

## Growth Hormone Directly Stimulates Testosterone and Oestradiol Secretion by Rat Leydig Cells *in vitro* and Modulates The Effects of LH and T<sub>3</sub>

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**Abstract.** The modulatory effect of GH on basal, LH and T<sub>3</sub> mediated secretion of testosterone and oestradiol by purified rat (60 day old) Leydig cells was studied *in vitro*. Percoll gradient purified Leydig cells ( $1 \times 10^3$ ) were cultured for 48 hours at 34°C in a medium containing different concentrations of rat GH (5–400 ng/mL), after an initial culture for 24 hours at 37°C. GH increased testosterone and oestradiol secretions in a dose dependent manner. While testosterone secretion reached the saturation point with 50 ng GH, oestradiol secretion reached the saturation point with 150 ng GH, followed by diminished secretions. Co-administration of minimum (10 ng) effective doses of GH with minimum (25 ng) or maximum (100 ng) effective doses of oLH significantly decreased the testosterone secretion. However, an increased secretion of testosterone was observed when maximum effective doses of rGH (50 ng) and oLH (100 ng) were co-administered. Minimum effective (25 ng) or maximum effective (50 ng) doses of T<sub>3</sub> inhibited GH mediated secretion of testosterone *in vitro*. Oestradiol concentration in the culture medium increased when either dose of rGH was co-administered with the minimum or maximum effective doses of oLH. T<sub>3</sub> 50 ng augmented the secretion of oestradiol by Leydig cells in the presence of GH. These results indicate that GH acts as a gonadotrophin to stimulate testosterone and oestradiol secretions by Leydig cells, and that it modulates LH or T<sub>3</sub> induced secretion of these steroids, depending on the intensity of their stimulation.

**Key words:** Oestradiol, Growth hormone, Leydig cells, Luteinizing hormone, Testosterone

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**EXPERIMENTAL** and clinical studies emphasize the importance of GH on male reproduction [1–3]. GH receptor immunoreactivity was demonstrated in rat testis [4]. Long term hypophysectomy regressed spermatogenesis and GH replacement reinitiated the same [5]. The importance of GH in regulating testicular steroidogenesis was underscored by the poor response of Leydig cells to exogenous hCG stimula-

tion in patients with isolated GH deficiency [6, 7] and by the potentiating effect of GH on hCG induced testosterone production in early childhood [7]. GH deficiency or resistance is associated with delayed puberty and poor Leydig cell response to LH/hCG *in vivo* [8]. GH induced testosterone secretion *in vivo* is mediated by IGF-I [2]. It is well known that PRL augmented LH mediated Leydig cell steroidogenesis [2]. Since PRL and GH are from the same genomic family [9], in the present study we examined whether GH has any modulatory effect on LH induced Leydig cell steroidogenesis. Early studies indicate that T<sub>3</sub> is one of the important non-classical hormones which involves the proliferation, maturation and function

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of Leydig cells [10–12]. GH synthesis and secretion is also mediated by  $T_3$  [13], so  $T_3$  mediated GH action on Leydig cell has also been studied. For the first time we demonstrated a direct stimulatory effect of rat GH on testosterone/oestradiol production by Percoll purified rat Leydig cells under basal conditions and a specific modulatory effect on oLH and  $T_3$  induced production of these steroids.

## Materials and Methods

### *Chemicals*

Bioactive rat GH (NIDDK-rGH-B-13; AFP-87401) and ovine LH (NIDDK-oLH-26; AFP-5551B) were a gift from the National Institute of Diabetes, Digestive and Kidney Diseases, Maryland, USA (award no. 32065). Collagenase (type IV), HEPES, BSA, fetal calf serum (FCS), minimum essential medium (MEM), Percoll, sodium pyruvate, sodium lactate, trypan blue,  $\beta$ -NAD, NBT, dehydroepiandrosterone and amphotericin B were purchased from Sigma Chemical Company, St. Louis, MO, USA. [1, 2, 6, 7- $^3H$ ] testosterone and [2, 4, 6, 7- $^3H$ ] oestradiol were from Amersham International PLC, Buckinghamshire, England. Testosterone antibody was a gift from Professor Med. E. Nieschlag, Director, Institute of Reproductive Medicine, University of Münster, Germany. Oestradiol antibody was a gift from Dr. C. Munroe and Dr. B. Lesely, University of California, Davis, USA.

### *Radioimmunoassay of testosterone and oestradiol*

Testosterone and oestradiol were quantified by liquid phase RIA [14]. Inter- and intra-assay variations were 6% and 4%, respectively for testosterone and 4.5% and 3.2%, respectively for oestradiol. Sensitivity of these assay was 17 fm/tube for testosterone and 12.5 fm/tube for oestradiol.

### *Isolation of Leydig cells*

Leydig cells were isolated from 60 day old Wistar rats and they have adult type of Leydig cells with peak LH receptor numbers [15, 16]. Leydig cells were isolated by using collagenase digestion method [17]. In brief, testes were decapsulated without

breaking the seminiferous tubules, under aseptic condition and incubated with 5 mL MEM (pH 7.4) containing collagenase (0.35 mg/mL), HEPES (1 mg/mL) and BSA (1 mg/mL) in 25 mL flasks for 15 minutes at 34°C and the flasks were gently shaken without damaging seminiferous tubules. After this, 10 mL MEM (pH 7.4) was added, allowed to stand for 5 minutes at room temperature and the supernatants were transferred to sterilized centrifuge tubes using Pasteur pipette. This procedure was repeated twice to obtain maximum Leydig cells. Leydig cells were washed repeatedly (at least thrice) by centrifuging ( $1000 \times g$ ) at 4°C 15 minutes, and the pellet was resuspended in 5 mL MEM. These crude Leydig cells were purified by discontinuous Percoll density gradient method [18] and identified by histochemical localization of  $3\beta$ -hydroxysteroid dehydrogenase ( $3\beta$ -HSD) [19]. The purity of Leydig cells was 80–85%. The principal contaminants were spermatogonia (12–15%), sperm (2%) and peritubular myoid cells (1–3%) which were removed by an initial 24 hours culture and 37°C. The viability of Leydig cells (90–95%) was assessed by trypan blue exclusion method [19].

### *Incubation of Leydig cells*

$1 \times 10^3$  Percoll-purified cells were plated in 96 well culture dishes at 37°C under a humidified atmosphere (5%  $CO_2$  and 95%  $O_2$ ). After the initial incubation for 24 hours, the medium was replaced with a fresh medium containing different concentrations (5, 10, 25, 50, 100, 150, 200, and 400 ng/mL) of bioactive rGH and incubated for 48 hours at 34°C (10 wells per treatment group in duplicate). From the dose response curve of testosterone secretion, the maximum and minimum effective doses of rGH (10 and 50 ng), LH (25 and 100 ng) and  $T_3$  (25 and 50 ng) were selected for combination studies. LH (25 & 100 ng) and  $T_3$  (25 & 50 ng) doses were selected from author's early observations [17]. The culture media were collected after 48 hours, centrifuged and used for the estimation of testosterone and oestradiol by liquid phase RIA.

### *Statistical analysis*

Statistical evaluation of the difference in mean values of testosterone and oestradiol among different

treatment groups was based on ANOVA. The data were subjected to multiple comparison using SNK's test [20].

## Results

### *Leydig cell response to rGH* *Testosterone*

GH stimulated testosterone secretion by Leydig cells in a dose dependent biphasic manner. Testosterone secretion increased in response to 10, 25 and 50 ng rGH, decreased sharply when Leydig cells were challenged with higher doses of rGH (100, 150 and 200 ng) and reached the nadir value stimulated with 200 ng rGH. Stimulation with 400 ng rGH produced a significant increase in testosterone concentration when compared with basal value. However, it was less than that of cultures stimulated with 10 ng rGH (Fig. 1).

### *Oestradiol*

Oestradiol concentraion in the culture media showed a dose dependent steady state increase when

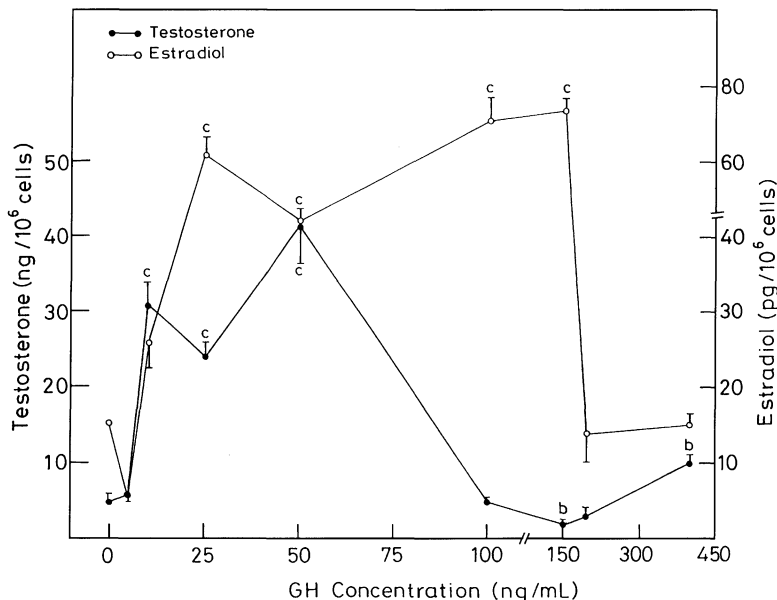
Leydig cells were stimulated with 10, 25, 50 and 100 ng, reaching the zenith in cultures stumulated with 150 ng rGH, except for a dip between 25 and 100 ng doses of GH. Oestradiol level in the culture medium of Leydig cells challenged with 200 or 400 ng rGH showed a steep fall, reaching the nadir value (Fig. 1).

### *Interaction of rGH and oLH in regulating the secretion of testosterone and oestradiol by Leydig cells*

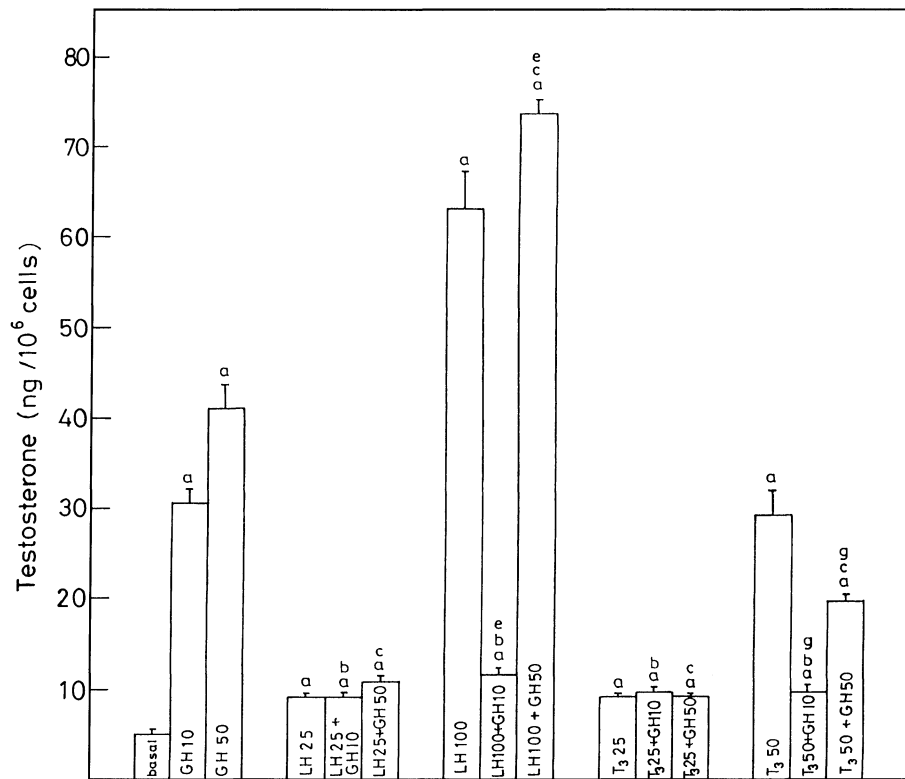
In order to test the modulatory effect of GH on LH mediated testosterone and oestradiol secretions and vice versa, Leydig cells were challenged with the minimum (25 ng) and maximum (100 ng) effective doses of oLH along with minimum (10 ng) or maximum (50 ng) effective doses of rGH.

### *Testosterone*

Co-administration of 10 or 50 ng rGH with the minimum effective dose of oLH diminished the secretion of testosterone by Leydig cells, when compared with cognate cultures stimulated with the respective dose of rGH alone. Nevertheless, the



**Fig. 1.** Dose response curves of testosterone and oestradiol secretions by rat Leydig cells stimulated with different doses of rGH *in vitro*. Each point represents the mean and the vertical line denotes the SEM (n=9) b-p<0.01; c-p<0.001 vs basal. Cells were cultured for 48 hours at 34°C after an initial culture for 24 hours at 37°C.



**Fig. 2.** Modulatory effects of GH on LH and T<sub>3</sub> mediated changes in testosterone secretion by rat Leydig cells *in vitro*. Each point represents the mean and the vertical line denotes the SEM (n=9). a-g denote the statistical significance of the difference between values compared. Differences were regarded as significant when P<0.05. a- vs basal; b- vs GH 10 ng; c- vs GH 50 ng; d- vs LH 25 ng; e- vs LH 100 ng; g- vs T<sub>3</sub> 50 ng. Cells were cultured for 48 hours at 34°C after an initial culture for 24 hours at 37°C.

levels of testosterone were comparable to that of cognate cultures stimulated with 25 ng oLH alone (Fig. 2).

Co-administration of the minimum effective dose of rGH (10 ng) with the maximum effective dose of oLH (100 ng) led to a sharp fall in testosterone concentration in the medium when compared with cognate culture stimulated with similar doses of rGH or oLH alone. On the other hand, co-administration of maximum effective doses of rGH and oLH enhanced the secretion of testosterone, when compared with cells challenged with either hormone (Fig. 2).

#### Oestradiol

Oestradiol concentration in the culture medium increased when 10 ng rGH was co-administered with 25 or 100 ng oLH. While the maximum effective doses of rGH with the minimum effective dose of oLH also produced a similar result, co-administra-

tion of maximum effective doses of rGH and oLH increased oestradiol secretion when compared with a cognate culture stimulated with oLH or rGH alone (Fig. 3).

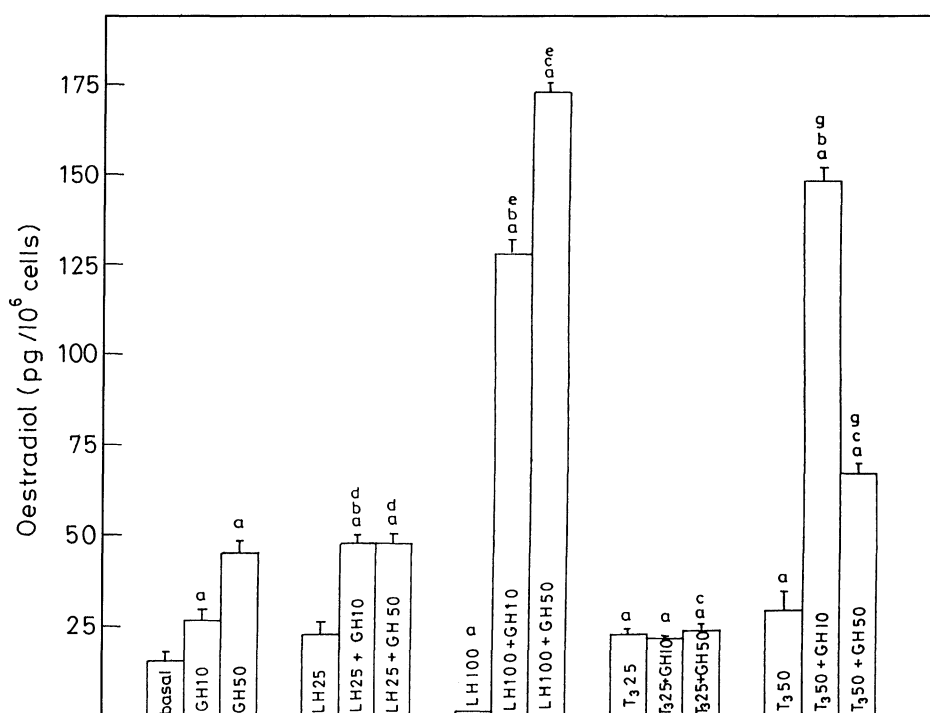
#### Interaction of rGH and T<sub>3</sub> on the secretion of testosterone and oestradiol by Leydig cells

##### Testosterone

Combination of the minimum and maximum effective doses of T<sub>3</sub> (25/50 ng) with 10 or 50 ng doses GH significantly decreased the secretion of testosterone by Leydig cells, when compared with cognate cultures stimulated with GH alone (Fig. 2).

#### Oestradiol

The minimum effective dose of T<sub>3</sub> did not modify the stimulatory effect of the minimum effective dose of rGH on oestradiol secretion, whereas the same



**Fig. 3.** Modulatory effects of GH on LH and T<sub>3</sub> mediated changes in oestradiol secretion by rat Leydig cells *in vitro*. Each point represents the mean and the vertical line denotes the SEM (n=9). a-g denote the statistical significance of the difference between values compared. Differences were regarded as significant when P < 0.05. a- vs basal; b- vs GH 10 ng; c- vs GH 50 ng; d- vs LH 25 ng; e- vs LH 100 ng; g- vs T<sub>3</sub> 50 ng. Cells were cultured for 48 hours at 34°C after an initial culture for 24 hours at 37°C.

dose of T<sub>3</sub> inhibited stimulatory effect of the maximum effective dose of GH induced oestradiol secretion. On the other hand, the maximum effective dose of T<sub>3</sub> augmented the stimulatory effect of GH on oestradiol secretion by Leydig cells (Fig. 3).

## Discussion

The present study provides evidence for the gonadotrophic action of GH on rat Leydig cells. The observed diametrically opposite responses of oestradiol and testosterone secretion by Leydig cells challenged with high dose of GH are similar to their response to LH stimulation Dufau [21] has reviewed that low doses of gonadotrophin increased testosterone secretion along with increased activity of steroidogenic enzymes, while higher doses decreases testosterone secretion by inhibiting steroidogenic enzyme activities, i.e. either reduced conversion of progesterone to androgens (late steroidogenic lesion) or cholesterol to pregnenolone (early steroidogenic

lesion). The early steroidogenic lesion is mediated by mitochondrial inhibitory protein located at the inner mitochondrial membrane of Leydig cells [22]. Late steroidogenic lesion is mediated by oestradiol [23–25]. The initial phase of the lesion in GH (100, 150 ng) mediated testosterone secretion appears to the result of enhanced secretion of oestradiol (late steroidogenic lesion), rather than due to a down regulation of GH receptors. However, the steroidogenic lesion in cells challenged with high doses of GH (200, 400 ng) is the result of a homologous down regulation or desensitization of GH receptors and such a lesion in steroidogenesis appears to be at a step prior to testosterone synthesis (early steroidogenic lesion) as concentration of both, testosterone and oestradiol decreased.

Rat GH induced testosterone secretion *in vivo* is mediated by IGF-I [22]. GH upregulates IGF-I secretion, IGF-I receptor numbers and LH receptors in Leydig cells *in vivo* [8, 26–29]. GH and IGF-I treated premature male rats had elevated level of serum testosterone [30]. A similar mechanism might

have been in operation in the present study on purified rat Leydig cells stimulated with rGH. Horikara *et al.* [31] reported that hGH had no effect on testosterone secretion whereas it enhanced the same in combination with hCG. Our results are in contradiction with the previous results of Horikawa *et al.* [31] since we used rat GH and 60 day old rat Leydig cells whereas they used hGH and 40 day old rats.

The data from *in vitro* studies using GH and LH combinations suggest the existence of an interaction between these two pituitary peptides on Leydig cell steroidogenic function. The inhibitory effect of the minimum effective dose of oLH on rGH mediated testosterone secretion may be due to an enhanced rate of aromatization of testosterone (late steroidogenic lesion) [23–25] and this modulatory effect of GH on LH mediated Leydig cell functions may vary depending upon the intensity of the stimulus from either. This could be evinced from the observed unaltered oestradiol concentration in the medium of cultures stimulated with 25 ng oLH plus 50 ng rGH (early steroidogenic lesion) [22]. Similarly while the minimum effective dose of rGH could inhibit the maximum effective dose of oLH induced testosterone secretion, there was a paradoxical increase in testosterone secretion when the maximum effective doses of rGH and oLH were co-administered. Such a paradoxical increase in testosterone appears to be the result of an augmented steroidogenic activity in Leydig cells [2] as oestradiol concentration also increased concomitantly. Taken together, the data on testosterone and oestradiol from Leydig cell cultures stimulated with oLH plus rGH point out the existence of co-operative effect of these two pituitary hormones [2] in regulating Leydig cell function, i.e. depending upon the stimulatory or inhibitory effect of either of them, the modulatory effect of the other varies.

A comparison of the response of oestradiol/testosterone secretions by Leydig cells co-stimulated with  $T_3$  and GH will reveal an inhibitory effect of

$T_3$  on GH induced testosterone secretion. As the minimum effective dose of  $T_3$  (25 ng) inhibits both testosterone and oestradiol, this should indicate that the steroidogenic lesion may be mediated by mitochondrial inhibitor protein (early steroidogenic lesion). However, the maximum effective dose of  $T_3$  (50 ng) inhibits testosterone secretion through elevated estradiol (late steroidogenic lesion). Maximum effective dose of LH (100 ng) in combination with GH stimulated both testosterone (LH 100 ng + GH 50 ng) and oestradiol (LH 100 ng + GH 10 ng) secretion, whereas that of  $T_3$  combination with GH stimulated only oestradiol secretion.

In conclusion, the present study demonstrates for the first time a direct stimulatory effect of GH on basal secretion of testosterone and oestradiol by rat Leydig cells. GH modulates LH and  $T_3$  induced testosterone and oestradiol production in a dose dependent manner. The exact site of GH action on the steroidogenic pathway in Leydig cells is not clear at present. Nevertheless, from the data on testosterone and oestradiol under various experimental conditions of the present study, it is suggested that aromatase activity in the cells is vulnerable to GH stimulation.

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