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Studies on Rat Ovarian Receptors for Lutropin (Luteinizing Hormone)

INTERACTION WITH β-SUBUNIT OF SHEEP LUTROPIN

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By using radioimmunoassay, the interaction of sheep lutropin (luteinizing hormone, LH) β-subunit with rat ovarian receptors was investigated. The binding of β-subunit was specific, although of much lower order than that of lutropin. Sheep lutropin β-subunit effectively inhibited the binding of human choriogonadotropin (chorionic gonadotropin, hCG) to the ovary, showing that both occupy the same sites. The binding of sheep lutropin β-subunit to ovary was not followed by any detectable increase in cyclic AMP. The ovarian response to lutropin in terms of cyclic AMP production was inhibited in the presence of free β-subunit. The α-subunit of lutropin, when used at concentrations where contamination with whole lutropin was negligible, enhanced the degree of binding of β-subunit; this did not lead to increased cyclic AMP in the tissue. Surprisingly, the binding of β-subunit *in vitro* was drastically decreased by the prior removal of all endogenous rat lutropin bound to receptors. The implications of these data are discussed in the light of the reported biological activity of the β-subunit.

The availability of the subunits of lutropin (luteinizing hormone, LH) in relatively pure form (Papkoff *et al.*, 1973) has permitted investigations of their biological activity. Although most of the reports appear to discount intrinsic biological activity in either of the subunits (Catt *et al.*, 1973; Lee & Ryan, 1973), some do claim appreciable activity for the free subunits. This includes the ability of the α-subunit to cause lipolysis (Gospodarowicz, 1971, 1973), the β-subunit to cause lactate production in pre-pubertal rat ovaries (Farmer *et al.*, 1973), to increase cyclic GMP in superovulated immature rat ovaries *in vivo* (Rao & Carman, 1973), to maintain elevated concentrations of plasma progesterone in these rats when given *in vivo* (Rao, 1973), to induce ovulation in hamsters (Yang *et al.*, 1972) and to maintain gestation in hypophysectomized rats (Yang *et al.*, 1973). In addition, antiserum to sheep lutropin β-subunit has been shown to terminate gestation in pregnant hamsters (Sairam *et al.*, 1974).

One of the initial events in the expression of hormonal activity is recognition and binding to receptors in the target tissue. Therefore the ability of the free β-subunit to bind to ovarian receptors was tested by using the new approach to receptor studies described in the preceding papers (Muralidhar & Moudgal, 1976*a*,*b*).

Materials and Methods

The sources of all chemicals, hormones and animals are as described by Muralidhar & Moudgal (1976*a*). In addition, sheep lutropin (NIH-LH-S19) was used for Expts. II and III described in Table 2. Sheep lutropin α-subunit (G3-213C) and β-subunit (G3-217P) were kind gifts of Professor H. Papkoff, Hormone Research Laboratories, San Francisco, CA, U.S.A.

The procedures for receptor-binding studies and cyclic AMP measurements were as described by Muralidhar & Moudgal (1976*b*). Tissue-bound sheep lutropin and β-subunit of lutropin were measured by a homologous radioimmunoassay for sheep lutropin. The procedure is same as that described for tissue-bound lutropin (Muralidhar & Moudgal, 1976*a*). Tissue-bound human choriogonadotropin (choriogonadotropin, hCG) was measured by a homologous radioimmunoassay for human lutropin by using the kit supplied by National Institute of Arthritis, Metabolic and Digestive Diseases (NIAMDD), National Institutes of Health (NIH), Bethesda, MD, U.S.A. Details of the assay are same as described for sheep lutropin (Muralidhar & Moudgal, 1976*a*) except that ^{125}I-labelled human lutropin and rabbit antiserum to human lutropin were used. Note that the cross-reaction of α-subunit with lutropin anti-

serum is less than 5%, whereas that of the β-subunit is more than 70% (Moudgal *et al.*, 1974). Therefore, the lutropin measured in α-subunit by the lutropin radioimmunoassay is due to contaminating lutropin and not to the α-subunit itself.

Results

Specificity of binding of β-subunit of lutropin

The binding of the β-subunit of lutropin to the ovary, like that of lutropin itself, withstood washing with buffer or normal rabbit serum, the binding increasing with increasing concentration of β-subunit in the medium. The tissue appeared to have a 6–8-fold greater capacity for binding lutropin than for β-subunit (Fig. 1). The β-subunit binds to ovaries from rats under different physiological states, the binding paralleling that of lutropin (Moudgal & Muralidhar, 1974). In addition, boiled ovarian tissue did not bind any significant amount of β-subunit when incubated under identical conditions as for the untreated ovary (K. Muralidhar & N. R. Moudgal, unpublished work; see also legend to Fig. 2).

State of binding of sheep lutropin, its β-subunit and human choriogonadotropin

Studies were performed to ascertain whether sheep lutropin β-subunit and human choriogonadotropin competed for the same sites on the ovary. Human choriogonadotropin is known to occupy binding sites for lutropin (Lee & Ryan, 1973). Although human choriogonadotropin can be distinguished from sheep lutropin β-subunit in a radioimmunoassay, the latter cannot be distinguished from sheep lutropin. Therefore human choriogonadotropin and sheep lutropin β-subunit were chosen for such competition studies. Flasks containing ovarian minces were incubated in duplicate with either sheep lutropin β-subunit or human choriogonadotropin and also with both the hormones together at the concentrations indicated in Table 1. The binding of sheep lutropin β-subunit and human choriogonadotropin was decreased significantly in the presence of the other (approx. 60% in each case), suggesting that the two hormones were competing for the same sites on the luteal tissue. It should be noted that human choriogonadotropin is bound much more than sheep lutropin β-subunit or even the lutropin itself (K. Muralidhar & N. R. Moudgal, unpublished work). Though the experiment was not done at saturating concentrations of sheep lutropin β-subunit or human choriogonadotropin, competition could still be observed. This suggested that when both the hormones were present together, their combined concentration was exceeding the saturation limit of either of the hormones for the ovarian receptor content.

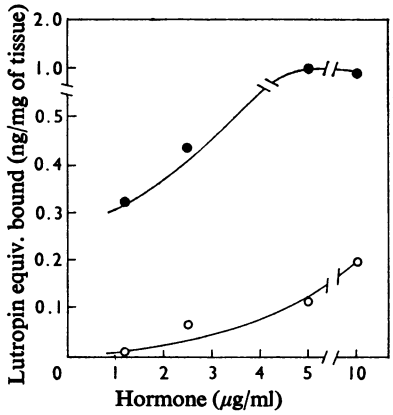

Fig. 1. *Comparison of the dose–response curves for binding of sheep lutropin* (●) *and its β-subunit* (○) *to minces of ovaries from superovulated immature rats*

Ovarian tissues from these rats were incubated with lutropin or β-subunit over a range of dose from 0 to 10 μg/ml for 1 h at 37°C. The hormone bound to the receptors was later measured by radioimmunoassay by procedures described by Muralidhar & Moudgal (1976*a*).

Effect of α-subunit on the binding of the lutropin β-subunit to ovarian receptors

We decided to investigate whether the presence of free lutropin α-subunit would in any way affect the binding of β-subunit. The tissue was incubated with a constant amount of β-subunit and increasing amounts of α-subunit. As shown in Fig. 2, the presence of α-subunit in the medium along with the free β-subunit resulted in an enhanced binding of β-subunit, or to be more precise by an increase in the amount of lutropin measurable in the tissue. The enhanced binding could be due to the contamination of α-subunit with lutropin, or the formation of whole lutropin, or true potentiation of binding of β-subunit to receptors by α-subunit.

In a separate experiment, the tissue exposed to α-subunit alone was assayed for any bound lutropin. As shown in Fig. 2, there was no significant amount of lutropin bound to tissue when α-subunit alone was used below a concentration of 5 μg/ml. The amount of contamination by lutropin in the α-subunit became significant at higher concentrations. Enhanced binding of β-subunit observed at low concentrations of α-subunit in the medium, was therefore considered significant, and due to potentiation of β-binding by α-subunit.

Response of rat luteal tissue to sheep lutropin β-subunit

When tested for ovarian response to the binding of β-subunit in terms of cyclic AMP concentration, the

Table 1. *Competition between sheep lutropin β-subunit and human choriogonadotropin for ovarian-receptor sites*

Minces of ovaries obtained from three rats were pooled before distribution into flasks, each flask containing 10–15mg of tissue and hormones as indicated in the Table. Lutropin β-subunit and human choriogonadotropin were added to the flasks at the same time. The tissue at the end of the incubation was analysed for receptor-bound human choriogonadotropin and β-subunit in two separate radioimmunoassay systems; the former was measured in a homologous human lutropin radioimmunoassay system and the latter in a homologous sheep lutropin radioimmunoassay system. Note that the human lutropin radioimmunoassay system measures sheep lutropin β-subunit also. Values are averages for duplicate flasks. Net binding refers to values obtained after subtracting the value for control flasks.

Hormone	Concentration (μg/ml)	Receptor-bound hormone (ng/mg of tissue)			
		Sheep lutropin radioimmunoassay	Net	Human lutropin radioimmunoassay	Net
None	—	0.02	0.00	0.85	0.00
Sheep lutropin β-subunit	2.5	0.70	0.68	1.58	0.73
Human choriogonadotropin	1.0	—	—	13.00	12.15
Sheep lutropin β-subunit+human choriogonadotropin	2.5+1.0	0.30	0.28	5.40	4.55

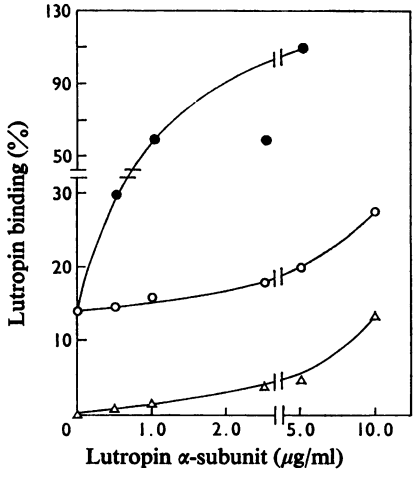

Fig. 2. *Interaction of lutropin, lutropin α-subunit and lutropin β-subunit with rat ovarian receptors*

Minced ovarian tissue pooled from a group of five rats was incubated in duplicate with either lutropin (2.5μg/ml), its α-subunit (△) at the concentrations specified in the Figure, or with a combination of the two subunits (●) (2.5μg of β-subunit/ml and increasing concentrations of α-subunit specified in the Figure). The tissue-bound hormone was measured by a radioimmunoassay for sheep lutropin. The degree of binding obtained when incubated with 2.5μg of lutropin was taken as 100% and the results of other binding assays were expressed as a percentage of this binding. The binding of β-subunit to boiled ovarian tissue was only 4%. ○, Calculated binding curve for β-subunit in the presence of increasing concentrations of α-subunit obtained by simple addition of the binding of the α- and β-subunits at each dose. Values are average of duplicate determinations.

binding of β-subunit to luteal receptors was not followed by any significant increase in the total cyclic AMP. Lutropin, under similar conditions could increase tissue cyclic AMP (Table 2).

The observation that the α-subunit enhanced the binding of β-subunit to ovarian receptors led us to investigate whether, associated with this, there was formation of whole lutropin. This should be reflected by an increased cyclic AMP concentration. As shown in Table 2, the addition of α-subunit at equivalent concentration, which enhanced binding of β-subunit to ovary, did not influence ovarian cyclic AMP concentration. Therefore the α-subunit at the concentration used did not contain lutropin as a contaminant sufficient either to give spurious lutropin values in the binding experiments or to increase cyclic AMP. Therefore the potentiation of β-subunit binding by α-subunit is not due to formation of whole lutropin. Further, it is known that recombination of α- and β-subunits to form lutropin takes 16h when dialysed together against slightly alkaline buffers (Papkoff *et al.*, 1973).

Effect of sheep lutropin β-subunit on ovarian response to lutropin

The absence of ovarian response to β-subunit in spite of the fact that it could compete with human choriogonadotropin for the receptor sites prompted us to test whether an excess of free β-subunit could inhibit the tissue response to lutropin in terms of cyclic AMP production. Tissue was initially exposed to free sheep lutropin β-subunit for 5min and then lutropin was added and the incubation continued for another 20min. Table 2 shows that an excess of free β-subunit could inhibit the ovarian response to lutropin, although the inhibition was variable depending on the model system used. In the three experiments shown in Table 2, the corpora lutea were of different age. The older the corpora lutea (Expt. I), the lower the response to lutropin and the greater the inhibition by β-subunit. However, in younger corpora lutea, which are more sensitive to lutropin (Muralidhar & Moudgal, 1976*b*), the inhibition by

Table 2. *Changes in ovarian cyclic AMP in response to lutropin and its subunits in vitro*

Corpora lutea from immature rats injected with pregnant-mare-serum gonadotropin and human choriogonadotropin were incubated in quadruplicate flasks without or with hormones as indicated in the Table. When present together, the subunits were added at the same time. A group of five rats were used for each experiment. The response in terms of cyclic AMP production was measured by procedures described in the Materials and Methods section. Values represent total cyclic AMP (tissue plus medium). Rats were used on days 5, 4 and 3 after human choriogonadotropin injection for Expts. I, II and III respectively. The concentrations of hormones used are; 2.5 μg of lutropin or α-subunit or β-subunit/ml for experiments I (groups I–IV), and 5.0 μg of the same/ml for experiments II and III. For group V, 5.0 μg of β-subunit/ml was used in experiment I and 10.0 μg of β-subunit/ml was used for experiments II and III. Values are means ± S.E.M. of quadruplicate flasks. Percentage inhibition in response was calculated from the formula:

$$100 - \left[\frac{\text{cyclic AMP in group V} - \text{group I}}{\text{cyclic AMP in group II} - \text{group I}} \times 100\right]$$

Group	Hormone	Expt. ...	Cyclic AMP (pmol/mg of tissue) I	II	III
I	None		3.5±0.21	4.1±0.95	2.3±0.59
II	Lutropin		6.3±0.45*	12.0±0.56*	9.8±2.4*
III	Lutropin β-subunit		3.0±0.23†	5.3±0.54†	4.5±0.5*
IV	Lutropin α-subunit+lutropin β-subunit		3.6±0.19†	3.9±1.12†	7.1±2.4†
V	Lutropin+lutropin β-subunit		4.0±0.51‡	6.1 ±0.39‡	5.7±1.7§
	Inhibition in response (%)		81	75	55

* Significantly different from group I, $P < 0.05$.
† Not significantly different from group I, $P > 0.1$.
‡ Significantly different from group II, $P < 0.05$.
§ Not significantly different from group II, $P > 0.1$.

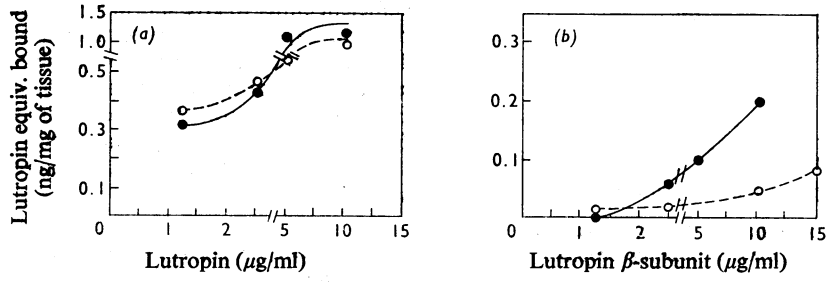

Fig. 3. *Effect of treatment of ovarian minces with lutropin antiserum on the subsequent binding of sheep lutropin and its β-subunit: comparison of dose–response curves for binding with lutropin (a) and β-subunit (b)*

○, Tissue treated with lutropin antiserum (diluted 1:1000) for 15 min at 4°C, subsequently washed free of antiserum and then incubated with lutropin or β-subunit respectively. ●, Tissue that was not exposed to lutropin antiserum before such incubation. The receptor-bound lutropin β-subunit and lutropin were measured by radioimmunoassay as described by Muralidhar & Moudgal (1976*a*). Values are average of duplicate flasks which contained tissue obtained from a group of five rats.

β-subunit was not so pronounced. In fact we observed that if immature rat ovaries (which are highly responsive to lutropin even at low concentration of lutropin) are used, the inhibition by free β-subunit is only 20% (K. Muralidhar & N. R. Moudgal, unpublished work). The free β-subunit appeared to have a slight contamination with lutropin, as shown in Expt. III, where young corpora lutea highly sensitive to lutropin and a large excess of β-subunit (10 μg) were used. However, in Expts. II and I, where older corpora lutea less sensitive to lutropin were used, the β-subunit had no activity even at 10 μg/ml whereas lutropin at 2.5 μg/ml increased tissue cyclic AMP.

Effect of washing ovarian tissue with lutropin antiserum in vitro on its ability to bind lutropin β-subunit

A comparison of the dose–response curves for binding of sheep lutropin and its β-subunit to ovarian

tissue washed with lutropin antiserum shows that whereas the binding of lutropin was not affected, that of the β-subunit was (Fig. 3). There was a 60–70% decrease in the amount bound to ovarian tissue when the tissue was washed with lutropin antiserum. Therefore the endogenous rat lutropin bound to ovarian tissue could be thought of as aiding the subsequent binding of sheep lutropin β-subunit in a co-operative fashion.

Discussion

Since the isolation of subunits of lutropin, conflicting reports have appeared on their biological activity. Most workers have interpreted the residual activity as being due to contamination with whole lutropin, suggesting the absence of intrinsic activity. Some reports, however, claim biological activity for the free β-subunit (Yang *et al.*, 1972, 1973; Rao, 1973). Recombination studies on hybrid molecules have also shown that the hormonal activity generated was always of the donor that provided the β-subunit. Expression of biological activity has to be naturally preceded by binding to tissue-specific receptors. By using ^{125}I-labelled β-subunit, no specific binding to target tissue has yet been reported. The present study, using unlabelled β-subunit of lutropin, has demonstrated the specific binding of β-subunit to ovarian receptors. The degree of binding of β-subunit was low compared with that of whole lutropin, and therefore it was not surprising that radioiodinated β-subunit lost this capacity, probably because of the presence of heavy iodine atoms. Therefore the equivalence of ^{125}I-labelled β-subunit to unlabelled β-subunit cannot be assumed and is perhaps difficult to assess in the absence of any known biological activity of β-subunit.

The β-subunit of lutropin, although it does not generate a response, appears to possess part of the active site(s) of lutropin. Its ability to inhibit the response to lutropin in terms of cyclic AMP production, and also its ability to inhibit the binding of human choriogonadotropin, would lead to such a conclusion. This could mean that in addition to free β-subunit, other factors (perhaps the α-subunit) contribute to it. Two possibilities exist regarding the make-up of the additional site(s). First, it could be that portions of α- and β-subunit, conformationally altered as a consequence of recombination, contribute to it. The second possibility is that the additional site is in the β-subunit itself, but is brought to the fore by the conformational alteration that it undergoes as a consequence of combination with α-subunit. Hence it seems that the 'active site' of lutropin has certain conformational rigidity, requiring the α- and β-subunits to be present as they are in whole lutropin.

The fact that the tissue washed with antiserum did not show appreciable binding of β-subunit, compared with the unwashed tissue, would mean that the endogenous lutropin bound to the ovary could in some way aid in the subsequent binding of β-subunit by providing a necessary 'threshold' and making the binding of β-subunit detectable by radioimmunoassay. The endogenous lutropin content appears to play a role similar to that of α-subunit in facilitating binding of β-subunit. Thus the binding of β-subunit is true and is due to β-subunit alone and not due to lutropin probably being present as a contamination.

It should be recalled that in all the cases where β-subunit has been claimed to possess activity, whole-animal models have been used. These systems were not free of endogenous lutropin. Therefore, to decide whether the β-subunit possesses intrinsic biological activity or not, it should be tested in a 'lutropin-free system'. However, the present study does not permit any definite conclusion regarding the role played by the endogenous lutropin in the interaction of β-subunit with lutropin receptors.

In conclusion, the β-subunit of sheep lutropin can be recognized by rat ovarian receptors to lutropin, although it has no biological activity as far as cyclic AMP production is concerned.

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