino rats (I.I.Sc. strain), by *i.v.* route (2×10^6 cpm [^{125}I]-inhibin/200 µl of phosphate buffered saline) and groups of rats autopsied at periods thereafter. The different tissues obtained at autopsy were carefully blotted, weighed to the nearest 0.2 mg and 50-70 mg portions of these transferred to tubes and monitored for radioactivity

in a Packard Autogamma spectrometer. In some cases, the tissue was homogenized with cold 10% TCA, and the radioactivity associated with the protein pellet determined.

Assessment of testicular function

Testicular function in immature male rats following chronic treatment (for 10 days starting from day 23) with inhibin was assessed using two biochemical parameters—*in vitro* ^{3}H-thymidine incorporation into testicular DNA and hyaluronidase activity—as indices of normal function. The methods used to determine the above are essentially similar to those described earlier for monkey testicular biopsy samples by Sheela Rani *et al.* (1978). For ^{3}H-thymidine incorporation studies, 30-50 mg of testicular tissue in triplicate was incubated in 1 ml of Krebs Ringer biocarbonate buffer, pH 7.4 containing 50 mM HEPES, 0.2% D-glucose and 1.0 µCi ^{3}H-thymidine (Radiochemical Center, Amersham, UK, specific activity 26 Ci/mmol). The tissue was incubated at 34°C for 4 h in a Dubnoff metabolic shaker. At the end of the incubation period 50 µg of non-radioactive thymidine (Sigma Chemical Co., USA) was added and the samples were processed for DNA by precipitating the macromolecules with 5% cold perchloric acid followed by hydrolysis with 10% perchloric acid at 90°C for 20 min. Aliquots of the filtrate were used for measurement of radioactivity in a Packard Tricarb Spectrometer as well as determination of DNA according to Burton's modified procedure (Giles and Myers, 1965). The results are expressed as cpm ^{3}H-thymidine incorporated/100 µg DNA per 4 h.

The hyaluronidase activity of the testes of rats was determined essentially according to the procedure of Males and Turkington (1970) as standardized in this laboratory (Sheela Rani *et al.,* 1978). The protein estimation was done according to the method of Lowry *et al.* (1951). The results are expressed as µmol of N-acetyl glucosamine (NAGA) released per mg protein per h.

All chemicals/biochemicals used in this study, unless otherwise mentioned, were of analytical grade and were purchased with Sigma Chemical Co., St. Louis, Missouri, USA or Sarabhai Chemical Co., India.

Statistical analysis

The results of this study were analyzed using the students '*t*' test.

Results

Characteristics of the inhibin preparation used

The final product (P-5) of purified inhibin on polyacrylamide disc gel electrophoresis (both at pH 8.2 and 4.5) showed one major and one/two minor bands (data not shown). The biactivity of the active fractions increased significantly with fractionation (figure 1). The amount needed to bring about 50% inhibition in uterine weight was reduced from an initial 7.7 mg (for P-l) to .019 mg of the P-5 fraction indicating that approximately 300-fold purification was achieved by this method.

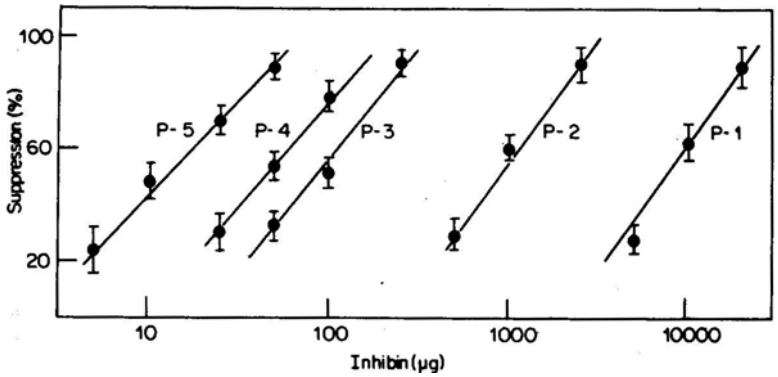

Figure 1. Dose response curves for inhibin activity as tested in the mouse uterine weight assay.

The different inhibin fractions (P1-P5) were tested at 3 dose levels and the results are expressed in terms of per cent suppression in uterine weight. This was calculated as follows:

$$100 \times \frac{(100\text{-uterine wt. of hCG + inhibin group}) - (\text{uterine wt. of control group})}{(\text{Uterine wt. of hCG group}) - (\text{uterine weight of control})}$$

Relative ability of different tissues of the rat to concentrate [^{125}I]-labelled inhibin

Groups of immature male rats, 23 days of age, were injected [^{125}I]-inhibin (P-5: 2×10^6 cpm/200 µl) *i.v.* and tissues obtained following sacrifice at ½ 1,2,4 and 8 h were analyzed for radioactivity. Of the various tissues analyzed, the pituitary appeared to retain maximal radioactivity for the longest period of time (figure 2).

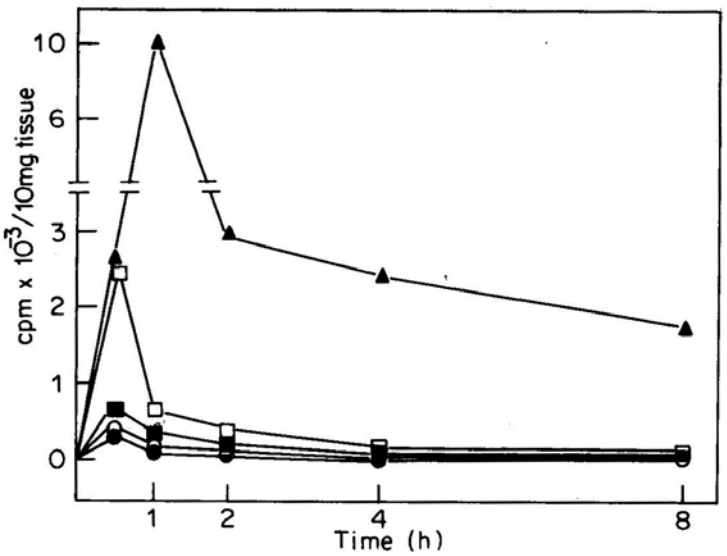

Figure 2. Distribution of [^{125}I]-inhibin into different tissues as a function of time, following its injection in immature rats.

23 day old immature male rats were injected 2×10^6 cpm[^{125}]-inhibin (P-5 by *i.v.* route). Animals were autopsied at various times thereafter and bound radioactivity (TCA precipitable fraction) in different tissues were monitored, *n* of each group = 4.

▲-Pituitary .□-Kidney. ■ -Adrenal. O— Liver and ●-Lung.

The radioactivity associated with the pituitary was precipitable with 10% TCA indicating that radiolabeled inhibin and not free iodine was being monitored. The ability of the pituitary to concentrate [^{125}I]-inhibin could be increased 3-fold by pretreating immature fats for 3 days with 1 mg testosterone per day (table 1).

Table 1. Effect of treating immature rats with testosterone on the uptake of [^{125}I]–labelled inhibin by the pituitary.

Treatment	cpm/10 mg tissue mean ± S.D.
Control	982±401
Testosterone	2917±914*

Testosterone propionate (1 mg/day) was injected s.c. to 23 day old immature intact male rats for 3 days in groundnut oil, controls being given vehicle only. 100,000 cpm of freshly labelled inhibin (P-5) in 200 µl of saline was injected *i.v.,* on day 26 of age and animals were sacrificed 1 h later, *n* of each group was 5.

★ Significantly different from the control P < 0.01.

Relative ability of testosterone and inhibin to suppress FSH levels of castrate rats

Within one week of castration, serum FSH levels of adult male rats were known to be significantly increased. While injection of 2 mg of testosterone per day for 3 days starting from day 7 of castration was unable to reduce the increased level of FSH of castrate rats, injection of 2 mg of inhibin preparation (P-3) for the same 3 day period did bring about a 20% reduction in serum FSH levels. The ability of inhibin to suppress serum FSH level, however, was further increased (by 43%) if administered along with testosterone (table 2).

Table 2. Ability of inhibin and testosterone to reduce serum FSH level in long term castrate rats.

Treatment	FSH* (µg/ml) mean±SD	Suppression (%)
Untreated controls	4.13±0.85	–
Testosterone	4.26±0.57	–
Inhibin	3.28±0.18	21
Testosterone + inhibin	2.35±0.09†	43

Adult male rats were castrated 7 days prior to start of therapy.

Inhibin (fraction P-3) and testosterone were injected, each at a dose 2 mg/day in two equal doses in the morning and evening for 3 days. Rats were sacrificed 16h after the last injection, *n* of each group = 5.

★ Expressed as rFSH-RPI NIH standard.
† Significantly different from the appropriate control P<0.001.

Effect of long term administration of inhibin on testicular function of immature rats

Immature male rats, 23 days of age, were administered s.c. inhibin (preparation P-3) at 1 mg/day for 10 days. The rats were autopsied 16 h after the last injection. The testis and accessory sex glands dissected and weighed to the nearest 0.2 mg showed no significant change in their weights (table 3). Analysis of serum for LH and FSH concentrations showed that inhibin treatment did bring about 33% reduction in FSH levels while LH levels remained unchanged (table 4). The two biochemical parameters used to assess testicular function–^{3}H-thymidine incorporation into testicular DNA and testicular hyaluronidase activity–showed significant reduction (by 25%) in their activities (table 5).

Table 3. Effect of long term administration of inhibin to immature male rats on the weight of testis and accessory sex glands.

Tissue	Weight of organs in mg (mean ±SD)	
	Control	Inhibin treated
Testis	802±39.9	721±23.1
Epididymis	88.9±6.89	103±8.65
Seminal vesicle	13.8±1.16	14.4±3.68
Prostate	33.0±3.71	40.2±5.25

Inhibin (fraction P-3,1 mg/day) was administered to immature male rats for 10 days starting from day 23 of age. Each tissue was excised, blotted and weighed to the nearest of 0.2 mg. The rats were autopsied 16 h after the last injection, *n* of each group = 6.

Differences not significant by *'t'* test.

Table 4. Effect of long term administration of inhibition to immature male rats on serum FSH and LH.

Treatment	FSH (μg/ml) mean±SD	LH (ng/ml) mean±SD
Control	2.2±0.28	69± 4.1
Inhibin	1.4±0.16*	60±11.9
Per cent change over control	33	—

For treatment schedule and duration of treatment see footnote to table 3. The FSH and LH are expressed in terms of rFSH and rLH NIAMDD standards.

n of each group = 6.

★ Significantly different from the control P = <0.01.

Table 5. Effect of long term administration of inhibin to immature male rats on testicular hyaluronidase activity and *in vitro* ^{3}H-thymidine incorporation into testicular DNA.

Treatment group	Hyaluronidase activity*	^{3}H-Thymidine incorp. (cpm × 10^{4}/100 µg DNA/4 h)
Control	610±74.5	23.7±2.3
Inhibin	449±27.6**	17.5±0.6**
Per cent change over control	26	25

See foot note of table 3 for details of experimentation.

n of each group = 6.

The hyaluronidase activity was determined in triplicates using one testis of each animal. The other testis from individual animals of a group were pooled and the *in vitro* ^{3}H-thymidine incorporation studies into testicular DNA were carried out using triplicate samples.

★Expressed as µmol of N-acetyl glucosamine (NAGA) released per mg protein per h.

★★ Significantly different from control ($P<0.001$).

Discussion

An attempt has been made here to study the biological effects of the FSH suppressing factor (inhibin) isolated in this laboratory from the ovine testes. The inhibin preparations as tested in immature female mice are able to inhibit the hCG-induced increment in uterine weight in a dose-dependent manner and the specific activity, as to be expected, increased with the progress of fractionation. The validity of using the mouse bioassay for measuring FSH suppressing activity has earlier been provided. Although the method of purification adopted here has not provided an electrophoretically homogenous product (P-5 shows the presence of one major and 1 -2 minor bands in disc gel electrophoresis), it was still felt desirable to biologically characterize the product using different systems which could monitor inhibin activity before improving on the fractionation procedure.

One of the recommended procedures for assaying inhibin activity is the pituitary cell culture method (Hudson *et al.,* 1979). This as well as other studies (de Jong *et al.,* 1979; Scott *et al.,* 1980) suggest that the pituitary could be the principle target organ of inhibin action and Sairam *et al.* (1981) have also described a receptor assay for inhibin using crude pituitary homogenates. The present studies with [^{125}I]-labelled inhibin essentially show that the pituitary, relative to other organs monitored, preferentially retains labelled inhibin and of particular interest is the observation that pre-treatment of rats with testosterone facilitates this ability of the pituitary to retain the labelled compound. This essentially supports the suggestion that the pituitary has receptors to inhibin. Vanage *et al.* (1980) have reported using [^{125}I]-labelled inhibin preparation isolated from human seminal plasma, that it is preferentially taken up in addition to the pituitary, by the pineal of rats. The uptake appears to be a function of the age of the animal, maximal uptake being exhibited by 18 day old male rats. Recently, by implanting highly purified inhibin preparation in the third ventricle of unanaesthatized intact and castrated

rats, a specific reduction in plasma FSH levels has been observed, suggesting thereby that the hypothalamus could also be one of the sites of action of inhibin (Lumpkin *et al.,* 1981; Nagesh Babu *et al.,* 1981).

The castrate rat model used here permits one to assess the intrinsic ability of inhibin to suppress serum FSH levels. Though testosterone by itself is unable to suppress the FSH levels of long term castrate rats, essentially confirming the observation of Morger (1976) and juneja *et al.* (1976), it appears to potentiate the ability of inhibin to do so. The mechanism whereby this is effected is presently unknown. Our earlier studies in the long term castrate monkey had similarly shown that inhibin has intrinsic ability to suppress FSH levels (Murthy *et al.,* 1980).

It is now known that in immature rats, neutralization of serum FSH with a specific FSH antibody results in significant inhibition of the spermatogenic process (Raj and Dym, 1976; Shivashankar *et al.,* 1977); the adult male rat, however, does not appear to be dependent upon FSH to support spermatogenesis (Lostroh, 1976). Administration of inhibin to immature male rats for a ten day period starting from day 23 of age also seems to result in inhibition of testicular function as adjudged by its effect on ^{3}H-thymidine incorporation into testicular DNA and testicular hyaluronidase activity, used here as indices of active spermatogenesis and tubular functionality respectively. These parameters have been shown earlier by us to be effected following FSH antiserum treatment of adult male monkey (Sheela Rani *et al.,* 1978) as well as of immature male rats (Dhanasekaran and Moudgal, unpublished observations).

Although the reduction observed in both these parameters is of the order of only 25%, on the basis of specific activity (35% if expressed per pair of testes) it was statistically significant ($P<0.001$). It is possible that instead of using whole testicular tissue as in the present case, if enriched tubular preparation had been used the reduction observed may have been more marked. Franchimont (1982) interestingly, has also observed that following injection of an inhibin preparation isolated from rete testicular fluid of sheep to immature rats, a reduction in ^{3}H-thymidine incorporation into testicular DNA as well as uptake by type B spermatogonia occurs. Using bovine follicular fluid treated with charcoal as a source of FSH suppressing factor, in immature male rats, de Jong *et al.* (1978) observed a retardation in spermatogenesis and in particular, decrease in the number of pachytene spermatocytes. In the monkey, an inhibition in ^{3}H-thymidine incorporation into testicular DNA and testicular hyaluronidase activity have been correlated with a marked reduction in total number of sperms voided and a loss in fertility (Murthy *et al.,* 1980). Although, inhibin injection to immature rats resulted only in 33% reduction in serum FSH levels (in contrast to total neutralization of FSH achieved by giving antiserum), this appears to be adequate to effect testicular function showing that there is a critical need for FSH to support spermatogenesis during the immature state. The specificity of the effect of inhibin on FSH-dependent function is evident from the fact that the weights of the accessory sex glands, a sensitive index of testosterone (and as such LH) availability did not show any change.

It is thus possible to conclude from the present study that the procedure used by us to isolate inhibin does provide a product which exhibits properties expected of a

factor which specifically suppresses FSH secretion. Preliminary data indicates that this factor does not possess FSH binding inhibiting activity (Bhat and Moudgal, unpublished observations).

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