Evaluation of relative roles of LH and FSH in regulation of differentiation of Leydig cells using an ethane 1,2-dimethylsulfonate-treated adult rat model

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Abstract

The relative role of LH and FSH in regulation of differentiation of Leydig cells was assessed using an ethane 1,2-dimethylsulfonate (EDS)-treated rat model in which endogenous LH or FSH was neutralized from day 3 to day 22 following EDS treatment. Serum testosterone and the *in vitro* response of the purified Leydig cells to human chorionic gonadotropin (hCG) was monitored. In addition RNA was isolated from the Leydig cells to monitor the steady-state mRNA levels by RT-PCR for 17α-hydroxylase, side chain cleavage enzyme, steroidogenic acute regulatory protein (StAR), LH receptor, estrogen receptor (ER-α) and cyclophilin (internal control). Serum testosterone was undetected and the isolated Leydig cells secreted negligible amount of testosterone on stimulation with hCG in the group of rats that were treated with LH antiserum following EDS treatment. RT-PCR analysis revealed the absence of message for cholesterol side chain cleavage enzyme and 17α-hydroxylase although ER-α and LH receptor mRNA could be detected, indicating the presence of undifferentiated precursor Leydig cells. In contrast, the effects following deprival of endogenous FSH were not as drastic as seen following LH neutralization. Deprival of endogenous FSH in EDS-treated rats led to a significant decrease in serum testosterone and *in vitro* response to hCG by the Leydig cells. Also, there was a significant decrease in the steady-state mRNA levels of 17α-hydroxylase, cholesterol side chain cleavage enzyme, LH receptor and StAR as assessed by a semiquantitative RT-PCR. These results establish that while LH is obligatory for the functional differentiation of Leydig cells, repopulation of precursor Leydig cells is independent of LH, and also unequivocally establish an important role for FSH in regulation of Leydig cell function.

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Introduction

Leydig cells are present in the interstitial compartment of the testes and their main function is to produce testosterone, which is essential for spermatogenesis and development of secondary sexual characteristics. The testicular secretion of androgens depends on the Leydig cell number and also on the activity of Leydig cells (Teerds 1996). This process involves Leydig cell proliferation and acquisition of the capacity to produce testosterone, which is associated with morphological changes, and multiple regulators govern this process. Luteinizing hormone (LH) is the key regulator of Leydig cell function and it is indispensable for functional differentiation and proliferation of Leydig cells (Benton *et al.* 1995, Sriraman *et al.* 2000) although its role in early development of Leydig cells remain unclear (Ge *et al.* 1996, Baker & O'Shaughnessy 2001).

Follicle-stimulating hormone (FSH) has also been implicated in Leydig cell function through its action on Sertoli cells (Saez & Lejeune 1996). The precise role of FSH in male reproduction still remains a debate, although several lines of evidence suggest that FSH is essential for initiation of spermatogenesis (Sharpe 1994). The suggestion that Sertoli cells could regulate Leydig cell function is based on the observation that following induction of damage to spermatogenesis, hypertrophy of neighboring Leydig cells was observed (Jegou & Sharpe 1993). Moreover, using ethane 1,2-dimethylsulfonate (EDS)-treated rats (EDS is an alkylating agent which specifically destroys Leydig cells in adult rats) it has been demonstrated that Leydig cells that are localized at the vicinity of seminiferous tubules which lack germ cells exhibit faster regeneration (Jegou & Sharpe 1993). Also, experimental induction of hypothyroidism led to an increase in Sertoli cells associated with a parallel increase in Leydig cells, thereby maintaining the ratio of Leydig cells to Sertoli cells constant (Cooke *et al.* 1992). Studies by administering FSH have also shown a stimulatory role in Leydig cell

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function, but the physiological importance of these findings is a matter of debate, since most of the preparations of FSH used for the study were contaminated with LH and hence the observed effects could be attributed to the trace quantity of LH that was present in the FSH preparation (Purvis *et al.* 1979, Moger & Murphy 1982, Lecerf *et al.* 1993, Saez & Lejeune 1996). However, using recombinant FSH preparations, a role for FSH in regulation of Leydig cell function was demonstrated. Constant infusion of recombinant human FSH for 7 days to immature hypophysectomized rats resulted in an increased steroidogenic responsiveness of Leydig cells (Vihko *et al.* 1991). Coculture of Leydig cells with Sertoli cells was also found to enhance testosterone production, and pretreatment of these cocultures with FSH further enhanced the capacity of Leydig cells to produce testosterone (Benahmed *et al.* 1985). In contrast, using EDS-treated hypophysectomized or testosterone-implanted rats to suppress LH, Molenaar *et al.* (1986) reported that the repopulation of Leydig cells is FSH-independent and it was speculated that mature Sertoli cells secreted factors independently of FSH, suggesting that FSH is not required in adult rats. In addition, it has been reported that FSH-β knockout male mice were fertile with normal serum testosterone levels (Kumar *et al.* 1997). On the contrary, Dierich *et al.* (1998) reported that FSH receptor knockout mice are subfertile and serum testosterone levels are reduced by 50% when compared with the wild type and suggested existence of an intercellular communication pathway between Sertoli cells and Leydig cells. Results of recent studies by Huhtaniemi *et al.* (1999) with a hypophysectomized rat model demonstrated that administration of recombinant FSH increases the Leydig cell androgen production and LH receptors. Based on several lines of evidence accumulated using the above models it has been suggested that if FSH has a role in Leydig cell function at all, it is essentially only a permissive role through the Sertoli cell-derived factors. Considering the controversies that still exist, neither the knockout nor hypophysectomized model is suitable to study the function of a particular hormone. In the knockout model there is a permanent loss of the gene product from the embryonic stage and it cannot be used to study a stage-specific function of a hormone. In a hypophysectomized rat model, there is surgical trauma with loss of all pituitary-derived hormones and all the pituitary hormones have been demonstrated to have a role in regulation of testicular function (Saez & Leujeune 1996).

The objective of the present study is to better understand the relative role of LH and FSH during early Leydig cell development, using model systems that provide an opportunity to monitor the role of the hormone by selective deprival for desired duration. A passive neutralization approach was employed in an EDS-treated rat model, where a highly specific antiserum to ovine LH (oLH) and FSH (oFSH) was used to neutralize endogenous LH and FSH to assess their relative roles in regulation of differentiation of Leydig cells during the course of regeneration after EDS treatment. The EDS-treated rat model has been widely used to study the hormonal regulation of the development of Leydig cells (Kerr *et al.* 1985, Molenaar *et al.* 1986, Morris *et al.* 1986); within 2–3 weeks after EDS treatment Leydig cell regeneration occurs and this could be compared with the postnatal development of Leydig cells in morphological and functional characteristics (Teerds 1996).

Materials and Methods

Reagents

EDS was synthesized in the laboratory of Prof. Sri Krishna, Department of Organic Chemistry, Indian Institute of Science, Bangalore from ethylene glycol and methansesulfonyl chloride as described by Jackson & Jackson (1984). The purity of the EDS employed was 98% as assessed by NMR spectroscopy. M-199, Percoll and soya bean trypsin inhibitor were purchased from Sigma, St Louis, MO, USA. Collagenase was purchased from Worthington, Lakewood, NJ, USA. DNAse and Dispase were obtained from Boehringer Mannheim Ltd, Mannheim, Germany. [^{3}H]testosterone, Moloney murine leukemia virus reverse transcriptase and dNTPs were obtained from Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK. Primers for PCR and Taq polymerase were obtained from Bangalore Genei, Bangalore, India. oLH and oFSH were prepared at the Clinical Research Institute of Montreal, Quebec, Canada. Rat LH and human chorionic gonadotropin (hCG) were kind gifts from Dr A F Parlow, National Hormone and Pituitary Program, NIDDK, Bethesda, MD, USA.

Production and characterization of antiserum to oLH and oFSH

LH antiserum Antiserum to highly purified oLH was raised in monkeys as described earlier (Sriraman *et al.* 2000). The antiserum was capable of binding 75% ^{125}I-rat LH *in vitro* at a dilution of 1:20 000. The ability of this antiserum to neutralize endogenous LH in rats was established by demonstrating that a single injection of 200 μl LH antiserum i.p. reduced the serum testosterone by 85–90% in 24 h when compared with the preimmune serum-treated animals ($0{\cdot}33 \pm 0{\cdot}04$ ng/ml vs $2{\cdot}87 \pm 0{\cdot}4$ ng/ml). Administration of this high-titer LH antiserum to adult male rats for 7 days leads to 30% reduction in testis weight and decreases the serum and testicular testosterone to undetected levels, demonstrating the complete neutralization of LH.

FSH antiserum Highly purified oFSH was used for immunization in adult bonnet monkeys (Selvaraj & Moudgal 1994). The FSH preparation was affinity purified

Table 1 Details of primers employed and expected product size of the PCR amplified cDNA

	Sequence of the primer	Product size (bp)
LH receptor	Forward primer 5′-ACT GCT GCG CCT TCA GGA ATT-3′ Reverse primer 5′-CCT AAG GAA GGC ATA GCC CAT-3′	257
Estrogen receptor-α	Forward primer 5′-CCG GGG AAG CTC CTG TTT G-3′ Reverse primer 5′-AGA GAT GCT CCA TGC CTT TGT TAC-3′	382
17α-hydroxylase	Forward primer 5′-TCA AAG ACG CCC GGT GCC AA-3′ Reverse primer 5′-ACA GTG ACT TGG CTT CCT GA-3′	415
Cholesterol side chain cleavage enzyme	Forward primer 5′-TCA AAG CCA GCA TCA AGG AG-3′ Reverse primer 5′-GCA GCC TGC AAT TCA TAC AG-3′	473
StAR	Forward primer 5′-TTG GGC ATA CTC AAC AAC CA-3′ Reverse primer 5′-ATG ACA CCG CTT TGC TCA G-3′	389
IGF-I	Forward primer 5′-GCC ACA GCC GGA CCA GAG ACC CTT-3′ Reverse primer 5′-CTA CAT TCT GTA GGT CTT GTT TCC-3′	327
Cyclophilin	Forward primer 5′-GTG GCA AGT CCA TCT ACG-3′ Reverse primer 5′-CAG TGA GAG CAG AGA TTA CAG-3′	380
Androgen binding protein	Forward primer 5′-ACA ATC TCT GGG CTC GGC TT-3′ Reverse primer 5′-TTG CAG GTC CAC ATC ACA GT-3′	330

to remove the trace quantities of LH by passing through an LH antibody affinity column. Before using it for immunization, the purity of FSH was established by checking its ability to stimulate testosterone production by purified Leydig cells which respond to added LH (10 ng) by producing 8- to 10-fold more testosterone over the basal levels. While addition of 1 μg FSH before purification to the Leydig cell preparation resulted in an 8-fold increase in testosterone production, following purification no increase over basal level was seen even after addition of 2 μg oFSH, thus establishing the absence of LH contamination.

The ability of antiserum to neutralize endogenous FSH in rats was established by the observation that when neonatal rats (10 days old) were administered 100 μl FSH antiserum i.p. for 7 days, there was a decrease in the testicular weight by 50% with significant reduction in seminiferous tubule diameter as assessed by histology of testes (data not presented). Also, when the steady-state mRNA levels of androgen-binding protein (ABP) were assessed by a semiquantitative RT-PCR analysis of the total RNA from the testis, a 75% reduction in ABP levels was observed in FSH-deprived animals (data not shown). ABP is synthesized in Sertoli cells and in the neonatal rat testis the expression of ABP is regulated by FSH (Tindall & Means 1976). It is also important to note that in the monkeys immunized with purified oFSH there was no detected effect on the nocturnal rise in serum testosterone, which is an LH-dependent parameter (Mukku *et al.* 1981). In addition, i.v. administration of 3 ml of this antiserum to adult male bonnet monkeys also did not have any effect on the nocturnal surge of serum testosterone (control: 31·5 ± 2·8 ng/ml, FSH antiserum-treated: 32 ± 2·4 ng/ml). In contrast, administration of LH antiserum resulted in complete abolition of the nocturnal rise in serum testosterone (LH antiserum-treated: 3·45 ± 1 ng/ml).

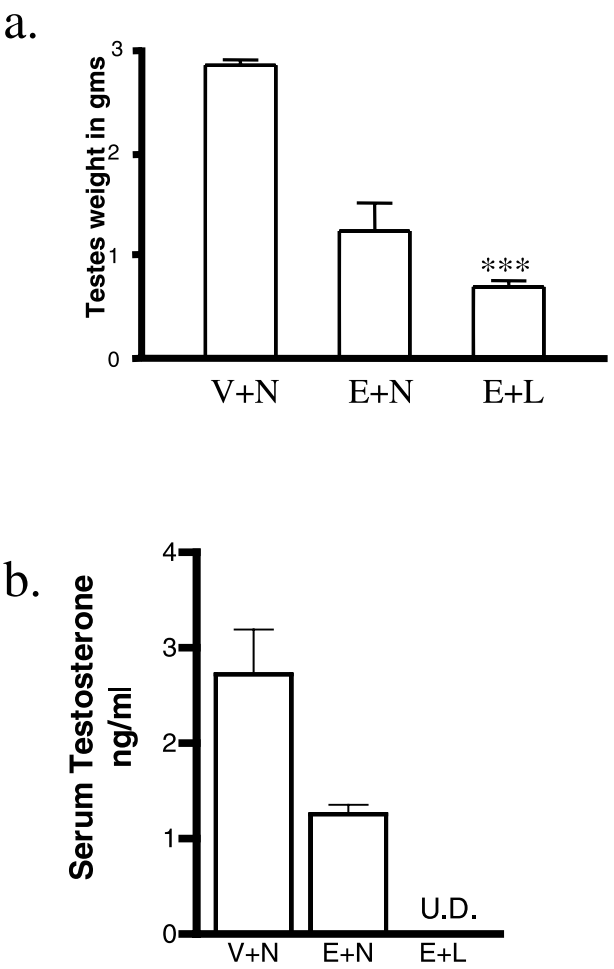


Figure 1 Effect of administration of LH antiserum in EDS-treated rats from day 4 to day 23 post-EDS treatment on (a) testes weight and (b) serum testosterone. Values represent means ± S.E. from three separate experiments for six animals in each group. Serum testosterone in each sample was estimated individually by RIA after ether extraction. U.D., undetected. ***P<0·001; E+N vs E+L. V+N, vehicle and NMS treated; E+N, EDS and NMS treated; E+L, EDS and LH antiserum treated.

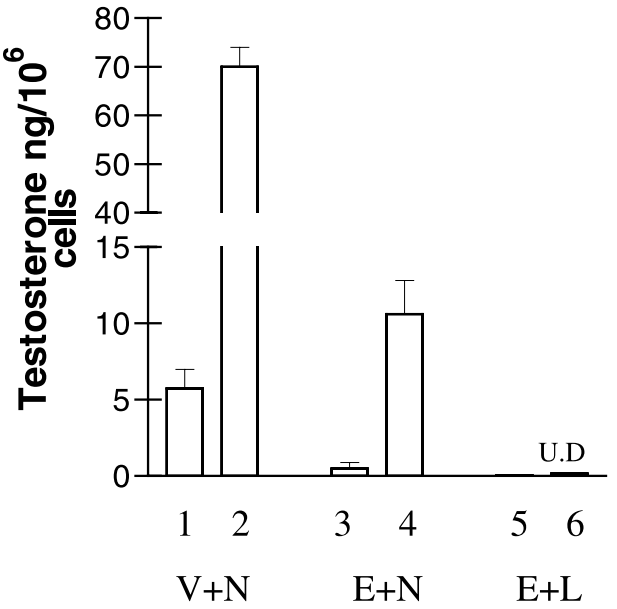


Figure 2 Effect of deprivation of endogenous LH on *in vitro* testosterone production by Leydig cells isolated from the EDS-treated rats. Bars corresponding to 1, 3, 5 and 2, 4, 6 indicate the testosterone values without (basal), and with, hCG respectively. Values represent means ± S.E. from three separate experiments analyzed in triplicate from six animals in each group. U.D., undetected; V+N, vehicle and NMS treated; E+N, EDS and NMS treated; E+L, EDS and LH antiserum treated.

Animals and antiserum treatment

Adult Wistar rats (90 day old) were obtained from the National Institute of Nutrition, Hyderabad, India. The animals were housed in an environmentally controlled facility with 12 h of light and 12 h of darkness and were allowed free access to food and water. Animals were administered EDS (75 mg/kg body weight.) in 50% DMSO i.p. and from 4 days after EDS treatment, the rats were injected with 500 µl LH antiserum (E+L) or FSH antiserum (E+F) for 18 days i.p. Control groups were injected with equal volumes of normal monkey serum (NMS) (E+N) and vehicle (DMSO)-treated rats also received normal monkey serum (V+N). Since our own studies and studies by other groups (Molenaar *et al.* 1986, Zhai *et al.* 1996) have demonstrated that by 3 weeks after EDS treatment there is a significant recovery in the levels of serum testosterone and *in vitro* testosterone production by Leydig cells, day 23 after EDS treatment was chosen as the end point of the study. On day 23 rats were killed, blood was collected for serum testosterone estimation, testes were weighed and Leydig cells were isolated to assess the *in vitro* testosterone production and for RNA isolation.

In order to ensure that the observed effects of LH or FSH neutralization is under a condition in which the hormone is neutralized to the maximum that could be achieved with this approach, the presence of surplus antibody in the serum was monitored, which could be used as an indicator of efficiency of neutralization (Medhamurthy *et al.* 1995, Meachem *et al.* 1998). Conventional RIA systems cannot be used to measure unneutralized free hormone in this system since the presence of free antibody in the serum will interfere with the assay. Serum from E+L rats showed 20–25% binding to ^{125}I-oLH at 1:50 dilution, demonstrating the presence of excess antibody which can neutralize the free LH that can arise in the circulation. Similar results were observed from the serum obtained from E+F with ^{125}I-oFSH. We have also observed the period required to recover partially from the LH deprival took more than 3 days as seen in the E+L+N group (EDS and LH antiserum treated and then allowed to recover by

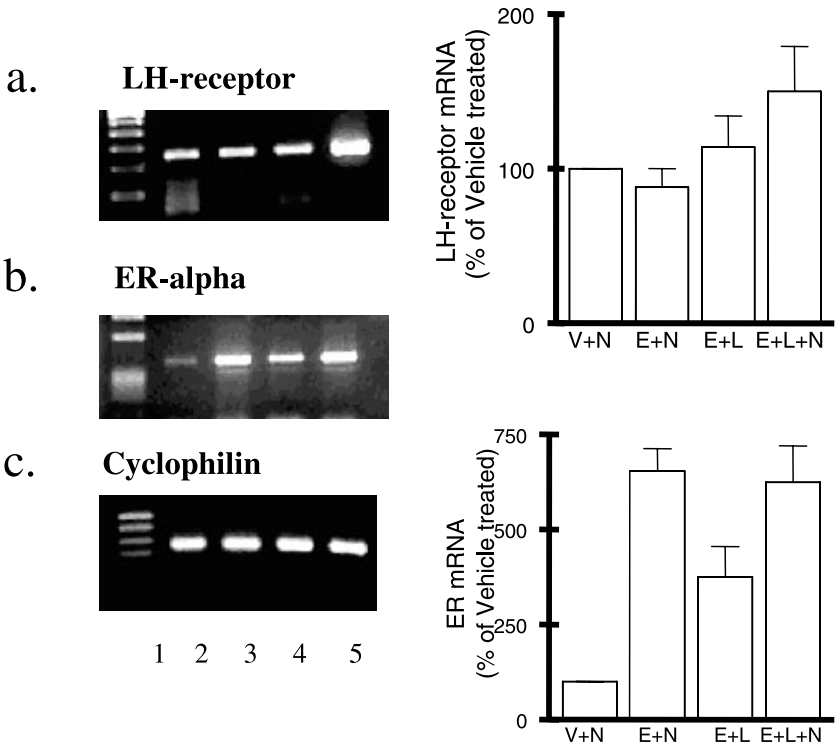


Figure 3 Effect of administration of LH antiserum in EDS-treated rats on the expression of ER-α and LH receptor. Total RNA isolated from purified Leydig cells was reverse transcribed and cDNA obtained was subjected to PCR for (a) LH receptor (b) ER-α and (c) cyclophilin (internal control). Lane 1, DNA markers, lanes 2–5, PCR-amplified products from V+N, E+N, E+L, E+L+N groups respectively. Values represent means ± S.E. ($n=6$). V+N, vehicle and NMS treated; E+N, EDS and NMS treated; E+L, EDS and LH antiserum treated; E+L+N, EDS and LH antiserum treated and then allowed to recover by administration of NMS for 5 days.

administration of NMS for 5 days) and importantly even after 5 days serum testosterone levels could not be restored. These results essentially demonstrate that the antibody was always administered in excess to ensure complete neutralization of LH or FSH, and hence the observed effects are under maximally saturated binding of LH or FSH antibodies with the circulating hormone.

Isolation of Leydig cells

Leydig cells were isolated according to the procedure of Klinefelter *et al.* (1987) and Risbridger & Davies (1994). Briefly, the decapsulated testes were subjected to collagenase digestion in the presence of DNAse. The released interstitial cells were pooled and subjected to Percoll density-gradient fractionation. The density gradient was formed by centrifugation at 20 000 ***g*** at 4 °C. When Leydig cells were isolated on day 23 following EDS and NMS or FSH antiserum treatment, cells which were positive for 3β-hydoxysteroid dehydrogenase (3β-HSD) staining were found in a fraction which had a density more than 1·068 and were 93% pure. However, in the EDS- and LH antiserum-treated group while the cells obtained in the same fraction were homogeneous they did not stain for 3β-HSD. Analysis for the Leydig cells at other densities in Percoll gradients revealed only erythrocytes at higher densities (1·084 and above). Damaged cells and macrophages were seen at lower densities.

Serum testosterone and in vitro *testosterone production*

All blood samples were processed within 2–3 h after collection, and serum separated was stored at –20 °C until further use. Testosterone concentrations in the serum samples were estimated individually after ether extraction using a sensitive RIA standardized in our laboratory (Rao & Kotagi 1989). In order to assess the testosterone-producing capacity of the isolated Leydig cells, 1×10^5 cells were incubated with or without 100 ng hCG (12 000 IU/mg) in 1 ml M-199 medium containing 0·1% BSA at 34 °C for 4 h in a shaking water bath, and testosterone secreted in the medium was determined by a specific RIA without extraction. In addition to the response of cells to hCG, the ability of cells to produce testosterone in the

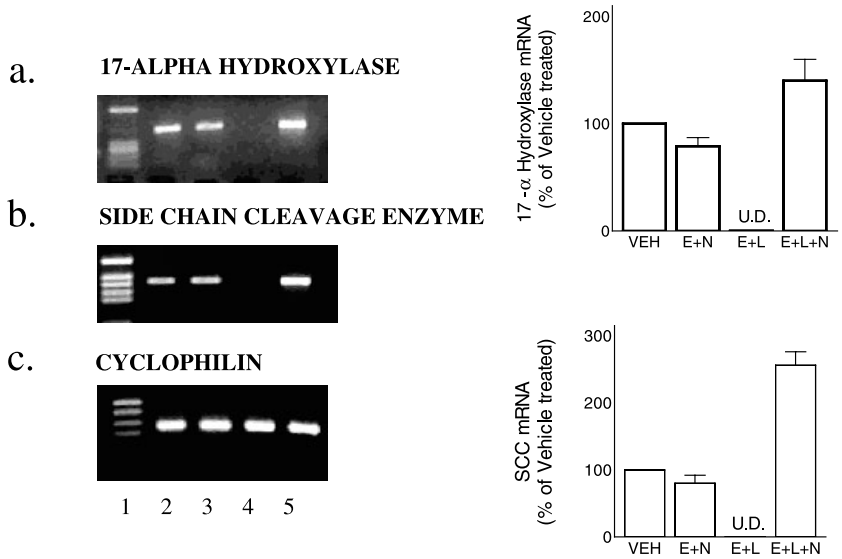


Figure 4 Effect of administration of LH antiserum in EDS-treated rats on the expression of 17α-hydroxylase and cholesterol side chain cleavage enzyme. Total RNA isolated from purified Leydig cells was reverse transcribed and cDNA obtained was subjected to PCR for (a) 17α-hydroxylase, (b) cholesterol side chain cleavage enzyme (SCC) and (c) cyclophilin (internal control). Lane 1, DNA markers, lanes 2–5, PCR-amplified products from V+N, E+N, E+L, E+L+N groups respectively. V+N, vehicle and NMS treated; E+N, EDS and NMS treated; E+L, EDS and LH antiserum treated; E+L+N, EDS and LH antiserum treated and then allowed to recover by administration of NMS for 5 days. Values represent means ± S.E. (n=6). U.D., undetected.

presence of 22(*R*)-hydroxycholesterol (20 μM) was also assessed (Sriraman *et al.* 2001).

RT-PCR

Total RNA was isolated from Leydig cells (5–8 × 10^6 cells) using Tri Reagent (Sigma) and intact RNA with an $A_{260/280}$ ratio 1·6 and above was used for RT-PCR. One microgram of total RNA was reverse transcribed by incubation at 37 °C for 1 h with 50 units of reverse transcriptase in a 20 μl reaction volume containing 200 ng random hexamers. PCR was carried out with specific primers within the linear range of amplification to assess the steady-state mRNA levels for 17α-hydroxylase, LH receptor, cholesterol side chain cleavage enzyme, steroidogenic acute regulatory protein (StAR), estrogen receptor (ER)-α and cyclophilin in a 50 μl reaction mixture with 2·5 μl RT mixture containing the cDNA. The details of the primers used and size of the PCR-amplified products are listed in Table 1. Each PCR product was sequenced for its authenticity (data not included). To visualize, the products were analyzed on 1·5% agarose gel with ethidium bromide in Tris–borate/EDTA buffer. The differences in intensity of the products following electrophoretic analysis were analyzed using an EDAS 120 Kodak Gel Documentation system.

Statistical analysis of data

The data were analyzed by Kruskal–Wallis ANOVA followed Neuman–Keuls test to identify significant differences in the group employing Graphpad software (San Diego, CA, USA), and a P value less than 0·05 was considered to be statistically significant. The data presented in the figures are representative of at least three independent experiments.

Results

Effect of administration of LH antiserum in EDS-treated rats

Testes weight and serum testosterone To assess the role of LH in regulation of development of Leydig cells, EDS-treated adult rats were deprived of endogenous LH by administering LH antiserum from day 4 to day 22 post-EDS treatment. On day 23, when analyzed for their testicular weight, there was significant decrease in the E+L group (compare E+L and E+N) (Fig. 1a) and serum testosterone was not detected in the E+L group (Fig. 1b). These results demonstrate that endogenous neutralization of LH in EDS-treated rats led to a decrease in serum testosterone to undetected levels and this was associated with drastic decrease in the testicular weight.

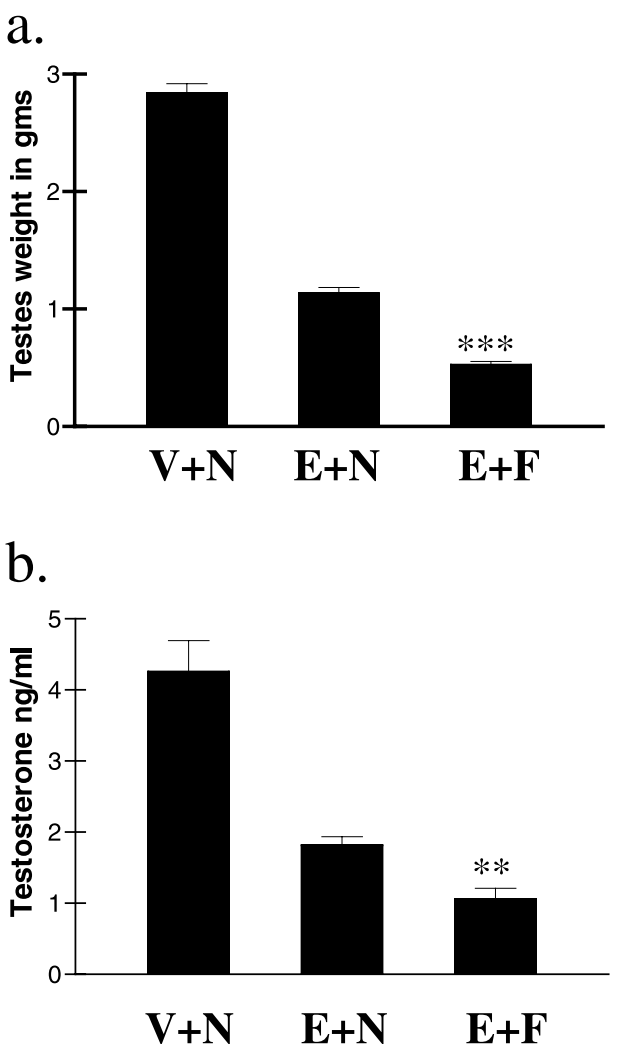


Figure 5 Effect of administration of FSH antiserum in EDS treated rats from day 4 to day 23 post-EDS treatment on (a) testis weight and (b) serum testosterone. Values represent means ± S.E. from three separate experiments for six animals in each group. Serum testosterone in each sample was estimated individually by RIA after ether extraction. ***P<0·001, **P<0·01; E+N vs E+F. V+N, vehicle and NMS treated; E+N, EDS and NMS treated; E+F, EDS and FSH antiserum treated.

***In vitro* testosterone production** Leydig cells were isolated from V+N, E+N and E+L groups and analyzed for their *in vitro* testosterone-producing capacity at basal and hCG-stimulated conditions. Leydig cells from V+N- and E+N-treated groups produced 10-fold more testosterone following addition of hCG, compared with their basal production (Fig. 2). On the contrary, upon stimulation of Leydig cells isolated from the E+L group with hCG, testosterone could not be detected in the medium. These results demonstrate that endogenous neutralization of LH in EDS-treated rats results in impaired response to

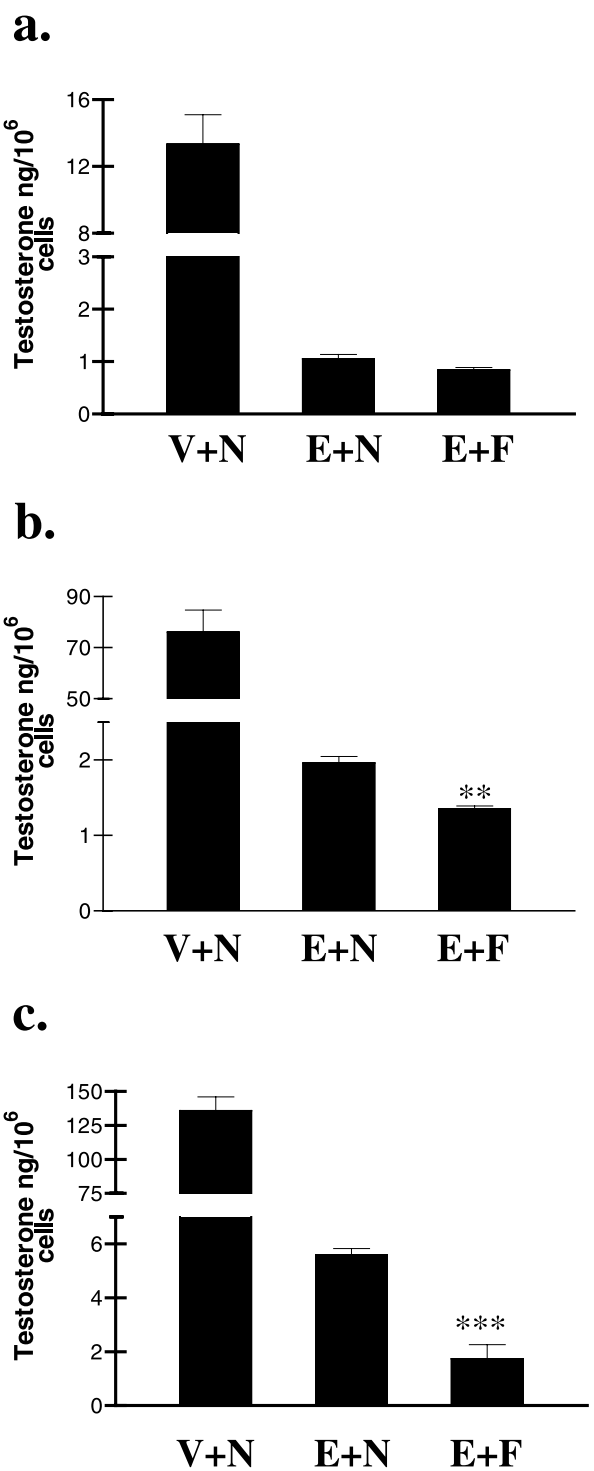
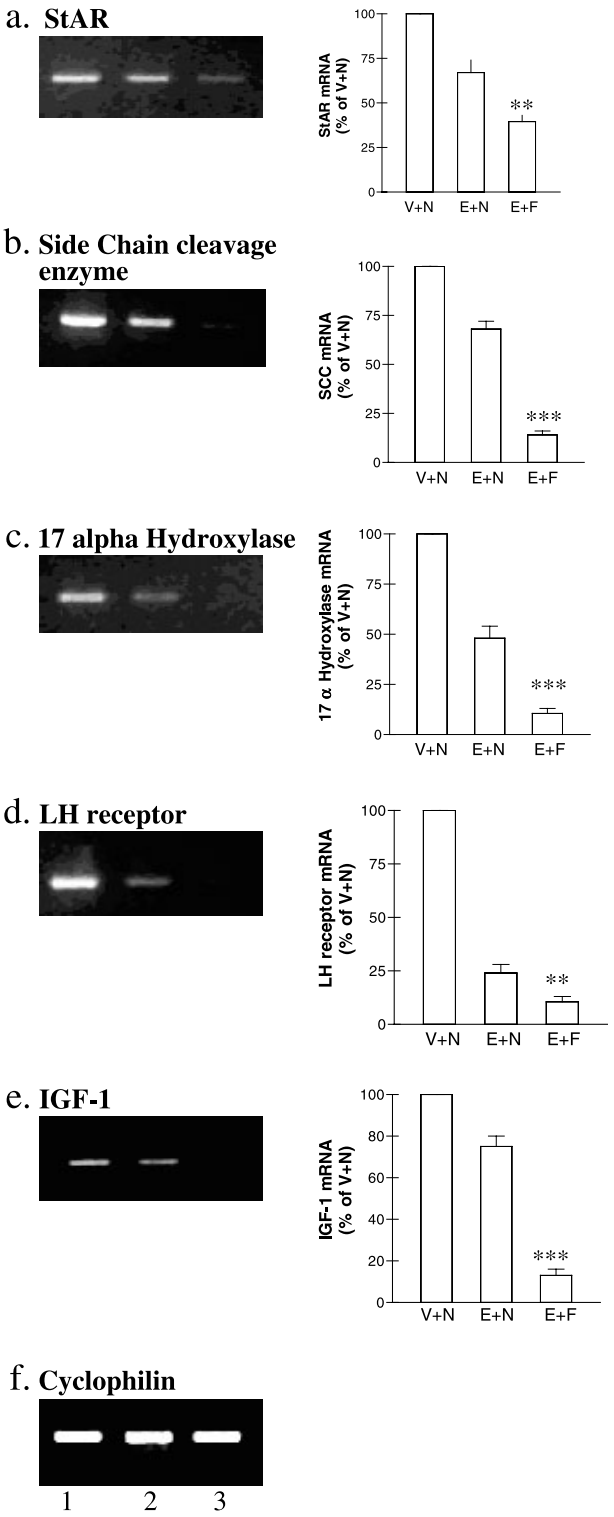


Figure 6 Effect of deprival of FSH in EDS treated rats on (a) basal, (b) hCG-stimulated and (c) 22(*R*)-hydroxycholesterol-saturated *in vitro* testosterone production. Values represent means ± S.E. from three separate experiments cultured in triplicate with six animals in each group. ***P<0·001, **P<0·01; E+N vs E+F. V+N, vehicle and NMS treated; E+N, EDS and NMS treated; E+F, EDS and FSH antiserum treated.



hCG-stimulated testosterone production *in vitro* and establish the absolute requirement for LH in testosterone production.

RT-PCR analysis for LH-receptor, ER-α, 17α-hydroxylase, and cholesterol side chain cleavage enzyme mRNA It has been well documented that LH receptor and ER-α are localized in the Leydig cells in the interstitial compartment of the testis and their presence can be used as marker for establishing the identity of Leydig cells. Moreover, 17α-hydroxylase and cholesterol side chain cleavage enzyme are the key steroidogenic enzymes involved in the testosterone biosynthetic pathway and hence assessing their steady-state mRNA levels would reflect the steroidogenic capacity of the Leydig cells. When the RNA isolated from Leydig cells purified from EDS-treated rats deprived of endogenous LH was analyzed for LH receptor (position 846–1101 of rat LH/hCG receptor DNA sequence, which is necessary for binding and signal transduction (Reiter *et al.* 1995)) and ER-α by RT-PCR (35 cycles), both the messages could be detected (Fig. 3). Similarly, mRNAs for LH receptor and ER-α could be detected in the V+N and E+N groups. However, when mRNA levels of 17α-hydroxylase and cholesterol side chain cleavage enzyme were monitored, no messages could be detected in the EDS- and LH antiserum-treated groups, despite amplification for 40 cycles (Fig. 4), although the message for 17α-hydroxylase and cholesterol side chain cleavage enzyme could be detected in V+N and E+N groups. Upon termination of LH antiserum treatment to recover from LH deprival from day 22 for 5 days (by administration of NMS; E+L+N), the messages for 17α-hydroxylase and cholesterol side chain cleavage enzyme could be detected (Fig. 4). These results demonstrate that precursor Leydig cells can repopulate in testes even in the absence of LH but require LH for their functional differentiation.

Effect of administration of FSH antiserum in EDS-treated rats

Testes weight and serum testosterone Following administration of FSH antiserum in EDS-treated rats from

Figure 7 Semiquantitative RT-PCR analyses in RNA obtained from purified Leydig cells following deprival of endogenous FSH in EDS-treated adult rats. In the left panels, total RNA isolated from the isolated Leydig cells was reverse transcribed and cDNA obtained was subjected to a semiquantitative PCR in the linear range of amplification for (a) StAR, (b) cholesterol side chain cleavage enzyme, (c) 17α-hydroxylase, (d) LH receptor, (e) IGF-I and (f) cyclophilin (internal control). Lanes 1–3 correspond to PCR-amplified products from V+N, E+N and E+F treated groups respectively. In the right panels, the intensity of the signals was quantified by densitometry and normalized to that of cyclophilin. The data provided are means ± S.E. from three separate experiments with six animals in each group. ***P<0·001, **P<0·01; E+N vs E+F. V+N, vehicle and NMS treated; E+N, EDS and NMS treated; E+F, EDS and FSH antiserum treated.

day 4 to day 22 post-EDS treatment there was a significant decrease in testis weight and serum testosterone (Fig. 5a and b). The observed reduction in serum testosterone was not as drastic as seen in the case of the LH-deprived group.

***In vitro* testosterone production** When Leydig cells isolated from EDS- and FSH antiserum-treated animals (E+F) were assessed for their *in vitro* testosterone-producing capacity, there was no change in their basal testosterone production but interestingly there was a significant decrease in hCG-stimulated and 22(*R*)-hydroxycholesterol-saturated testosterone production when compared with the E+N group (Fig. 6). These results establish that endogenous neutralization of FSH results in a significant decrease in steroidogenic capacity of Leydig cells, although the effects are not as drastic as seen in the case of LH-deprived animals.

RT-PCR analysis of 17α-hydroxylase, cholesterol side chain cleavage enzyme, StAR, LH receptor and insulin-like growth factor-I (IGF-I) mRNA In order to assess the effect of neutralization of endogenous FSH in EDS-treated rats on the steroidogenic machinery, the steady-state mRNA levels of 17α-hydroxylase, cholesterol side chain cleavage enzyme, StAR, LH receptor and IGF-I were assessed by a semiquantitative RT-PCR within the linear range of amplification employing cyclophilin as internal control (30 cycles for all the parameters except for cyclophilin, which was amplified for 26 cycles). The linear range and the number of cycles were determined by measuring the amplification of the cDNA at different cycles as described previously (Sriraman *et al.* 2000). Analysis revealed a significant decrease in both cholesterol side chain cleavage enzyme and 17α-hydroxylase (Fig. 7). There was also a decrease in steady-state mRNA of StAR (Fig. 7) but the extent of decrease was not as much as that seen in the case of cholesterol side chain cleavage enzyme and 17α-hydroxylase. In addition, assessment of steady-mRNA levels of LH receptor and IGF-I (Fig. 7) also revealed a significant decrease. These results demonstrate that endogenous neutralization of FSH in EDS-treated rats resulted in reduction in expression of key molecules involved in steroidogenesis and the factors that regulate this process.

Discussion

Earlier studies have established a role for LH in Leydig cell regeneration after EDS treatment, but the precise role of LH in the developmental events associated with regeneration and the requirement of FSH in the differentiation process have not been well studied. Towards this a passive neutralization approach was employed in EDS-treated rats and 23 days after EDS treatment was chosen as an end point of the study since steroidogenic capacity of Leydig cells could be monitored easily.

The results obtained in the present study confirm that there is an LH-independent stage in the development of Leydig cells and this is contrary to the absolute requirement of LH for functional differentiation. Administration of LH antiserum to EDS-treated rats resulted in reduction of serum testosterone and *in vitro* testosterone-producing capability of purified Leydig cells to not-detected levels. Analysis for steady-state mRNA levels of cholesterol side chain cleavage enzyme and 17α-hydroxylase by RT-PCR in isolated Leydig cells after EDS and LH antiserum treatment revealed its total absence. In contrast, the message for LH receptor and ER-α could be detected and it is generally agreed that LH receptor (Benton *et al.* 1995, Tena-Sempere *et al.* 1997) and ER-α (Nozuk *et al.* 1981, Lin *et al.* 1982, Zhai *et al.* 1996) are localized in Leydig cells of the interstitial compartment of the testis. Earlier studies indicate that the Leydig cell capacity for testosterone synthesis correlates with the levels of cholesterol side chain cleavage enzyme and 17α-hydroxylase; it has been well documented that LH can influence its expression (Payne 1990, Lin 1996, Payne & Shaughnessy 1996). These results establish that while precursor Leydig cells repopulated in the testes in an LH-independent manner, there is an absolute requirement of LH for their functional differentiation. It is interesting to note here that Leydig cell precursors expressed LH receptor in the absence of LH, which is essential for subsequent maturation steps including the onset of steroidogenesis under the influence of LH. These results confirm and extend the previous reports on the evidence for the presence of LH receptor in Leydig cell precursors (Moore & Morris 1993, Tena-Sempere *et al.* 1997), which remained controversial. Careful analysis of the results of the present study and our earlier studies (Sriraman *et al.* 2000) with other reports (Teerds 1989*a*,*b*) reveal two waves of Leydig cell proliferation, one which occurs independently of LH which subsequently forms progenitor Leydig cells, and the next wave of proliferation occurs under the control of LH and other factors that determine the final Leydig cell number in the testes. The observed requirement of LH in adult Leydig cell development is different from development of fetal Leydig cells, which proliferate and differentiate independently of LH (Baker & O'Shaughnessy 2001).

Endogenous neutralization of FSH led to a reduction in steroidogenic capacity of Leydig cells with a decrease in the steady-state mRNA levels of 17α-hydroxylase and cholesterol side chain cleavage enzyme. The observed reduction in steroidogenesis was not as drastic as that of LH deprival. There was also a significant reduction in steady-state mRNA levels of StAR, which transports cholesterol from the cytosol to the mitochondria, and it is a key regulatory step in acute regulation of steroidogenesis (Stocco 1996). Moreover, a reduction in the steady-state mRNA levels of LH receptor and IGF-I was observed following FSH neutralization. It has been well documented that IGF-I secreted by the Leydig cells can act in

an autocrine fashion to induce LH receptor and enhance proliferation and steroidogenesis (Lin 1996), and that FSH causes the prepubertal rise in IGF-I in the testis (Closset *et al.* 1989). Thus it is possible that neutralization of FSH resulted in a decrease in the stimulatory factors derived from Sertoli cells which are capable of enhancing the message for LH receptor and IGF-I, which in turn stimulates steroidogenesis in the Leydig cells. It is necessary to emphasize here that the observed effects are only due to FSH neutralization and more importantly the antiserum employed had no contaminating LH antibodies, which was established by the lack of any effect on the nocturnal rise in serum testosterone when administered to adult bonnet monkeys, which is sensitive to LH deprival (Mukku *et al.* 1981). It is important to note that the LH levels are very high following EDS treatment (peaks by 3 weeks about 6-fold over the normal levels and starts declining thereafter to reach normal levels by 6 weeks) due to lack of feedback inhibition by testosterone (Tena-Sempere *et al.* 1997) and interestingly the decrease in Leydig cell function observed following FSH deprivation is when the levels of LH are high due to reduction in testosterone levels following EDS treatment. Previous studies employing EDS-treated rats have clearly shown that negative feedback of testicular factors on LH secretion is mediated completely through changes in gonadotropin-releasing hormone action via testosterone (Tena-Sempere *et al.* 1995) and hence the observed effects are not due to alterations in LH levels following FSH antiserum treatment. These results clearly establish that FSH has an important role in regulation of Leydig cell function indirectly during regeneration following EDS treatment, and this is in clear contradiction to the results obtained by Moolenar *et al.* (1986).

It is important to note that the earlier studies employed EDS-treated models to investigate the role of LH and FSH by hypophysectomy or administering steroids, but all these approaches suffer from one or more disadvantages. By administering steroids one cannot control the extent of inhibition of LH and the direct effect due to steroids. The passive neutralization approach has the unique advantage of depriving a specific hormone for a desired period of time. The usefulness of specific antibodies to investigate the role of LH and FSH in rats and monkeys has been well established earlier (Rao *et al.* 1970, Moudgal *et al.* 1974, Selvaraj & Moudgal 1994). Earlier studies have also demonstrated that immunoneutralization leads to complete neutralization of the endogenous hormone, bringing the free hormone levels below the level of detection and the administered excess antibody stays in the circulation (Medhamurthy *et al.* 1995, Meachem *et al.* 1998, 1999). Considering the fact that levels of LH and FSH were high after EDS treatment, the antiserum was always administered in excess, which was confirmed by the detection of free antibodies in serum. Moreover, all the studies were carried out with purified Leydig cells from EDS-treated rats by a method validated by Klinefelter *et al.* (1987) and Risbridger & Davies (1994), in contrast to the other studies which were carried out with interstitial cells or testicular slices. To conclude, the present study has provided conclusive evidence for a stage in Leydig cell development which is independent of LH and the absolute need for LH in functional differentiation. Also, the importance of FSH in regulation of Leydig cell function through its action on the Sertoli cells has been demonstrated.

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