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Extra-gonadal secretion of an inhibin-like substance in the laboratory albino rat

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Abstract. Acetone powder preparations of ventral prostates of adult albino rat exhibit an inhibin-like activity. *In vitro* cultured ventral prostate explants secrete a substance possessing similar activity which appears to be independent of testicular androgens for its elaboration.

Keywords. Inhibin-like substance; ventral prostate acetone powder; coupled bioassay.

Introduction

Ever since the demonstration by Franchimont and coworkers (Franchimont, 1972; Franchimont *et al.,* 1975a) of the presence of inhibin-like activity in human and bovine seminal plasma, there has been lively interest in the question of its origin. Accepting the premise of Setchell and Sirinathsinghji (1972) as to the testicular origin of inhibin and its subsequent mode of entry into circulation, Franchimont *et al.* (1975b) suggested that seminal plasma inhibin represents that fraction of the testicular output, brought *via* the rete testis fluid to the head of the epididymis, that failed to be absorbed at this site for subsequent transfer to general circulation. Such a possibility was rendered unlikely when Le Lannou and Chambon (1977) showed that, in the laboratory rat, inhibin-like activity of seminiferous tubule origin is found in the caput but not the cauda epididymidis. The suspicion that seminal plasma inhibin may not be of testicular origin was further strengthened by the observation of Peek and Watkins (1979) that seminal plasma collected from bulls one year after vasectomy still had measurable inhibin activity. Around the same time, Vaze *et al.* (1979) reported that the human prostate has significant amounts of radioimmunoassayable inhibin. The availability, in this laboratory, of a procedure for differential diagnosis and assay of inhibin-like activity, based on the modification by Ramasharma *et al.* (1979) of the 'reversed Steelman-Pohley assay', stimulated us to investigate the possibility that extra-gonadal sites for synthesis of such material may exist in many mammalian species. The data pertaining to the laboratory rodent is presented in this report.

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Abbreviations used: hCG, Human chorionic gonadotropin; hMG, human menopausal gonadotropin; FSH, follicle stimulating hormone FSH-BI, FSH receptor-binding inhibitor.

Materials and methods

Tissue

The donors of ventral prostate were Holtzman-derived albino rats from our animal colony, maintained under standard conditions (Mohapatra, S. K., Duraiswami, S. and Chari, S., unpublished observations). They were used following surgical manipulation, by standard procedures, for either bilateral epididymectomy or bilateral orchidectomy. The prostates were recovered following a few weeks of recovery (as detailed later) and when assayed were compared with age-matched controls. At autopsy, the ventral prostate was removed by dissection, freed of adhering connective tissue, quickly weighed on a torsion balance and plunged into crushed ice. When enough material had been collected, the tissue was transferred to a mini-Waring blendor, pre-cooled to 4°C, for the preparation of steroid-free crude extract (Chari *et al.,* 1978). All subsequent Operations were carried out at 4°C.

Preparation of crude extract

To the tissue in the Waring blendor, nine volumes of ice-cold saline (0·9 %) was added. Following homogenization, the material was transferred to a suitable, pre-cooled container and absolute ethanol, chilled to – 20°C, was added to a concentration of 86 % (v/v). The sample was stored in the deep freeze (– 50°C) for 48 h to ensure completion of precipitation. The precipitate was removed by centrifugation at – 10°C and washed successively, twice with chilled absolute ethanol and thrice with acetone (– 20° C). The pellet obtained after the last centrifugation was lyophilized to yield the 'crude extract', also referred to as 'acetone powder'.

Assay of inhibin activity

The acetone powders were processed as follows for purposes of assay. Following homogenization of an appropriate amount of a sample in 0·9 % saline, the material was centrifuged and the supernatant recovered. Dosages were computed on the basis of the ratio: A_{280nm}/A_{260nm} (Layne, 1957).

The assay described by Ramasharma *et al.* (1979) based on the 'reversed Steelman–Pohley' design has been used throughout this study. For the sake of convenience and in order to distinguish from the related 'coupled assay' (see below), this assay will be referred to as the 'Moudgal assay'. The gonadotropin preparations used in the bioassays were:

hCG – Pregnesin/Serono: hMG – Pergonal/Serono

Since, on its own, the 'Moudgal assay' cannot discriminate between an agent (inhibin) acting at the level of the pituitary, to prevent follicle stimulating hormone (FSH) secretion, from another (FSH-BI) acting at the level of the ovary, to prevent FSH action on target cells, the 'coupled assay' developed in this laboratory (Mohapatra *et al.,* 1983) was used in the preliminary studies on rat ventral prostate, in order to establish that indeed inhibin-like activity was being investigated. This assay follows closely the design

of the 'Moudgal assay', but differs from it only to the extent that the uterine response is assessed not only in hCG-treated but also in hMG-treated animals. A preparation is considered to be inhibin if a dose-dependent inhibition of uterine response is seen in the hCG-treated but not in the hMG-treated animals. The rationale behind this assay has been described in detail elsewhere (Mohapatra, 1983).

Synthesis of inhibin by ventral prostrate explants in vitro

Ventral prostates from 2-month-old males were cultured according to the modified Trowell's procedure of Laznitski (1964) using Medium – 199 supplemented with horse serum (5 %) and fetal calf serum (10 %). The media and its constituents were purchased from Centron Research Laboratories (Bombay). The explants were maintained in culture (37°C in a B.O.D.) for a period of 6 days at the end of which they were examined histologically, after appropriate fixation and staining. The culture medium was renewed once at the end of 3 days. The spent media were appropriately pooled, lyophilized and checked for inhibin activity by the 'Moudgal assay', following reconstitution in saline.

Results

Inhibin activity of rat ventral prostate

Saline extracts of acetone powders from 90-day old males were assessed for biological activity using the 'coupled assay'. Figure 1 depicts the results in the form of a histogram. When administered to hCG-primed animals, a dose-dependent suppression of uterine weight gain was seen. In contrast, none of the administered doses had any effect on uterine weight gain in mice treated with hMG, thus clearly identifying the test material as inhibin-like in nature. Ventral prostate acetone powder of 45 day old males evoked a dose dependent suppression of uterine weight gain when administered to hCG-primed animals. Expressed as per cent inhibition over the hCG-controls, this turns out to be 10·8, 29·4 and 45·7 respectively for the test doses of 40, 80 and 120 μg. It is further apparent that by the time of completion of the first cycle of spermatogenesis by the rats, which in our colony occurs on the 43rd day post-natum, there is definite evidence for the presence of inhibin in the ventral prostate.

Inhibin activity as a function of age

Saline extracts prepared from acetone powders of ventral prostates from animals aged 21, 30, 37, 45, 60 and 90 days were tested for activity using the 'Moudgal assay'. The data are presented as per cent inhibition of the hCG-induced response relative to the dose (figure 2) and suggest that inhibin activity in the ventral prostate is ontogeny-related. From low levels at weaning (21 days), the activity increases gradually. Adult levels are reached coincidental with the completion of the first cycle of spermatogenesis. For at least the period investigated, *viz.* 90 days, significant activity persists.

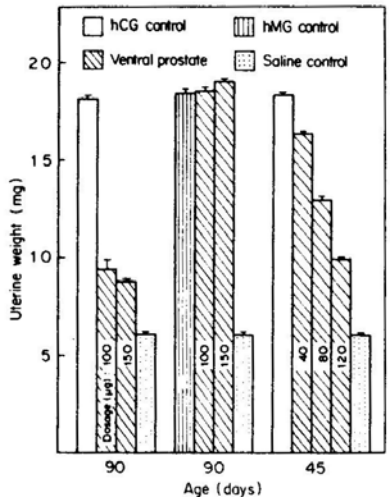

Figure 1. Inhibin activity of rat ventral prostate. The bar chart represents the mean ± S.E.M, n = 4. Briefly the assay involves injection of indicated dose of test material, intraperitoneally, into 24 ± 1 day old Swiss albino mice (12–12·5 g body wt.) in two equal volumes at 9 a.m. and 6 p.m. followed by injection of either 40 I.U. of hCG or 5 I.U. of hMG subcutaneously at 10 a.m. and 7 p.m. also in two equal volumes. The mice were autopsied 15 h after the last injection. The uteri were removed, freed from adhering fat and weighed to the nearest 0.2 mg. Saline extract of ventral prostate acetone powder inhibited the hCG response significantly (F-test, P <0·001). At the levels tested (100 μg and 150 μg) no dose dependent response was observed (P ~ 0·l). However, dosages 40 μg, 80 μg, and 120 μg evoked significant dose dependent suppression of the uterine wt. gain in hCG-primed animals (P < 0·001).

The saline extract of ventral prostate acetone powder did not inhibit the uterine response in hMG treated animals (P > 0·75).

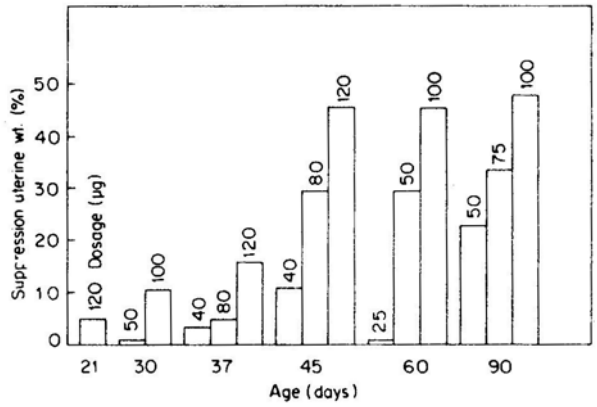

Figure 2. Inhibin-like activity of ventral prostate as a function of age. Saline extracts of acetone powders prepared from ventral prostates of rats of specified age were assayed. The results are expressed as per cent suppression of uterine weight:

$$100 \times \frac{\text{Uterine wt of hCG group} - \text{Uterine wt of inhibin group}}{\text{Uterine wt of hCG group}}$$

Source of the prostatic inhibin

Two-month old rats were subjected to bilateral epididymectomy. Four weeks later, ventral prostates from these animals, together with those from age-matched controls, were processed and assayed. It is evident (figure 3A) that surgical removal of the epididymis has no effect on prostatic inhibin activity, thus eliminating testis as the source.

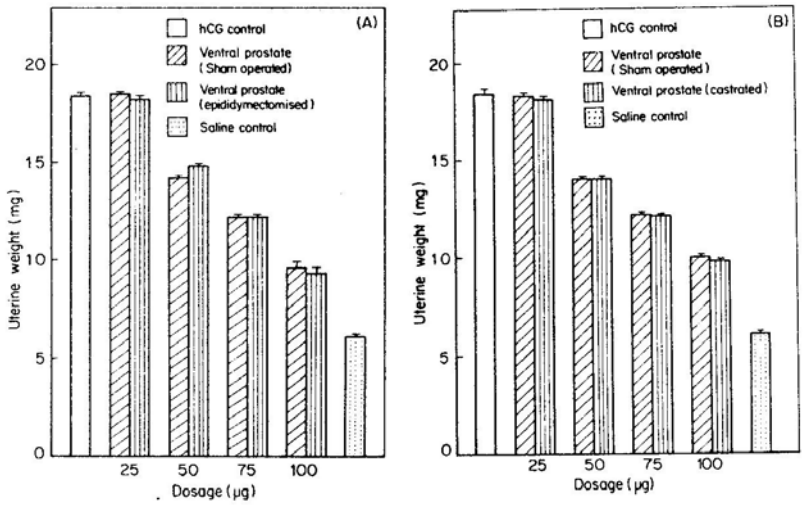

Figure 3. A. Effect of epididymectomy on production of rat prostatic inhibin.
The bar chart represents the mean ± S.E.M., $n = 4$. Epididymectomy has no effect on prostatic inhibin activity compared to sham-operated controls (F-test, $P > 0{\cdot}75$). A dose dependent suppression of uterine wt was observed compared to hCG control ($P < 0{\cdot}001$) from the ventral prostate extracts of both sham-operated and epididymectomised rats.
B. Effect of castration on production of rat prostatic inhibin.
The bar chart represents the mean ± S.E.M., $n = 4$. Castration does not affect the prostatic inhibin activity compared to sham-operated controls (F-test, $P < 0{\cdot}25$). A dose dependent effect was observed ($P < 0{\cdot}001$).

Is prostatic inhibin production androgen-dependent?

Having excluded the testis as the source of prostatic inhibin, it was of interest to inquire whether this androgen-dependent organ requires this hormone for the elaboration of inhibin also. To this end, 45-day-old rats were bilaterally orchidectomized and their ventral prostates recovered 6 weeks later and processed for assay, together with those from age-matched controls. The data summarized in figure 3B demonstrate that castration does not in anyway influence inhibin production by the ventral prostate.

Ability of the rat ventral prostate to synthesize inhibin in vitro

To avoid possible systemic effects and, specifically, to eliminate the possible contribution of adrenal androgens to prostatic inhibin synthesis in castrates, explants of

ventral prostate were studied *in vitro*. The results are depicted in figure 4. The stimulatory effect, on the mouse uterus, of the reconstituted control medium is noteworthy, though not surprising in light of the presence of horse and fetal calf serum. The inhibition of uterine weight gain seen in animals treated with reconstituted spent medium must therefore be an under estimate of the inhibin activity secreted by the explant into the culture medium. True to expectations, histological examination showed that the secretory epithelial cells lining the alveoli of the exocrine gland had indeed involuted, (to be reported elsewhere).

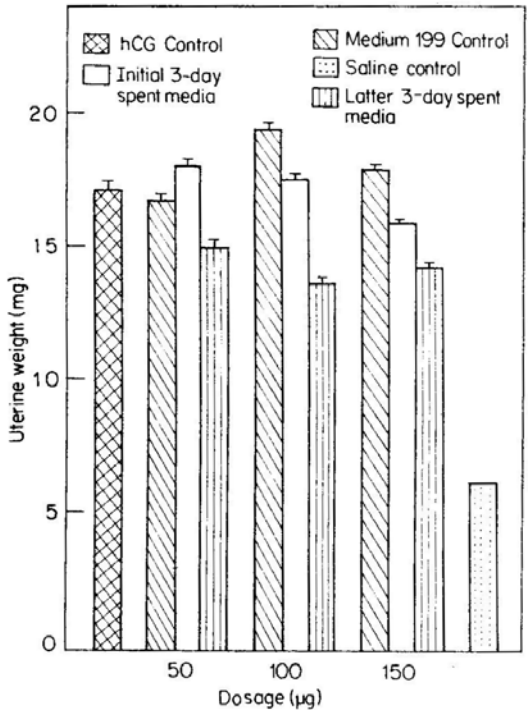

Figure 4. Secretion of inhibin-like substance into the spent media by ventral prostate explants *in vitro*.

The bar chart represents the mean ± S.E.M., $n = 4$.

Medium 199 control refers to the group of animals that received Medium 199, lyophilized and reconstituted, as in the case of the spent media, *in lieu* of a sourse of inhibin.

The initial 3-day and the latter 3-day spent medium are significantly different from the control medium (F-test, $P < 0{\cdot}001$).

The latter 3-day spent medium was significantly more potent in inhibiting the uterine wt. in hCG-primed animals than the initial 3-day spent media ($P < 0{\cdot}001$). The control medium is significantly uterotropic ($P < 0{\cdot}001$) over hCG control.

Discussion

On the identity of prostatic inhibin

The currently accepted definition of inhibin is that of a water-soluble feedback signal of gonadal origin, acting to suppress pituitary FSH output. Consequently, assessment, *in*

vivo or *in vitro,* of the inhibitory effect of the test substance on pituitary FSH secretion has been the basis for the various bioradioimmunoassays currently in vogue (Baker *et al.,*1981, for a review). It was felt, however, that a simple bioassay which is specific and sufficiently sensitive for use with tissue extracts rather than serum samples and which also has a short 'turn -around' time between assays would be essential for detecting and measuring inhibin produced by tissues *in vivo* and *in vitro.* Based on the experience of atleast three laboratories (Ramasharma *et al.,* 1979; Sairam *et al.,* 1981; Mohapatra S. K., Duraiswami, S., Chari, S., Daume, E. and Sturm, G., unpublished observations), the 'Moudgal assay' appears to meet such a requirement. Some reservation has been expressed by some workers (see for example, Grady *et al.,* 1982) as to its specificity, since the assay end point is a 'second order' effect and hence is not directly related to FSH output by the test animals. The argument is that the dose-dependent suppression of uterine weight gain can be mimicked by any factor that, unlike inhibin, acts directly at the level of the ovary to prevent stimulation of estradiol production by gonadotropin.

The validity of the above argument became evident to us in our attempts to isolate what were presumed to be isohormones of inhibin from bull seminal plasma (Mohapatra, S. K., Duraiswami, S., Chari, S., Daume, E. and Sturm, G., unpublished observations). The two peptides isolated, *viz.* α and β, were equipotent in their ability to suppress uterine weight gain in the 'Moudgal assay'. However, α alone could suppress serum FSH levels when administered to acutely castrate adult male rats. When these two peptides were examined by the 'coupled assay' design, β was found to suppress uterine weight gain not only in the hCG-treated, but also in the hMG-treated mice. In contrast, α effected such a response only in animals exposed to the hCG regimen.

The justification for the 'coupled assay' design is derived from the early observations of Lamond and Emmons (1959). According to these authors, while the influence of hCG on the ovary of the immature mouse is mediated by the animal's own pituitary, *via* release of endogenous FSH, hMG acts directly to stimulate the ovary. Inhibition of hMG-stimulated uterine weight gain would be characteristic for a factor acting at the level of the ovary. The 'coupled assay', then, depending on hMG-treated *vis-a-vis* hCG-treated mice, enables distinguishing between inhibin-like extracts acting to suppress pituitary FSH output and those acting to prevent ovarian response to FSH. A substance belonging to the latter category would be expected to suppress uterine weight gain in animals treated with either hCG or hMG, whereas inhibin-like material would elicit a response only in those mice exposed to hCG. A differential diagnosis is thus possible and hence a definitive identification of inhibin.

In light of the above considerations, it is justifiable to conclude that the biological activity exhibited by saline extracts of rat ventral prostate (figure 1) is inhibin.

Two observations in this study appear worthy of further comment. The first is that what could be presumed to be adult levels of inhibin activity, in the ventral prostate, are attained coincident with the completion of the first cycle of spermatogenesis. The significance of this relationship is rather obscure at this stage, in as much as it is difficult to visualize 'prostatic inhibin' entering circulation and acting as a feedback signal.

Secondly, the fact that elaboration of this factor by the ventral prostate is not androgen dependent is in contrast to the otherwise complete androgen-dependence of this organ. This immediately raises the question, for which there is no ready answer, of

the cell type responsible for this particular activity.

A more fundamental question is the biological significance of the ability of the ventral prostate to elaborate inhibin-like activity. While this may serve to explain the observations, in the bull, of Peek and Watkins (1979), its role remains a conundrum, especially when considered in light of the additional observation, to be presented elsewhere, that the seminal vesicles also are capable of androgen-independent elaboration of this factor.

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