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T3 Directly Stimulates Basal

Testosterone and Oestradiol

Cells in vitro

and Modulates LH Induced

Production by Rat Leydig

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Abstract. The effect of T3 on basal and LH mediated synthesis and secretion of testosterone and oestradiol by
puberal rat Leydig cells was studied in vitro. Percoll gradient purified Leydig cells (1 x 103) were cultured for 48 hours
at 34 ° C in a medium containing a range of 5-400 ng/mL concentration of T3 or ovine LH after 24 hours initial culture at 37 °C. T3 increased testosterone and oestradiol secretions in a dose dependent manner which reached the saturation point with 50 ng dose. While the minimum effective dose of T3 (25 ng) potentiated the stimulatory effect
of the minimum effective dose of LH (25 ng) on testosterone secretion, it suppressed the effect of the saturation dose of LH (100 ng). Fifty ng T3 quelled the stimulatory effect of either dose of LH. Both doses of T3 increased oestradiol secretion, irrespective of the dose of LH. Addition of androstenedione (500 ng/mL) to the culture medium enhanced 25 ng T3 induced testosterone and oestradiol secretions. While androstenedione potentiated the stimulatory effect of T3 (25 ng) on LH (25 ng) induced testosterone and oestradiol secretions, it reversed the inhibitory effect of 50 ng T3 on LH mediated testosterone secretion which was accompanied by a decrease in oestradiol secretion. Puromycin (35 pg/mL) suppressed the stimulatory effect of T3 on basal and LH mediated testosterone and oestradiol production.
Taken together, the present results indicate a direct stimulatory effects of T3 on basal production of testosterone and oestradiol by Leydig cells and its modulatory effect on LH mediated steroidogenic activity varies depending upon the intensity of LH stimuli.

Key words: Leydig cells, LH, Oestradiol, T3, Testosterone
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MAMMALIAN testis, which has specific T3 recep-
tors (TR) is considered to be a prime target organ of
thyroid hormones [1-3]. The majority of existing reports consider immature, rather than mature rat testis to be vulnerable to thyroidal influence, as TR concentration decreases significantly in adult rats [3]. Though TR-al expression in the testis ceases at prepuberal age, expression of TR-a2 persists till puberty but at a reduced rate [1, 2]. On the contra-

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ry, the genomic locus of c-erb Aa, the protooncogene that encodes TR-a and its variant transcribes another

mRNA called Rev-erb Aa (Rev) during prepuberal age of rats with maximal expression in adults [3]. Modulatory effects of thyroid hormones on testi-cular steroidogenesis are well known [4-6]. An in-crease in Leydig cell number with reduced steroido-genic activity was reported in adult rats subjected to transient neonatal hypothyroidism [7] . Recently, we have shown enhanced Leydig cell number accompa-nied by steroidogenic activity in puberal rats sub-jected to transient neonatal hypothyroidism during the first two weeks of postnatal period [8]. Early studies identified Sertoli cells as the only testicular target for T3 in view of the presence of specific TR [3, 9]. However, recent studies have shown the presence

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of T3 binding sites in adult rat and goat Leydig cells [10-13]. Hardy et al. [14] demonstrated the expres-
sion of TR-al and TR-a2 mRNA in rat Leydig cells
but the functional significance of TR in Leydig cells
is yet to be understood. We have shown subdued Leydig cell function in rats subjected to hypothy-roidism from prepuberal age under basal and LH induced conditions [6]. Valenti et al. [15] reported resistance of normal and hypothyroid adult rat Ley-dig cells to T3 stimulation in vitro. This paper is the first to report a direct dose dependent stimulatory effect of T3 on basal testosterone and oestradiol

production and a dose dependent modulatory effect on LH induced testosterone production by purified Leydig cells of puberal rats in vitro.

Chemicals

Materials and Methods

Collagenase (type IV), HEPES, bovine serum al-bumin (BSA), fetal calf serum (FCS), minimum es-sential medium (MEM), Percoll, sodium pyruvate, sodium lactate, trypan blue, j3-nicotinamide adenine dinucleotide (3-NAD), nitro blue tetrazolium (NBT), dehydroepiandrosterone (DHEA), puromycin, an-drostenedione, amphotericin and L-triiodothyronine were purchased from Sigma Chemical Company, St. Louis, MO, USA. Bioactive ovine luteinizing hor-mone (NIDDK-LH-26; AFP-5551 B) was a gift from the National Institute of Diabetes, Digestive and Kidney Diseases, Maryland, USA. [1, 2, 6, 7-3H] Testosterone, and [2, 4, 6, 7-3H] oestradiol were purchased from Amersham International PLC, Buckinghamshire, England. Testosterone and oes-tradiol standards were provided by World Health Organization (WHO), Geneva, Switzerland under SMA programme. Testosterone antibody was a gift from Dr. Med. E. Nieschlag, Director, Institute of Reproductive Medicine, University of Munster, Germany. Oestradiol antibody was a gift from Dr. C. Munroe and Dr. B. Lesely, University of California, Davis, USA.

Isolation of Leydig cells

Sixty day old Wistar rats were used for Leydig cell harvest as puberal maturation of the testis is com-

plete by this age with predominance of adult type Leydig cells having the maximum number of LH receptors [16, 17].
Leydig cells were isolated by using collagenase digestion method [18] with slight modifications. Testes were decapsulated without breaking semi-niferous tubules under an aseptic condition and in-cubated with 5 mL MEM (pH 7.4) containing col-lagenase (0.35 mg/mL), HEPES (1 mg/mL) and BSA (1 mg/mL) in 25 mL flasks for 15 minutes at 34°C, while gently shaking the flasks without damaging the 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 trans-ferred to sterilized centrifuge tubes using a Pasteur pipette. This procedure was repeated twice to obtain the maximum number of Leydig cells. Pooled Leydig cells were washed repeatedly (at least thrice) with MEM by centrifuging (1000 x g) at 4°C for 15 minutes, and the pellet was resuspended in 5 mL MEM. These crude Leydig cells were purified by Percoll density gradient method [19] and identified by histochemical localization of 3 j3-hydroxysteroid de-hydrogenase (3 j9-HSD) [20] . The purity of Leydig cells was 80-85%. The principal contaminants were spermatogonia (12-15%), sperm (2%) and peritubu-lar myoid cells (1-3%) which were removed by an initial 24 hours culture at 37°C (described under cell culture). The viability of Leydig cells (90-95% was assessed using trypan blue exclusion method [20].

Cell culture studies

Purified Leydig cells (1 x 103) in MEM 300 iL containing 4% FCS were plated in 96 well flat bottom culture plates and incubated under 5% CO2 and 95% 02 at 37°C for 24 hours. At the end of this initial

incubation, most of the germ cell contaminants floated and were discarded along with the medium. This step helped to improve the purity of Leydig cells and to remove residual hormones secreted by the cells into the culture medium. After the initial incuba-

tion, the culture was replenished with fresh medium containing different concentrations (5, 10, 25, 50, 100, 150, 200 and 400 ng/mL) of bioactive ovine LH or T3 at 34°C. The culture media were collected af-

ter 48 hours, centrifuged and used for the estimation of testosterone and oestradiol by radioimmunoassay (RIA). The viability of Leydig cells in each culture,

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checked at the end of 48 hours incubation was 90-95%.

Modulatory effect of T3 on LH induced testoster-one and oestradiol secretion by Leydig cells

From the dose response curve of testosterone, the maximum (saturation) and minimum effective doses of LH (25 ng and 100 ng) and T3 (25 ng and 50 ng) were selected for combination studies involving LH plus T3. Then 1 x 103 cells were cultured for 48 hours after an initial 24 hour culture in a medium contain-

ing the following combination of T3 and LH: 25 ng T3 + 25 or 100 ng LH; 50 ng T3 + 25 or 100 ng LH. Concentrations of testosterone and oestradiol in the

medium were quantified by RIA.

Testosterone and oestradiol production from an-drostenedione

In order to check the influence of T3 on testoster-

one and oestradiol production, Leydig cells were cultured for 48 hours (after the initial culture) at 34°C as described above with a medium containing 500 ng/mL androstenedione [21] and challenged with the minimum or maximum effective doses of LH/ T3 alone or in combination as described earlier. Concentrations of testosterone and oestradiol in the

culture media were quantified after 48 hours.

Effect of puromycin on LH or T3 induced changes in Leydig cell function

To check the involvement of new protein synthesis if any, in mediating T3 induced changes in Leydig cell steroidogenic activity, a set of cultures was challenged with LH or T3 in the presence of puromycin [22]. Then 1 x 103 Leydig cells were in-cubated in a fresh medium containing puromycin (35 ttg/mL) along with minimum or maximum effec-tive doses of LH or T3 and incubated for 48 hours at 34°C after the initial culture and concentrations of

testosterone and oestradiol in media were quantified.

RIA of testosterone and oestradiol

Testosterone and oestradiol concentrations in cul-ture media were measured (for testosterone 10 t€L media was taken and for oestradio125 tsL media was

taken extracted in diethyl ether and quantified) by li-quid-phase RIA [23]. The sensitivity of these assays were 14 fm/tube for testosterone and 7 fm/tube for

oestradiol. Inter- and intra-assay variations were: 7% and 7.2%, respectively for testosterone; 5% and 3.2%, respectively for oestradiol.

Statistical Analysis

Data were subjected to analysis of variance (ANOVA). If the F ratio was significant, the data were subjected to multiple comparison using Student-Newman-Keul's (SNK) test to assess the in-dividual significance [24].

Results

LH induced testosterone and oestradiol secretion
by Leydig cells
Testosterone

A clear dose dependent biphasic effect of LH on
testosterone secretion by purified Leydig cells was
observed in vitro. Leydig cells stimulated with 25, 50 and 100 ng LH responded with increased secretion of
testosterone in a graded manner, reaching the satu-
ration point with 100 ng LH stimulation. On the other hand, a significant slump in testosterone secre-tion was evident when Leydig cells were challenged with 150, 200 or 400 ng LH, reaching the nadir level after stimulation with 200/400 ng LH (Fig. 1).

Oestradiol

LH induced oestradiol secretion presented a di-ametrically opposite picture to that of testosterone (Fig. 1). An initial marginal but significant increase in oestradiol secretion by cells stimulated with 50 ng LH was followed by a slump, reaching the nadir value when stimulated with 100 ng LH. This was followed by significantly increasing concentrations of oestradiol in media of Leydig cells challenged with higher doses of LH, reaching the zenith in response to 200/400 ng LH (Fig. 1).

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T3 induced testosterone and oestradiol secretions
by Leydig cells
Testosterone

Testosterone secretion by Leydig cells stimulated
with T3 showed a dose dependent biphasic response. Testosterone concentration increased in response to stimulation with 25 ng T3 and reached the saturation point with 50 ng T3. This was followed by a slump in the concentration of testosterone in media of cultures

challenged with 100, 150, 200 and 400 ng T3 (except for cultures stimulated with 200 ng T3/mL) but these testosterone levels were higher than that of Leydig cells stimulated with the minimum effective dose

(25 ng) of T3 (Fig. 2).

Oestradiol

Unlike testosterone, oestradiol concentration in culture media of Leydig cells stimulated with 5 ng T3 showed a significant decrease, whereas stimulation with all other doses of T3 increased oestradiol level in culture media. Nevertheless, there was a significant decrease in oestradiol secretion in culture media of cells stimulated with 150 or 200 ng T3, when com-

pared with the peak value obtained in response to 100 ng T3 but oestradiol secretion regained the maxi-mum level when challenged with 400 ng T3 (Fig. 2).

Interaction of T3 and LH on testosterone/oes-

tradiol secretions by Leydig cells
Testosterone

When minimum effective doses of T3 (25 ng) and LH (25 ng) were administered together, the quantum of testosterone secreted by Leydig cells doubled from that of cognate cultures stimulated with minimum effective dose of either of these hormones. On the

contrary, co-administration of the minimum effective dose of T3 with the saturation dose of LH (100 ng) perceptibly decreased (82%) the concentration of testosterone in the medium when compared with cultures challenged with the saturation dose of LH alone. Similarly, when the saturation dose of T3
(50 ng) was co-administered with either 25 or 100 ng
LH, there was a conspicuous decrease (35% and
92%, respectively) in testosterone secretion, when
compared with cognate cultures stimulated with
either dose of LH alone (Fig. 3).

Fig. 1. Dose response curves of testosterone and oestradiol secretions by rat Leydig cells stimulated with different doses of oLH in vitro. Each point represents the mean and the vertical line denotes the SEM (n = 9). a-p < 0.05; b-p < 0.01; c-
p<0.001 vs basal level. Cells were cultured for 48 hours at 34 °C after an initial culture for 24 hours at 37 °C.

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Fig. 2. Dose response curves of testosterone and oestradiol secretions by rat Leydig cells stimulated with different doses of T3 in vitro. Each point represents the mean and the vertical line denotes the SEM (n=9). a-p<0.05; b-p<0.01; c-p<0.001
vs basal level. Cells were cultured for 48 hours at 34 °C after an initial culture for 24 hours at 37 °C.

Fig. 3. Modulatory effects of T3 on LH mediated changes in testosterone secretion by rat Leydig cells in vitro in the presence and absence of androstenedione (500 ng/mL). Each point represents the mean and the vertical line denotes the SEM (n=9). A-androstenedione. a-i denote the statistical significance of the difference between values compared. Differences were
regarded as significant when P < 0.05. a-vs basal; b-vs androstenedione; c-vs T325; d-vs T350; e-vs LH25; f-vs LH 100;
g-vs LH25+T325; h-vs LH25+T350; i-vs LH100+T350. Cells were cultured for 48 hours at 34 °C after an initial
culture for 24 hours at 37 °C.

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Oestradiol

When 25/50 ng T3 was co-administered with either
25 or 100 ng LH, it consistently increased oestradiol secretion when compared with cognate cultures stimulated with the respective dose of LH alone.
The important finding being the reversal of the in-hibitory effect of 100 ng LH on oestradiol secretion by T3 (Fig. 4).

Effct of T3 on basal and LH induced testosterone

and oestradiol production from androstenedione by Leydig cells
Testosterone

LH induced increase in testosterone concentration

was enhanced significantly when androstenedione (500 ng) was added to the culture medium, indicating LH mediated conversion of androstenedione into testosterone. Unlike LH, the effect of T3 on testo-sterone production from androstenedione was dose de-pendent. While 25 ng T3 increased the production of testosterone from androstenedione, 50 ng T3 did not produce any appreciable change in the same when compared with cognate cultures incubated in a

medium containing androstenedione alone (Fig. 3). The stimulatory effect of 25 ng T3 on 25 ng LH in-duced testosterone production increased significantly in the presence of androstenedione in the culture medium. The inhibitory effect of 25 ng T3 on 100 ng LH induced testosterone secretion persisted even in the presence of androstenedione. On the other hand, the inhibitory effect of 50 ng T3 on 25 ng LH induced testosterone secretion was reversed by the addition of androstenedione to the culture medium and the concentration of testosterone in the medium

was perceptibly higher than those cultures stimulated with either or both 25 ng LH and 50 ng T3. The in-hibitory effect of 50 ng T3 on 100 ng LH induced testosterone secretion was also reversed by the addi-tion of androstenedione to the culture medium but

the level of testosterone was significantly less than that of cognate cultures stimulated with 100 ng LH or
50 ng T3 alone (Fig. 3).

Oestradiol

Presence of androstenedione in the culture medium
increased the production of oestradiol in response to
25 ng LH stimulation and reversed the inhibiting

Fig. 4. Modulatory effects of T3 on LH in mediated changes in oestradiol secretion by rat Leydig cells in vitro in the presence and absence of androstenedione (500 ng/mL). Each point represents the mean and the vertical line denotes the SEM
(n=9). A-androstenedione. a-h denote the statistical significance of the difference between values compared. Differences were regarded as significant when P < 0.05. a-vs basal; b-vs androstenedione; c-vs T325; d-vs T350; e-vs
LH25; f-vs LH 100; g-vs LH25 +T325; h-vs LH25 +T350. Cells were cultured for 48 hours at 34 ° C after an initial
culture for 24 hours at 37 °C.

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effect of 100 ng LH, leading to a perceptible increase in the concentration of oestradiol in the culture

medium. While the stimulatory effect of 25 ng T3 on oestradiol production was augmented by the presence of androstenedione in the culture medium, no ap-preciable difference was noticed in the stimulatory effect of 50 ng T3. Androstenedione augmented the stimulatory effect of 25 ng T3 on 25 ng LH induced oestradiol production alone whereas, it decreased the potentiating effect of 50 ng T3 on 25 ng LH induced oestradiol production. The stimulatory effect of 25/50 ng T3 on 100 ng LH induced oestradiol secre-tion did not undergo any appreciable change due to the presence of androstenedione in the medium (Fig. 4).

Effect of puromycin on T3 induced changes in testosterone/oestradiol secretion
Testosterone

Presence of puromycin in the culture medium did not affect basal testosterone secretion. However, presence of this protein synthesis inhibitor blocked LH (25/100 ng) and T3 (25/50 ng) induced testoster-one secretions. Puromycin also decreased the con-centration of testosterone in media of Leydig cell cultures stimulated with the minimum effective dose

of T3 plus either dose of LH. Puromycin did not produce any appreciable effect on the inhibitory effect of 50 ng T3 on 25/100 ng LH induced testosterone secretion (Table 1).

Oestradiol

Addition of puromycin to the culture medium decreased the concentration of oestradiol secreted by Leydig cells stimulated with 25 ng LH and 25 or 50 ng T3 whereas there was a significant increase in the concentration of oestradiol in media of cells

challenged with 100 ng LH. Marked reduction in the concentration of oestradiol was also evident in cul-

ture media of Leydig cells challenged with 25 ng or 100 ng LH plus 25 or 50 ng T3 in the presence of puromycin (Table 1).

Discussion

The present study proves the existence of a direct effect of T3 on steroidogenic activity of adult type rat Leydig cells obtained from puberal rats. To the best of our knowledge, this is the first report to show a direct stimulatory effect on T3 on purified rat Leydig cells. Early reports explained the effect of thyroid hormones on Leydig cell steroidogenesis as an in-direct one through altered secretion of paracrine regulators from Sertoli cells [3, 25] which have specific TR [9]. This is mainly because of the in-ability of early researchers to show the presence of specific TR in Leydig cells [3, 9]. Valenti et al. [15] reported that T3 or T4 does not influence basal or LH mediated testosterone production by purified rat Leydig cells. Our results are inconsistent with the above study. This inconsistency may be explained by the differences in age of animals and culture con-

Table 1. Modulatory effect of T3 (25 or 50 ng/mL) on LH (25 or 100 ng/mL) mediated changes in testosterone
and oestradiol secretions by rat Leydig cells in the
presence and absence of puromycin (35 ig/mL).

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ditions between the two studies.
The dose response curves of testosterone/oes-
tradiol production under LH stimulation in vitro demonstrates the biological potency of oLH used and
the functional viability of the Leydig cells in the in
vitro system employed. Viability of cells was also confirmed after 48 hours incubation as explained earlier. The mechanism underlying LH-induced testosterone secretion has been studied in depth and reviewed by Dufau et al. [26], Dufau [27], Huhtaniemi [28].
Adult type Leydig cells respond to LH stimulation
in a dose dependent manner. Leydig cells showed increased steroidogenic activity in terms of testoster-one production when stimulated with low and medi-um doses of LH and with impaired steroidogenic ac-tivity when stimulated with high doses of LH. The latter effect is due to down regulation and desensiti-zation of LH receptors [26-29]. Down regulation of LH receptors involves sequestration of the LH receptors from plasma membrane, internalization and loss of binding (homologous down regulation) accompanied by a decrease in some other membrane receptors also (such as heterologous down regulation of lactogen receptors) [26-28]. Other evidence can be drawn from the study of Pakarinen et al. [30] which showed decreased LH receptor mRNA con-centration in adult rat Leydig cells challenged with high dose of hCG.
In vivo [31] and in vitro [32] studies have shown that higher doses of LH or hCG induce up regulation of Leydig cell LH receptors and testosterone secre-tion in fetal Leydig cells, whereas an opposite effect was observed in adult Leydig cells. The reason be-hind this differential effect of LH/hCG is the lack of

oestradiol receptors and aromatase activity in fetal Leydig cells [29] . Mature Leydig cells aromatize an-drogens into oestradiol [33, 34]. In rats, there occurs a shift in aromatization with maturation. In imma-ture rat, the Sertoli cell is the site of aromatization, while the Leydig cell is the site of 5a-reduction of testosterone, whereas in adult rats, the Leydig cell becomes the site of aromatization of testosterone in the testis [35].
An important finding of the present study is the increased oestradiol secretion and response of Leydig cells to high doses of LH stimulation. Early studies on stimulation of Leydig cells with LH have shown increased aromatase activity and oestrogen forma-

tion [33, 34, 36, 37]. LH-induced steroidogenic le-sions in the adult type Leydig cells include reduced conversion of cholesterol to pregnenolone (early le-sion) and progesterone to testosterone (late lesion). The late steroidogenic lesion caused by LH/hCG was shown to be oestrogen mediated by inhibiting steroidogenic enzymes 17,20-lyase and 17a-hydroxy-lase [31, 33, 34]. These are oestrogen-induced steroidogenic lesions mediated by the synthesis of a specific protein (27 kD) [36, 38]. The present study clearly shows that diminished secretion of testoster-one by Leydig cells challenged with high dose of LH is due to enhanced estradiol secretion.

A direct effect of T3 on Leydig cells appeared dur-ing the first half of this decade when specific T3 binding to Leydig cells of adult rats [11], and the expression of TR in the interstitium of rats [10] were demonstrated. The proposal for direct T3 action on Leydig cells was strengthened by a recent demonstration of TR-al and a2 mRNA expression in immature and mature rat Leydig cells [14]. In general, the thyroid hormone acts through ,0 form receptors. Testis is the unique organ wherein the thyroid hormones act through a form receptors [3]. The results from in vitro studies are consistent with

early in vivo studies that hypothyroidism decreased and hyperthyroidism increased serum and intrates-ticular testosterone concentration [6, 39, 40]. The present in vitro study on purified adult type Leydig cells from sixty day old rats provides clear evidence for a direct effect of T3 on Leydig cell steroidogenesis. An important aspect of the present finding is a partial desensitization or negative modulatory effect on steroidogenesis, rather than a complete down regulation of T3 receptors in Leydig cells challenged with high doses of T3. This could be evinced from the sustained elevation of testosterone secretion by Leydig cells challenged with T3 doses higher than its saturation dose (50 ng), albeit a decrease in the quantum of testosterone secreted. This partial decrement in testosterone production by Leydig cells is attributed to the accompanying enhanced secretion of oestradiol. This could be deduced from the di-

ametrical secretory responses of testosterone and oestradiol secretion observed in Leydig cells challenged with most of the high doses of T3 (100, 150 and 400 ng) used. This shift in Leydig cell response to T3 stimulation is comparable to that of LH. The difference between LH and T3 stimulation

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is that the high doses of the former depleted testosterone concentration, while the latter had only a partial effect.
The present study points out a clear stimulatory effect of T3 on Leydig cell aromatase activity though there was an inhibitory effect of the lowest dose of T3 employed (5 ng). To the best of our knowledge, this is the first report to show the dose dependent biphasic direct effect of T3 on oestradiol production by Leydig cells. This is consistent with in vivo stu-dies [40] when T4 administration increased serum oestradiol titres. The quantum of oestradiol secre-tion by Leydig cells stimulated with T3 suggest that Leydig cell aromatase activity is more sensitive to thyroid hormones rather than LH. T3 inhibits the Sertoli cell aromatase activity and granulosa cell aromatase mRNA [41-43], whereas it stimulates the production of oestradiol in Leydig cells, suggesting cell specific differential role of thyroid hormones on testicular oestradiol production. Per the authors' knowledge there is no report available on the effect of thyroid hormones on Leydig cell aromatase expres-sion, and further studies are required to confirm the effect of T3 on Leydig cell aromatase gene expression. The exact mechanism underlying the inhibitory effect of 5 ng T3 dose on oestradiol production is not clear at present. A recent study from this laboratory has shown the inhibition of oestradiol production by T3 when Leydig cells are co-cultured with Sertoli cells [44]. This finding suggests that T3 stimulates certain specific Sertoli cell paracrine factors which has an inhibitory effect on Leydig cell aromatase activity. Results from studies on Leydig cells stimulated with LH plus T3 attest to the stimulatory effect of T3 on Leydig cell aromatase. T3 favors enhanced aromatization of testosterone by Leydig cells stimu-lated with LH. This effect of T3 is also associated

with stimulation of testosterone production when the effect of LH is minimal i.e. when Leydig cells were stimulated with 25 ng LH. Taken together, the data on testosterone and oestradiol suggest that the in-hibitory effect of T3 on LH mediated testosterone secretion is not due to a lesion in its production; rather it is a result of enhanced rate of conversion of testosterone into oestradiol. Since oestradiol is a

potent inhibitor for the synthesis of testosterone [27], the enhanced production of oestradiol might have also decreased the testosterone secretion.

Data on testosterone and oestradiol production

from androstenedione by Leydig cells stimulated with 25 ng T3 would indicate that T3 induced reduction in testosterone concentration is the result of enhanced

aromatization of the same. Since aromatase activity seems to reach the zenith in Leydig cells challenged with 50 ng T3, there was no added effect on the same due to the presence of exogenous androstenedione in the medium. Therefore, the difference in testoster-one production in response to minimum and maxi-mum effective doses of T3 may be attributed to the dose dependent variation in the response of Leydig cell aromatase activity to T3 stimulation.
Data from in vitro studies on testosterone and

oestradiol production from androstenedione also suggest a dose dependent biphasic effect of T3 on Leydig cell 173-hydroxysteroid dehydrogenase (173-HSD) as minimum and maximum effective doses of T3 produced opposite results. Early studies from our laboratory [6] and others [5] showed decreased ac-tivity of Leydig cell 173-HSD in thyroidectomized rats. These in vivo studies also showed normal ac-

tivity of 173-HSD in those hypothyroid rats sup-plemented with T4, suggesting a stimulatory effect of thyroid hormones.
Studies on testosterone/oestradiol production from androstenedione by Leydig cells costimulated with LH + T3 provide additional evidence for a modulatory effect of T3 on LH action at the level of 173-HSD activity. This effect of T3 may vary de-pending upon the dose of LH used as addition of androstenedione to the culture medium failed to

reverse the inhibitory effect of T3 on 100 ng LH in-duced testosterone production. The increased level of oestradiol in the culture medium of Leydig cells stimulated with 100 ng LH plus T3 has probably blocked the activity of 173-HSD. There appears to be a dose dependent shift in the site of T3 effects on Leydig cell testosterone production.
Data from puromycin studies indicate that stimulatory effect of T3 on Leydig cell steroidogenic activity is mediated by new protein synthesis. At this juncture, it may be worthwhile to recall the finding of Jana and Bhattacharya [12] and Jana et al. [13] who showed the synthesis of a new protein during an acute stimulation of crude goat Leydig cell preparations with T3. It is now well established that steroidogenic acute regulatory protein (StAR) is one of the major regulators of Leydig cell steroidogenesis [45]. It is not clear at present whether T3 induced steroidogenic

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activity in Leydig cell is mediated by StAR protein.
This could be inferred from the reduction in T3 mediated increase in basal and LH induced

testosterone/oestradiol secretions due to puromycin. Recently Manna et al. (46) reported that T3 induced a
3.6 fold increase in StAR protein mRNA expression and progesterone production by mouse Leydig tumor cell line. In light of these observations, T3 induced steroidogenesis in rat Leydig cells may be mediated by StAR protein.
In conclusion, the present study reveals that (i) T3 has a direct stimulatory effect on basal steroido-genic activity in puberal rat Leydig cells, and (ii) T3 also modulates LH mediated steroidogenesis in a dose dependent manner.

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References

Jannini EA, Dolci S, Ulisse S, Nikodem VM (1994) Developmental regulation of the thyroid hormone
receptor al mRNA expression in the rat testis. Mol Endocrinol 8: 89-96.
Jannini EA, Ulisse S, Carosa E, Graziano FM, Piersanti D, D'Armiento M (1994) Variant thyroid hormone receptor (THR) isoforms in developing rat testis. In: Androli M (ed) Highlights in Molecular and Clinical Endocrinology. Frontiers in Endocrinology. Ares Serono Symposium Publishers, Rome, Vol. 9, 77-80.

Jannini EA, Ulisse S, D'Armiento M (1995) Thyroid hormone and male gonadal function. Endocr Rev 16: 443-459.
Ando S, Panno ML, Beraldi E, Tarantino G, Salerno M, Palmero S, Prati M, Fugassa E (1990) Influence of hypothyroidism on in vitro testicular steroidogenesis in adult rats. Exp Clin Endocrinol 96: 149-156. Biswas NM, Ghosh PK, Biswas R, Ghosh D (1994) Effect of thyroidectomy, and thyroxine and a2 ~- globulin replacement therapy on testicular steroido-genic and gametogenic activities in rats. JEndocrinol 140: 343-347.

Antony, FF, Aruldhas MM, Udhayakumar RCR, Maran RRM, Govindarajulu P (1995) Inhibition of Leydig cell activity in vivo and in vitro in hypothyroid rats. J Endocrinol 144: 293-300.
Hardy MP, Kirby JD, Hess RA, Cooke PS (1993) Leydig cells increase their numbers but decline in

8.

9.

10.

11.

12.

13.

steroidogenic function in the adult rat after neonatal hypothyroidism. Endocrinology 132: 2417-2420.
Maran RRM, Arunakaran J, Jeyaraj DA, Ravichan-dran K, Ravinsankar B, Aruldhas MM (2000) Tran-sient neonatal hypothyroidism alters plasma and testicular sex steroids concentration in puberal rats. Endocr Res (in press).
Palmero S, Maggiani S, Fugassa E (1988) Nuclear triiodothyronine receptors in rat Sertoli cells. Mol Cell Endocrinol 58: 253-256.
Macchia E, Nakai A, Janiga A, Sakurai A, Fisfalen ME, Gardner P, Soltani K, DeGroot LJ (1990) Characterization of site-specific polyclonal antibodies to C-erbA peptides recognizing human thyroid hor-mone receptors al, a2 and ,3 and native 3,5,3-triiodo-thyronine receptor, and study of tissue distribution of the antigen. Endocrinology 126: 3232-3239. Tagami T, Nakamura H, Sasaki S, Mori T, Yoshioka H, Yoshida H, Imura H (1990) Immunohistochemical localization of nuclear 3 triiodothyronine recep-tor proteins in rat tissues studied with antiserum against C-ERB A/T3 receptor. Endocrinology 127: 1727-1734.

Jana NR, Bhattacharya S (1994) Binding of thyroid hormone to the goat Leydig cell induces the genera-tion of a proteinaceous factor which stimulates an-drogen release. J Endocrinol 143: 549-556.
Jana NR, Halder S, Bhattacharya S (1996) Thyroid hormones induce a 52 kDa soluble protein in goat

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14.

15.

16.

17.

18.

19.

20.

21.

22.

23.

24.

25.

26.

testis Leydig cell which stimulates androgen release. Biochim Biophys Acta 1292: 209-214.
Hardy MP, Sharma RS, Arambepola NK, Sottas
CM, Russell LD, Bunick D, Hess RA, Cooke PS (1996) Increased proliferation of Leydig cells induced
by neonatal hypothyroidism in the rat. J Androl 17: 231-238.
Valenti S, Guido R, Fazzuoli L, Barreca A, Giusti M, Giordano G (1997) Decreased steroidogenesis and CAMP production in vitro by Leydig cells isolated from rats made hypothyroid during adulthood. Int J Androl 20: 279-286.
Sinha MK, Dash RJ, Chakravarti RN, Atreja SK (1979) Age related changes in gonadotropin receptors in testes of rats. Ind J Exp Biol 17: 855-858. Bortolussi M, Zanchetta R, Belvedere P, Colombo L

(1990) Sertoli and Leydig cell numbers and gonado-tropin receptors in rat testis from birth to puberty. Cell Tissue Res 260: 185-191.
Djakiew D, Onoda M (1993) Multichamber cell cul-ture and directional secretion. In: Russell LD, Griswold MD (eds) The Sertoli cell. Cache River Press, Clearwater, Florida, 181-194.
Lefevre A, Saez JM, Finaz C (1983) hCG responsive-ness of purified Leydig cells from immature and ma-ture rats. Horm Res 17: 114-120.
Aldred LF, Cooke BA (1983) The effect of cell damage on the density and the steroidogenic capacity of rat testis Leydig cells. Using an NADH exclusion test for determination of viability. J Steroid Biochem 18: 411-414.
Sordoillet C, Chauvin MA, Hendrick JC, Fran-chimont P, Morera AM, Benahmed M (1992) Sites of interaction between epidermal growth factor and transforming growth factor-91 in the control of steroidogenesis in cultured porcine Leydig cells. Endocrinology 130: 1352-1358.
Hollinger MA, Hwang F (1972) Effect of puromycin and actinomycin D on glucose-stimulated protein and RNA labeling in vitro, in rat testis. Biochim Biophys Acta 262: 336-343.
Sufi SB, Donaldson A, Jeffcoate SL (1986) Testo-sterone and oestradiol assays. In: World Health Organisation Collaborating Centre for Research and Reference Services in the Immunoassay of Hormones in Human Reproduction. 10th ed., 57-69, 85-96. Zar JH (1974) Biostatistical analysis. Prentice Hall Inc, Englewood Cliffs, NJ, 151-181.
Palmero S, Prati M, Barreca A, Minuto F, Giordano G, Fugassa E (1990) Thyroid hormone stimulates the production of insulin-like growth factor I (IGF-I) by rat Sertoli cells. Mol Cell Endocrinol 68: 61-65. Dufau ML, Winters CA, Hattori M, Aquilano D, Baranao JLS, Nozu K, Baukal A, Catt KJ (1984) Hormonal regulation of androgen production by the

Leydig cell. J Steroid Biochem 20: 161-173.
27. Dufau ML (1988) Endocrine regulation and com-municating functions of the Leydig cell. Ann Rev Physiol 50: 483-508.
28. Huhtaniemi I (1993) Hormonal control mechanisms of Leydig cells. In: DeKretser D (ed) Molecular Biol-ogy of Male Reproduction. Academic Press, San Diego, 383-410.
29. Huhtaniemi I, Pelliniemi LJ (1992) Fetal Leydig cells: Cellular origin, morphology, life span and special functional features. Proc Soc Exp Biol Med 201: 125-140.
30. Pakarinen P, Niemimaa T, Huhtaniemi I, Warren DW (1994) Transcriptional and translational regula-tion of LH, prolactin and their testicular receptors
by hCG and bromocriptine treatments in adult and neonatal rats. Mol Cell Endocrinol 101: 37-47.
31. Huhtaniemi IT, Warren DW, Catt KJ (1984) Func-tional maturation of rat testis Leydig cells. Ann NY Acad Sci 438: 283-303.
32. Warren DW, Dufau ML, Catt KJ (1982) Hormonal regulation of gonadotropin receptors and steroido-genesis in cultured fetal rat testes. Science 218: 375-377.

33. Tsai-Morris C-H, Aquilano D, Dufau ML (1985) Cellular localization of rat testicular aromatase activ-

ity during development. Endocrinology 116: 38-46.
34. Tsai-Morris C-H, Aquilano D, Dufau ML (1985) Gonadotropic regulation of aromatase activity in the adult rat testis. Endocrinology 116: 31-37.
35. Dorrington JH, Khan SA (1993) Steroid production, metabolism and release by Sertoli cells. In: Russell LD, Griswold MD, (eds) The Sertoli cell. Cache River Press, Clearwater, Florida, 538-549.
36. Nozu K, Dufau ML, Catt KJ (1981) Estradiol recep-tor-mediated regulation of steroidogenesis in gonado-tropin-desensitized Leydig cells. J Biol Chem 256: 1915-1922.
37. Valladares LE, Payne AH (1981) Effects of hCG and cyclic AMP on aromatization in purified Leydig cells
of immature and mature rats. Biol Reprod 25: 752-758.
38. Nozu K, Dehejia A, Zawistowich L, Catt KJ, Dufau ML (1982) Gondadotropin-induced desensitization of Leydig cells in vivo and in vitro estrogen actgion in
the testis. Ann NY Acad Sci 383: 212-230.

39. Maran RRM (1997) Modulatory effect of thyroid hormones on Leydig cell activity. Ph.D. thesis University of Madras, Chennai, India.
40. Maran RRM, Aruldhas MM, Udhayakumar RCR, Subramanian S, Rajendiran G, Antony FF, Arunakaran J, Govindarajulu P (1998) Impact of altered thyroid hormone status on prostatic glyco-sidases. Int J Androl 21: 121-128.
41. Panno ML, Beraldi E, Pezzi V, Salerno M, De Luca

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G, Lanzino M, Lepera M, Sisci D, Prati M, Palmero
S, Bolla E, Fugassa E, Ando S (1994) Influence of
thyroid hormone on androgen metabolism in
peripuberal rat Sertoli cells. J Endocrinol 140: 349-
355.
42. Ulisse S, Jannini EA, Carosa E, Piersanti D, Graziano FM, D'Armiento M (1994) Inhibition of aromatase activity in rat Sertoli cells by thyroid hor-mone. J Endocrinol 140: 431-436.
43. Cecconi S, Rucci N, Scaldaferri ML, Masciulli MP, Rossi G, Moretti C, D'Armiento M, Ulisse S (1999) Thyroid hormones effects on mouse oocyte matura-
tion and granulosa cell aromatase activity. Endo-crinology 140: 1783-1788.

44. Maran RRM, Arunakaran J, Govindarajulu P, Aruldhas MM (1999) T3 stimulates rat Leydig cell testosterone, estradiol secretion and it decreases es-tradiol secretion only when Leydig cell is co-cultured with Sertoli cell. XVth Testis workshop on the testis: From stem cell to sperm function, Louisville, KY, USA, April 7-10 (abstract p. 55).
45. Clark BJ, Stocco DM (1996) StAR-A tissue specific acute mediator of steroidogenesis. Trends Endocrinol Metab 7: 227-233.

46. Manna PR, Tena-Sempere M, Huhtaniemi IT (1999) Molecular mechanisms of thyroid hormone-stimulat-
ed steroidogenesis in mouse Leydig tumor cells. J Biol Chem 274: 5909-5918.