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A Rapid Bioassay for Measuring Inhibin Activity

K. RAMASHARMA, H. M. SHASHIDARA MURTHY and N. R. MOUDGAL

Laboratory of Endocrine Biochemistry,
Department of Biochemistry,
Indian Institute of Science,
Bangalore 560 012, India

ABSTRACT

A sensitive and rapid bioassay of inhibin is described. Swiss albino female mice, 27 days old, weighing 25–30 g, were primed with 20 IU hCG, given in 2 equal doses, administered at 0900 h and 1800 h. An increment of 200% in uterine weight could be observed when the animals were autopsied the next day at 1000 h. The validity of the endpoint chosen, as a function dependent on FSH, was shown by the fact that neutralization of endogenous FSH by FSH antiserum led to an inhibition of uterine weight increase. Further injection of the inhibin material caused an actual reduction in the heightened levels of FSH (ng/ml) brought about by hCG: Control, 207 ± 13.6; hCG, 365 ± 28.8 and hCG + inhibin, below the detection level of radioimmunoassay.

Inhibin preparation from ovine testicular extract when tested at 3 dose levels showed a dose dependent and significant suppression in uterine weight increase. The assay was statistically valid $\epsilon = 2.5$; slope = 23.9; $\lambda = 0.104$ and intra- and interassay variation = 8.3% and 11.7%, respectively. Since the mouse uterine weight assay is specific, has greater sensitivity and is uniformly reproducible, it is suggested that this assay procedure is ideally suited to assess the activity of different inhibin test preparations as well as to follow the purification of inhibin activity from crude material.

INTRODUCTION

Assaying the biological activity of inhibin-like material has posed problems for the investigators in this area for quite some time. Some of the assays proposed though specific and sensitive have been found to be cumbersome and time consuming for routine usage (Baker et al., 1976; Nandini et al., 1976; and Eddie et al., 1977).

Recently, Chari et al. (1976) reported an assay procedure which is dependent on the ability of inhibin preparations to suppress the ovarian weight increase seen in immature rats given 50 IU hCG. Being essentially a 24 h assay, this procedure has proved attractive. The investigators, however, while describing the method have not provided adequate reasons for assuming that the reduction in ovarian weight seen is due to suppression in FSH levels consequent to inhibin administration.

The assay procedure we report here employs an essentially similar model system, but uses the immature mouse as the experimental animal and the inhibition of uterine weight increment as an index of inhibin activity. This procedure is very specific as well as more sensitive compared to the rat ovarian weight method. The validity of using this endpoint for assaying inhibin activity has also been adequately substantiated.

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MATERIALS AND METHODS

General Methodology

Swiss albino female mice, 27 days of age, weighing 25–30 g bred in the Central Animal Facility of this Institute were used. The animals were housed under a light:dark schedule of 14 h:10 h with pelleted feed (Hindustan Lever, Bombay) and water provided *ad libitum*. Wistar strain of rats 27 days of age, weighing approximately 40 g, bred in this Institute, were also used.

A highly purified preparation of hCG (gift of Dr. Canfield) was used for priming the animals. The antiserum to ovine FSH used was raised in monkeys and characterized for specificity according to methods described earlier (Sheela Rani and Moudgal, 1977). FSH in the mouse serum was assayed using the NIAMDD rat FSH radioimmunoassay (RIA) kit. The validity of using this assay system for measuring mouse serum FSH has been established by Murr et al. (1973).

Preparation of Ovine Testicular Inhibin Material

The method used to obtain a crude extract was similar to that described by us earlier (Nandini et al., 1976). Some of the fractions used for testing the

activity were obtained after further processing of the crude extract. The details of fractionation have been described elsewhere (Shashidhara Murthy et al., 1979). The preparations tested were crude ammonium sulphate fraction (P1), a fraction obtained after chromatography on Sephadex G-75 column (P2) and that obtained by negative absorption on DEAE cellulose (P3).

Assay Protocol

The test material, dissolved in 0.2% gelatin, was injected s.c. in 2 equal doses to 27-day-old, immature mice or rats at 0800 h and 1700 h. The hCG also dissolved in 0.2% gelatin was administered s.c. in 2 equal doses at 0900 h and 1800 h of the same day. The animals were autopsied at 1000 h of the next day and the ovaries or the uteri removed, trimmed free of fat and weighed on a torsion balance to the nearest 0.2 mg. Each mouse received a total dose of 20 IU hCG while the dose per rat was 60 IU.

Statistical Analysis

The results were analyzed for sensitivity, precision, parallelism and intra- and interassay variation according to the standard procedures of Cornfield (1970) and Finney (1964).

RESULTS

Response to hCG as a Function of Dose and Age of the Animal

Prior to testing the ability of inhibin to block the response to hCG and to standardize the age of the mice as well as the dose of hCG to be used, the following 2 pilot studies not involving the use of inhibin were undertaken. In the first study a group of mice of different ages ranging from 22–29 days were injected s.c. with 20 IU of hCG in 2 equal doses and the uterine weight increment 24 h after the first injection was recorded. Compared to the vehicle controls, hCG treatment resulted in significant increment when mice of 27 and 29 days of age were used (Fig. 1). Since Day 30 coincides with the day of vaginal opening, mice 27 days of age were used in all subsequent studies to avoid confusion and ambiguity. It is evident from Fig. 2 that the increment in uterine weight is directly proportional to the dose of hCG administered, 20 IU of hCG providing optimal response.

Validity of Using the Inhibition in Uterine Weight Increase as an Endpoint of Inhibin Activity

Two experiments were designed to ascertain the mechanism by which hCG induces an increase in uterine weight. In the first experiment, simultaneous injection of FSH antiserum and hCG at 2 different s.c. sites led to an inhibition in uterine weight increase, suggesting that for expression of hCG activity, endogenous

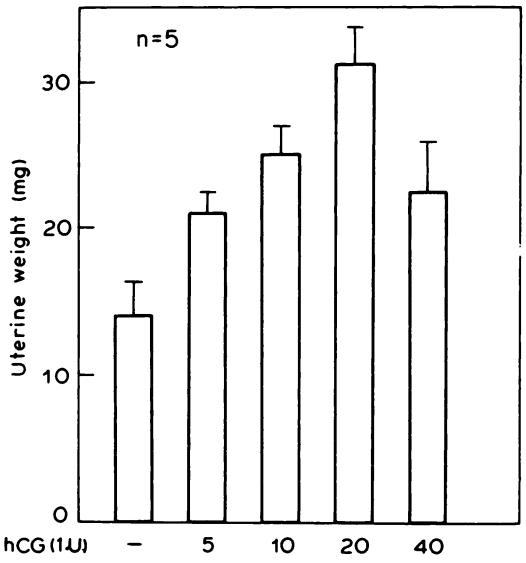

FIG. 1. Age dependent response to hCG. Immature mice 22–29 days of age were primed with 20 IU hCG given in 2 equal doses. The animals were autopsied 16 h after the last injection of hCG and uterine weights (mg mean ± SD) determined. (See text for further details.)

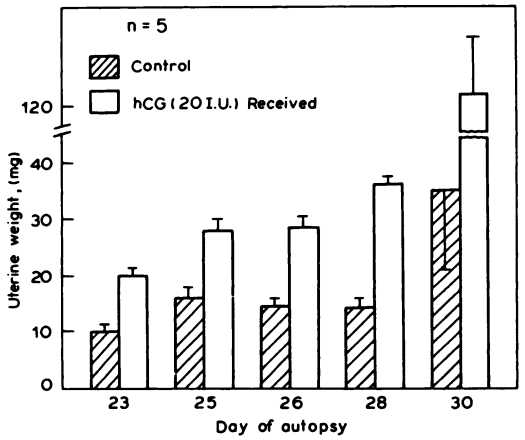

FIG. 2. Dose dependent response to hCG using mouse uterine weight (mg mean ± SD) as an endpoint. Immature 27-day-old mice were used. The animals were autopsied 16 h after the last injection of hCG which was administered in 2 equal doses. (See text for further details.)

FSH is needed (Fig. 3). The FSH antiserum used has been shown earlier by Jagannadha Rao and Moudgal (1970) to neutralize mouse FSH, but not to cross react with hCG.

In the second experiment, measurement of FSH at stipulated time intervals after hCG injection showed that within 1 h there was a significant increment in endogenous FSH level (Fig. 4). Injection of inhibin preparation 1 h prior to injection of hCG resulted in abolition of this increase (Table 1).

Use of the Mouse Assay to Estimate the Relative Activity of 3 Inhibin Preparations

The credibility of the assay for estimating rapidly the relative activity of different fractions obtained during purification of inhibin was tested by determining the activity of 3 preparations P1, P2 and P3. It is evident from Fig. 5 that these preparations can bring about suppression of uterine weight increase in a dose dependent manner. With increased purification, the amount of material needed to bring about significant suppression is also reduced. The activity contained in as little as 50 μg of the purified preparation (P3) could be safely estimated. Assuming that the unit of activity is that amount needed to bring about 50% suppression in uterine weight, the preparation P3, per mg protein, has relatively 1400 x the activity of the crude or 15 units/mg protein.

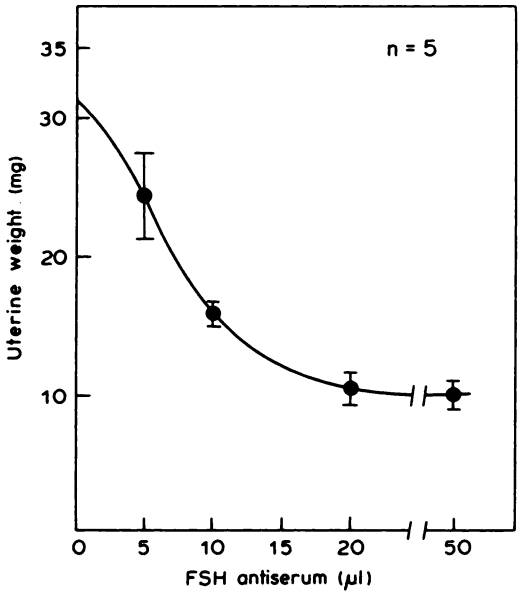

FIG. 3. Effect of antiserum to o-FSH injected 1 h prior to hCG injection on uterine weight (mg mean ± SD) increase in mice. Antiserum dose shown here is the total dose administered in 2 equal doses, 1 at 0800 h and the other at 1700 h. The remainder of the experimental protocol is the same as in Fig. 2.

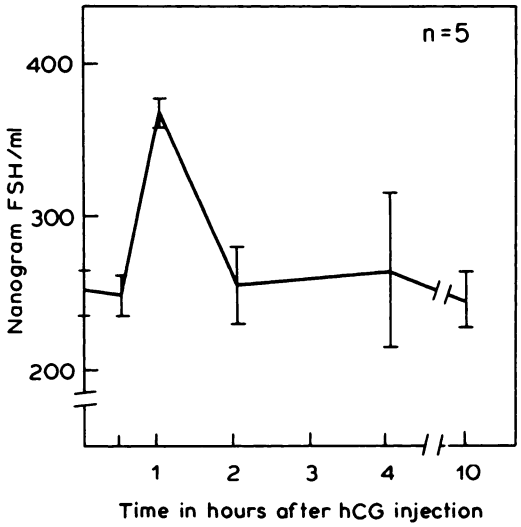

FIG. 4. Effect of hCG injection on serum FSH levels. The hCG was administered at Oh and serum samples collected at different time intervals thereafter. The FSH (ng/ml; mean ± SD) in the serum was assayed using the rat FSH RIA kit (NIAMDD, Bethesda MD) (See text for further details.)

Statistical Evaluation of the Assay

The validation of assay procedure has been checked using various statistical parameters (Table 2). The method is highly sensitive and gives reproducible results.

TABLE 1. Ability of inhibin to suppress the increment in FSH levels brought about by hCG.

Treatment	FSH (ng/ml)[a]
Control	207 ± 13.6
hCG	365 ± 28.8
Inhibin (1 mg P2) + hCG	N.D.[b]

[a]Mean ± SD.

[b]Not detectable; below the sensitivity of the radioimmunoassay using 100 μl of plasma. The sensitivity of the assay is 10 ng/tube. See text for details.

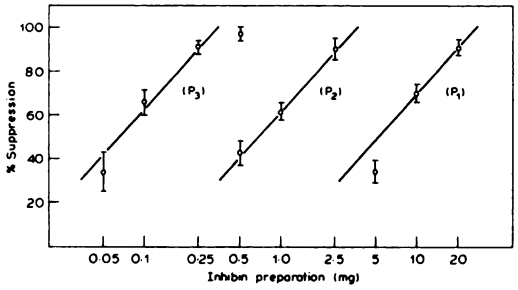

FIG. 5. Comparison of relative inhibin activity of 3 inhibin preparations (P1, P2 and P3). The results are expressed in terms of per cent suppression of increase in uterine weight.; 100 - [Uterine weight (hCG + inhibin) - uterine weight (control)] ÷ [Uterine weight (hCG) - uterine weight (control)] X 100.

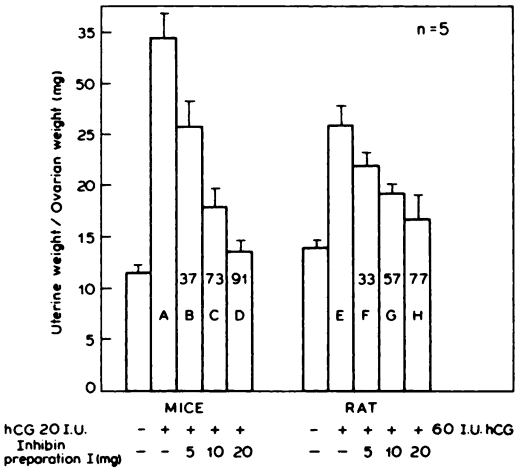

FIG. 6. Comparison of mouse uterine weight assay with the rat ovarian weight assay. Preparation P1 was tested at 3 dose levels in both the models. Note that the suppression in uterine weight was significantly different when compared with each dose level. P value for A:B, B:C and C:D was 0.001. The rat ovarian weight assay was relatively less sensitive with the P value for the different groups being E:F 0.001, F:G 0.01, G:H not significant and F:H 0.01. Numbers within the columns indicate per cent suppression.

Comparison of Rat Ovarian Weight Assay Method with Mouse Uterine Weight Assay Method

Fig. 6 shows that the mouse uterine weight assay method is much more sensitive than the rat ovarian weight assay, each dose of preparation (doubling dose) administered to the mice producing a response which is significantly different from the other. In the rat ovarian weight assay, the response becomes significant only when the dose is quadrupled.

DISCUSSION

The foregoing describes a simple bioassay procedure for estimating the activity of inhibin preparations. That the parameter used to assess inhibin activity, namely the suppression in uterine weight of mice treated with hCG and inhibin preparation, is a valid one has been demonstrated by using two different criteria.

TABLE 2. Statistical evaluation of the validity of mouse uterine weight assay.

Parameter	Value
Sensitivity	50 μg[a]
Intra-assay variation	8.3%
Interassay variation	11.7%
Sigma	2.5
Slope	23.9
(G) Departure from parallelism	0.610
(λ) Index of precision	0.104
(r) Regression coefficient	0.76

[a]Refers to the most highly purified preparation presently available to us.

Injection of hCG appears to result in an acute discharge of FSH which perhaps by stimulating follicular maturation and synthesis of estrogen causes an increment in uterine weight. The abolition of the hCG effect by simultaneous injection of FSH antiserum corroborates the above conclusion. Finally, injection of test inhibin material brings about abolition of the increment in FSH levels at 1 h after hCG injection which is reflected in a significant suppression in uterine weight at 24 h. The above provides the rationale for using suppression of uterine weight of mice treated with hCG as an index of inhibition of FSH secretion.

The inhibition of uterine weight increase observed is dose dependent with respect to the test material and the effective dose needed to bring about 50% suppression is significantly reduced, as is to be expected, with the progressive purification of the product. The dose response curves obtained with different preparations are parallel to each other which proves the validity of the assay (Table 2). The smallest dose of a highly purified preparation capable of giving significant suppression over the hCG control was 50 μg. We believe that this could be lowered with further purification. The values for precision of the assay, the intra- and inter-

assay variation etc., provided in Table 2, clearly show that all the indices obtained are within the range acceptable for a valid bioassay.

The superiority of the mouse uterine weight assay over the rat ovarian weight assay becomes evident when one observes that the difference between the dose levels (doubling dose) is significantly higher in the former. Inhibin preparations from three different laboratories have been tested by this method. All of them have shown a dose dependent response and it has been possible to obtain a relative activity index with reference to our preparation. This method is recommended as a good reliable procedure for following the purification of material having FSH suppressing activity. Even though the method is less sensitive when compared to the tissue culture assay procedure of Eddie et al. (1977), it is a fast, simple and reliable procedure which is not at all cumbersome. Finally, use of mice is much more economical in terms of animal cost and amount of hCG used.

ACKNOWLEDGMENTS

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RECOMMENDED REVIEWS

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