LH contamination may explain FSH effects on rat Leydig cells

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Summary. Treatment of immature, hypophysectomized male rats with 50 µg ovine FSH (NIH-FSH-S12) twice a day for 5 days stimulated the maximum quantity of 17β-hydroxyandrogen produced by isolated Leydig cells in response to hCG. Pretreatment of the FSH preparation with an LH antiserum in one study markedly reduced and in another study completely abolished this stimulatory effect of FSH, but only slightly impaired the capacity of the hormone to stimulate the Sertoli cell in vivo (epididymal androgen-binding protein). Administration of another highly potent FSH preparation (LER-1881) had no discernible effects on the dose–response characteristics of the Leydig cells but was superior to the NIH-FSH-S12 in its capacity for stimulating the Sertoli cell. When all hormone preparations were tested for their ability to stimulate steroid secretion from normal Leydig cells in vitro, a close correlation was obtained between their Leydig cell-stimulating activity (a measure of LH contamination) and their capacity to alter Leydig cell responsiveness after in-vivo treatment. FSH treatment had no effects on specific LH binding per 10^6 Leydig cells. It is concluded that the stimulatory influence of FSH on rat Leydig cells may to some extent be a result of the LH contaminating the hormone preparation.

Introduction

During the past few years a growing number of investigators have suggested that FSH, in addition to its stimulatory influence on the seminiferous tubules (Sertoli cells) may also exert effects on Leydig cell steroidogenic function. This consideration has largely been prompted by the finding that FSH treatment of hypophysectomized rats results in an increase in the maximum quantity of LH-stimulated androgen secretion by Leydig cells, both in vivo (Odell & Swerdloff, 1975; Selin & Moger, 1977) and in vitro (Chen, Payne & Kelch, 1976; van Beurden, Roodnat, de Jong, Mulder & van der Molten, 1976; Chen, Shaw & Payne, 1977). Furthermore, FSH also appears to stimulate the number of LH receptors in testis tissue (Chen et al., 1977). However, interpretation of these apparent effects of FSH is made difficult in light of the fact that the Leydig cell does not possess FSH receptors (de Kretser, Catt & Paulsen, 1971) and in addition that all of the currently available FSH preparations are contaminated to various degrees with LH.

There are two major aims of the present study: (1) to confirm the stimulatory influence of FSH on Leydig cell function in the hypophysectomized immature rat, and (2) to determine whether this influence could be partly or wholly explained by contamination of the hormone preparation with small quantities of LH.

Materials and Methods

Animals and treatments

Immature male rats, hypophysectomized on Day 29 after birth, were purchased from Hormone Assay Laboratory, Chicago. Hormone treatments began immediately on the day of arrival (Day 31), 2 days after hypophysectomy.
Study 1. Hypophysectomized rats (50) were divided equally into 5 groups and treated with the following: Group A (controls), saline (9 g NaCl/l); Group B, 50 µg ovine FSH (oFSH, NIH-S12); Group C, 50 µg oFSH pretreated with LH antiserum; Group D, 1 µg ovine LH (oLH, NIH-S20); Group E, 1 µg oLH pretreated with LH antiserum. All preparations were injected subcutaneously in 0-1 ml volumes twice a day for 5 days. All the animals were killed 24 h after the last injection and the testes were removed for the preparation of enriched Leydig cell suspensions.

Study 2. Hypophysectomized rats (48) were divided equally into 4 groups and treated with the following: Group A (controls), saline; Group B, 50 µg oFSH (NIH-S12); Group C, 50 µg oFSH pretreated with LH antiserum; Group D, 1-6 µg purified oFSH (LER-1881). All preparations were administered as described in Study 1.

Treatment with LH antiserum

The LH antiserum was raised against oLH in rabbits and 50 µl of this serum neutralized 10 µg NIH-LH-S18. Details concerning the monospecificity of this antiserum are presented elsewhere (Madhwa Raj & Moudgal, 1970). Since most NIH-FSH preparations have LH contamination of <1% it was assumed that the 50 µg oFSH dose contained less than 0-5 µg contaminating LH. The freeze-dried oFSH (10 mg) was first dissolved in 500 µl phosphate-buffered saline (PBS), pH 7-4, and then divided into equal volumes. One half was incubated with 250 µl LH antiserum at 4°C for 3 h whilst the remaining half was incubated under identical conditions with the same volume of normal rabbit serum. After incubation, the LH–antibody complexes were precipitated by centrifugation (4000 g, 10 min) and the supernatants were then diluted with PBS containing 0-1% bovine serum albumin (BSA) to give a final concentration of 50 µg oFSH/0.1 ml. It was assumed that no loss of FSH had occurred during the incubation. In the case of the antibody-treated oLH, the volume of antiserum was adjusted to neutralize all of the LH activity in the preparation (1 ml antiserum incubated with 200 µg oLH in 0.1 ml).

Choice of hormone doses to be injected

Ovine FSH was administered at a dose level (50 µg/injection) which had earlier been shown to elicit a half-maximal response from the Sertoli cell (androgen-binding protein secretion) in vivo under similar conditions (Hansson et al., 1976b). It was assumed that this dose of FSH would contain less than 0-5 µg LH contamination (<1%). A slightly higher dose of oLH (1 µg/ injection) was administered for comparison. The purified oFSH preparation (LER-1881) has been shown to have a bioassay potency (Steelman–Pohley assay) approximately 30 times higher than that of the crude oFSH. The dose of this potent preparation was therefore adjusted so as to give effects in vivo comparable to those of 50 µg oFSH, i.e. 1-6 µg LER-1881/injection.

Preparation of enriched Leydig cell suspensions

Enriched Leydig cell suspensions were prepared as described previously (Purvis, Clausen & Hansson, 1978b; Purvis, Clausen, Brandtzaeg & Hansson, 1978a). The number of Leydig cells present in each incubation tube was calculated after a nucleated cell count and determination of the percentage of 3β-hydroxysteroid dehydrogenase (3β-HSD) positive cells (i.e. Leydig cells) present. The androgen response could thus be expressed per 10⁶ Leydig cells.

Binding of ¹²⁵I-labelled hLH to Leydig cells

Aliquots (200 µl) of the cell suspensions were incubated with 100 000 c.p.m. of iodinated human LH (¹²⁵I-labelled hLH: sp. act. 50 µCi/µg) with various quantities of unlabelled human
LH (LER 960) at 33°C for 2.5 h in a total volume of 350 μl. After this time, the cells were washed twice in 2 ml ice-cold phosphate-buffered saline, pH 7.4, containing 0.1% BSA, and centrifuged at 1500 g for 30 min. The pellets were counted in a Searle 1285 Gamma counter with 70% efficiency. Incubation of the tracer in the presence of 10 μg unlabelled LH (NIH-LH-S19) provided an assessment of non-specific binding. Calculation of the number of binding sites in the preparation was by Scatchard analysis (Scatchard, 1949) and the results were expressed as fmol LH bound at saturation per 10^6 Leydig (3β-HSD positive) cells. The hLH (15 000 i.u./mg determined by radioimmunoassay, (see Sand & Torjesen, 1973 for details)) was iodinated by using the sodium hypochlorite method (Redshaw & Lynch, 1974), and when 20 000 c.p.m. 125I-labelled hLH were exposed to an excess of testicular particles (27 000 g pellet of testis homogenate), approximately 60–70% of the tracer was specifically bound.

**In-vitro assay of Leydig cell-stimulating activity of the hormone preparations**

To assess the LH activity of the different hormone preparations an aliquot of the solutions to be injected was added in different dilutions to a Leydig cell suspension prepared from normal 30-day-old rats. The incubations were performed in triplicate at 34°C for 3 h in a shaking incubator in 95% O₂:5% CO₂. The quantity of androgen produced by the Leydig cells in response to the different preparations was determined as described below. On this occasion the number of Leydig cells present in each incubation tube was not determined and the response was simply expressed in terms of androgen produced per flask.

**Measurement of epididymal androgen-binding protein (ABP)**

The epididymides were removed from each animal, defatted, pooled and weighed. A cytosol was then prepared from an homogenate of the pooled organs and subjected to polyacrylamide gel electrophoresis in triplicate for the measurement of ABP. The details of this technique have been published elsewhere (Ritzén, French, Weddington, Nayfeh & Hansson, 1974; Purvis & Hansson, 1978).

**Assay of androgen**

The steroidogenic response was evaluated by measuring the production of 17β-hydroxyandrogen. A previous study (Purvis et al., 1978b) showed that the steroid secreted by Leydig cells from immature intact and short-term hypophysectomized male rats is predominantly 5α-androstane-3α,17β-diol and not testosterone. The measurement of 17β-hydroxyandrogens therefore involved the determination not only of testosterone but also the 5α-reduced androgens, 5α-androstane-3β,17β-diol and dihydrotestosterone. The assay of 17β-hydroxyandrogen produced in response to the hCG was then expressed per 10^6 3β-HSD-positive cells.

**Other analytical techniques**

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

**Results**

**In-vitro response of Leydig cells to hCG**

**Study 1.** The effects of the different hormone preparations on the subsequent in-vitro response of isolated Leydig cells to hCG are shown in Text-fig. 1(a). Treatment with oFSH (Group B) in vivo almost doubled the quantity of androgen produced by the Leydig cells under
conditions of maximum stimulation with hCG in vitro. Pretreatment of the FSH with LH antiserum (Group C) markedly reduced this stimulatory effect. Similar treatment with oLH (Group D) evoked similar alterations in the maximum quantity of androgen produced by the Leydig cells, but treatment with the LH antiserum (Group E) did not abolish this stimulatory influence, since the antiserum did not absorb all the ovine LH under the conditions used (see below). None of the hormone preparations had obvious effects on the sensitivity (dose of hCG eliciting a half-maximal response, ED$_{50}$) of the Leydig cell to hCG.

Study 2. As found in Study 1, treatment with 50 µg oFSH (Group B) stimulated (by approximately 70%) the maximum quantity of androgen produced, and, absorption of the oFSH with the LH antiserum (Group C) completely abolished the effect (Text-fig. 1b). The purified FSH preparation (Group D) had no detectable effects at the dose level used.

![Text-fig. 1](https://example.com/figure1.png)

Text-fig. 1. The androgen response in vitro to increasing doses of hCG of Leydig cells from hypophysectomized rats after treatment (twice daily for 5 days) with various hormones in (a) Study 1 and (b) Study 2. (a) Group A, ■ = saline control; Group B, ○ = 50 µg oFSH; Group C, □ = 50 µg oFSH pretreated with LH antiserum; Group D, ● = 1 µg oLH; Group E, ▲ = 1 µg oLH pretreated with LH antiserum. (b) Group A, ○ = saline control; Group B, ▲ = 50 µg oFSH; Group C, △ = 50 µg oFSH pretreated with LH antiserum; Group D, ● = 1.6 µg purified oFSH. The values are mean ± s.d. of triplicate incubations.

Binding of $^{125}$I-labelled hLH to Leydig cells

Study 1. Scatchard analysis revealed no major changes in the average number of LH receptors per Leydig cell (as indicated by the intercepts of the plots with the x axes) after the different hormone treatments (Text-fig. 2). The parallel slopes of all Scatchard plots indicated that no alteration in the affinity of the LH receptors had occurred as a result of the hormone treatments. The average $K_d$ was $9 \times 10^{-10}$ M, assuming a molecular weight for LH of 40 000 and that all the LH was biologically active.
FSH effects on rat Leydig cell

**Text-fig. 2.** Scatchard plots of binding of $^{125}$I-labelled hLH to isolated Leydig cells from hypophysectomized rats treated with (a) saline—Group A; (b) 50 µg oFSH—Group B; (c) 50 µg oFSH pretreated with antiserum to LH—Group C; (d) 1 µg oLH—Group D; (e) 1 µg oLH pretreated with antiserum to LH—Group E. (f) Diagram summarizing the data from the Scatchard analyses.

**Effects on Sertoli cell function (Table 1)**

**Study 1.** Although there were no discernible effects on ABP secretion (Sertoli cell function) in Groups D and E, FSH caused a marked stimulation in the levels of ABP (Group B), an effect which was only slightly reduced by prior treatment of the preparation with the LH antiserum (Group C).

**Study 2.** Treatment with the LH antiserum had little effect on the biological activity of the FSH preparation in vivo (Groups B and C). The effect of the LER-1881 preparation (Group D) was greater than that of the oFSH used in Group B.

**Leydig cell-stimulating activity of hormone preparations**

**Study 1.** The LH-like activity of the hormone preparations in vitro is demonstrated in Text-fig. 3. The level of the LH contamination in the oFSH (Group B) amounted to only 0.031% of the mass of hormone present (650 ng oFSH stimulated a response from the Leydig cells equivalent to 0.22 ng oLH), suggesting that only about 15 ng oLH were administered for each injection of 50 µg oFSH. Pretreatment of the oFSH with the LH antiserum (Group C) removed all trace of Leydig cell-stimulating activity, but pretreatment of the oLH with LH antiserum (Group E) did not completely remove all the LH from the preparation: approximately 2% of the LH activity still remained (22 ng) and this was sufficient to mimic the effect of 50 µg oFSH (NIH-S12).
Table 1. Epididymal levels of androgen-binding protein (ABP) in hypophysectomized rats treated with various hormone preparations

<table>
<thead>
<tr>
<th>Study</th>
<th>Group</th>
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<td>A</td>
<td>Control</td>
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<tr>
<td></td>
<td>B</td>
<td>oFSH (NIH-S12)</td>
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</tr>
<tr>
<td></td>
<td>C</td>
<td>oFSH + LH antiserum</td>
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</tr>
<tr>
<td></td>
<td>D</td>
<td>oLH</td>
<td>0.11</td>
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<tr>
<td></td>
<td>E</td>
<td>oLH + LH antiserum</td>
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<tr>
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<tr>
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<tr>
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<td>D</td>
<td>oFSH (LER 1881)</td>
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* Determinations were carried out in triplicate on pools containing the epididymides from at least 8 rats. The coefficient of variation of replicates never exceeded 5%.

Text-fig. 3. The LH-like activity of the various hormone preparations used in Study 1 (see legend to Text-fig. 1a). Activity was assessed by exposing normal Leydig cells from immature rats to various dilutions of the preparations in vitro. The vertical arrows indicate the quantity of LH present in each preparation relative to the activity of the oLH (see summary in inset figure). Vertical bars represent s.d. of triplicate incubations.

Study 2. As shown in Text-fig. 4 the LER-1881 preparation (Group D) had slightly more LH activity on a weight basis than did the oFSH (Group B). Pretreatment of oFSH with the LH antiserum (Group C) removed approximately 98% of the LH contamination.

Discussion

The present study confirms the observation that FSH treatment of hypophysectomized rats in vivo results in an enhancement in the maximum quantity of androgen secreted by the Leydig cells in vitro in response to hCG. However, two observations suggest that this positive influence is a manifestation of small quantities of LH contaminating the hormone preparation. Pretreatment of the FSH with an LH antiserum abolished these stimulatory effects and this loss in efficacy was correlated with the complete removal of the capacity of FSH to stimulate steroid...
secretion directly from normal Leydig cells in vitro (a measure of its LH contamination). The fact that this antiserum treatment only slightly impaired the capacity for the FSH to stimulate Sertoli cell function in vivo in the same animals (as revealed by the quantity of ABP present in the epididymides) rules out the possibility that this loss of effect was due to a non-specific removal of biologically active FSH by the LH antiserum. The small decrease in FSH efficacy observed after antiserum treatment may simply reflect the withdrawal of an androgen stimulus (via removal of the contaminating LH). The second observation which made the possibility of FSH–Leydig cell interaction unlikely was that the preparation with the greatest FSH potency in vivo (LER-1881) with regard to Sertoli cell function had no discernible effects on the in-vitro dose–response characteristics of the Leydig cells in the same animals. Although the level of LH contamination in this preparation was high, if not higher than that of the oFSH when expressed per unit weight, its much greater FSH potency enabled it to be used at a dose level approximately 1:30 that of the crude preparation, thus reducing the LH content to negligible levels. The fact that in both studies the LH antiserum was effectively able to remove almost all the LH activity from the FSH preparation suggests that the Leydig cell-stimulating activity of these preparations is mainly due to LH contamination and not to an intrinsic LH-like activity.

It is difficult to provide a single explanation for the apparent discrepancies between our work and that of others. Van Beurden et al. (1976) showed that continuous administration of the expected contaminating amount of oLH (600 ng/day) in the oFSH preparation had no effect on the in-vitro response of Leydig cells from immature, hypophysectomized rats to LH nor did it increase the effect observed with a supposedly pure rat FSH preparation (containing <0·1% LH). Apart from the fact that the dose of LH administered was lower, it may be significant that the preparation was injected daily and not, as in our case, twice daily. The possibility that such small doses of LH are rapidly metabolized (unless some degree of protection is conferred on it by being injected as a contaminant in a much larger quantity of FSH) is very real, and multiple injections may be necessary to achieve an adequate blood level of LH. The rats were also hypophysectomized at an earlier age (21–25 days) than the present study; this is a critical period for Leydig cell development and these small differences in age may therefore be important. Selin & Moger (1977) found that 1.5 µg LH/day, a dose comparable to that which we used, resulted in a significant increase in the subsequent testicular response of hypophysectomized rats to LH in vivo. However, the effect was not as great as that with FSH (42·5 µg/day) which contained this quantity of LH as contamination. The problem with testing the response of the Leydig cells in vivo is that it is also potentially subject to effects via the seminiferous tubules. In hypophysectomized rats the tubules are atrophic and the Leydig cell response to FSH +
containing LH may differ from that induced by LH alone. Another major difference between our own work and that of others is that in all other studies testosterone has been used as the indicator of steroid response rather than 17β-hydroxyandrogen secretion, which measures a greater proportion of the androgen secreted. It may well be that FSH causes a qualitative change in the conversion of testosterone to dihydrotestosterone and androstenediol.

If the observed FSH effects can be explained by contaminating LH, the extremely small quantities of LH which are involved in this priming effect are of great interest. With the in-vitro Leydig cell bioassay, 50 μg crude oFSH had quantities of contaminating LH equivalent to 15 ng oLH (approximately 0-03%) which were apparently sufficient to account for the observed effects on the Leydig cell response. Moreover, in one of the control preparations in Study 1, although the LH antiserum removed more than 95% of the biologically active LH from the oLH preparation, enough LH still remained (amounting to 20 ng/injection) to have a pronounced effect on the response of the Leydig cells. Such priming effects of small doses of LH on Leydig cell responsiveness have recently been reported for the immature monkey (Arslan, Zaidi & Qazi, 1978). In the study of Arslan et al. (1978), the doses of LH were not large enough to stimulate peripheral plasma testosterone levels but did cause a stimulation in the maximum steroidogenic response to LH from the testis tissue in vitro. There has been much speculation that, at least in the rat, the gradual acquisition of LH responsiveness by the Leydig cell during early development is the result of an increase in FSH secretion. It now appears that normal Leydig cell responsiveness to LH may be the result of small, priming doses of LH secreted during the early phases of development, possibly acting synergistically with prolactin (Negro-Vilar, Krulich & McCann, 1973). Such priming probably takes place before 30 days of age since by this time the rat Leydig cell has already acquired its maximum responsiveness to LH (Purvis et al., 1978b).

Indeed, it has long been recognized that several of the major steroidogenic enzymes in the Leydig cell are under LH control (Samuels & Helmreich, 1956; Menon, Dorfan & Forchielli, 1967; Shikita & Hall, 1967; Purvis et al., 1973) and it is therefore not surprising that exposure to low doses of this hormone may have beneficial effects. On the other hand, higher doses of LH may be inhibitory and cause an attenuated response from the Leydig cell (Tsuruhara, Dufau, Cigorraga & Catt, 1977; Purvis, Torjesen, Haug & Hansson, 1977; Purvis et al., 1978b; Sharpe & McNeilly, 1978). This desensitization phenomenon is now becoming increasingly accepted as a physiological process by which the target tissue can adjust its responsiveness depending on the levels of trophic hormone. It therefore becomes important to distinguish between priming doses of LH and the higher doses which are responsible for these negative effects. Chen et al. (1977) have shown that FSH treatment increases the number of LH receptors in testis tissue from hypophysectomized rats, but we could not confirm this observation in the present study. Chen et al. (1977) used testis tissue and not isolated cells and therefore had no control over the number of Leydig cells under investigation. It is possible that the contaminating LH in the FSH preparation may cause the appearance of new Leydig cells with additional receptor complements. Another possibility is that FSH treatment of hypophysectomized animals may reduce proteolytic activity in the tubules and thus cause an apparent increase in the number of LH receptors measured. Although the above studies tend to suggest that FSH has no direct effect on the Leydig cell, they obviously do not preclude the possibility that FSH may exert an indirect effect on the Sertoli cell at least at some stage in development. The influence of the Leydig cell on Sertoli cell function (via androgens) is well established (Hansson, Calandra, Purvis, Ritzén & French, 1976a) and it is an attractive hypothesis that some local feedback mechanisms may operate in the opposite direction to co-ordinate tubular events with androgen secretion.

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References


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