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Corticosterone Induces Steroidogenic Lesion in Cultured Adult Rat Leydig Cells by Reducing the Expression of StAR Protein and Steroidogenic Enzymes

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Abstract The present study was designed to investigate the dose-dependent direct effect of corticosterone on adult rat Leydig cell steroidogenesis in vitro. Leydig cells were isolated from the testis of normal adult male albino rats, purified on discontinuous Percoll gradient and plated in culture plates/flasks overnight at 34°C in a CO_2 incubator under 95% air and 5% CO_2 using DME/F12 medium containing 1% fetal bovine serum. After the attachment of cells, serum-containing medium was removed and cells were exposed to different doses (0, 50, 100, 200, 400, and 800 nM) of corticosterone using serum-free fresh medium for 24 h at 34°C. At the end of exposure period, cells were utilized for assessment of the activities and mRNA expression of steroidogenic enzymes (cytochrome P_{450} side chain cleavage enzyme, 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and cytochrome P_{450} aromatase) and steroidogenic acute regulatory protein gene expression. Testosterone and estradiol production were also quantified. Activities of cytochrome P_{450} side chain cleavage enzyme, 3β- and 17β-hydroxysteroid dehydrogenases were declined significantly in a dose-dependent manner after corticosterone exposure, while their mRNA expression were significantly reduced at higher doses of corticosterone exposure. The activity and mRNA expression of cytochrome P_{450} aromatase registered a significant increase at 100 nM dose of corticosterone whereas at 200–800 nM doses both the activity as well as the mRNA levels was significantly reduced below the basal level. StAR protein gene expression was significantly inhibited by higher doses of corticosterone employed. At all doses employed, corticosterone significantly reduced the production of testosterone by Leydig cells, while estradiol level registered a significant increase at 50 and 100 nM doses but at higher doses, it registered a significant decrease when compared to basal level. It is concluded from the present in vitro study that the molecular mechanism by which corticosterone reduces the production of Leydig cell testosterone is by reducing the activities and mRNA expression of steroidogenic enzymes and steroidogenic acute regulatory protein.
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Key words: Leydig cell; corticosterone; steroidogenic enzymes; steroidogenic acute regulatory protein; testosterone production

In male mammals, testicular Leydig cells are the pre-eminent source of testosterone, and the primary regulator of testosterone biosynthesis in Leydig cells is the gonadotropin, LH [Odell et al., 1974]. Opposing the stimulatory action of LH, corticosterone directly inhibits Leydig cell steroidogenesis through a glucocorticoid receptor-mediated process [Welsh et al., 1982; Stalker et al., 1989; Orr and Mann, 1992]. The inhibition by corticosterone occurs independently of changes in circulating levels of LH [Gao et al., 1996]. T levels decline during stress, when glucocorticoid levels are increased [Monder et al., 1994]. The decline in testosterone production is due in part to a direct stress-induced effect of corticosterone on Leydig cells [Cumming et al., 1983; Cooke et al., 1992; Monder et al., 1994; Gao et al., 1996].

The steroidogenic capacity of individual Leydig cell and the total number of Leydig cells

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per testis set the levels of testosterone in the circulation. Glucocorticoid inhibits the expression of testosterone biosynthetic enzymes [Payne and Sha, 1991; Orr et al., 1994] and thereby affects the steroidogenic capacity of Leydig cells. Addition of dexamethasone decreased the 3β-hydroxysteroid dehydrogenase (3β-HSD) activity in long-term culture of Leydig cells from adult rat testis [Agular et al., 1992; Agular and Vind, 1995]. In addition, it is reported that dexamethasone reduced the basal 3β-HSD mRNA content of mouse Leydig cells [Payne and Sha, 1991].

Glucocorticoids specifically decrease the constitutive and cAMP-induced synthesis of cytochrome P_{450} side chain cleavage (P_{450}scc) protein as well as the accumulation of its mRNA in mouse Leydig cell cultures. Cells cultured with as little as 10 nM dexamethasone resulted in a 50–60% decrease in the rate of synthesis of P_{450}scc protein and mRNA content [Hales and Payne, 1989]. On the contrary, glucocorticoid has been shown to impart an opposite effect in MA-10 cells (Mouse Leydig Tumor cells) causing a 1.7-fold increase in the rate of P_{450}scc synthesis and a 2.1-fold increase in the amount of P_{450}scc mRNA [Hales et al., 1990]. Acute immobilization stress, which results in increased endogenous corticosterone production, inhibited the activities of 17α-hydroxylase and 17, 20-lyase in Leydig cells of rats without affecting the binding of LH/hCG receptors [Orr et al., 1994]. Previous studies from our laboratory have shown that chronic corticosterone administration to adult rats resulted in diminished activities of Leydig cell 3β- and 17β-HSDs, inhibition of LH-stimulated cAMP and testosterone production and reduced number of cell surface LH receptors [Sankar et al., 2000a,b].

Dexamethasone (1–1,000 ng/ml) was shown to inhibit the LH (10 ng/ml)-induced steroidogenic acute regulatory (StAR) protein synthesis in rat preovulatory follicles in a dose-dependent manner. It also reduced the level of StAR protein induced by forskolin (10^{-5} M) and 8-bromo cAMP (0.5 mM) in rat preovulatory follicles [Huang and Shirley, 2001]. Dexamethasone treatment increases endogenous Dax-1 expression and concordantly decreases StAR expression in H295 human adrenocortical carcinoma cells as well as in primary culture of adrenal cells derived from B6/SJL F2 mice [Gummow et al., 2006].

Eventhough the above-mentioned reports point out the adverse effects of glucocorticoid on one or two steroidogenic enzymes in Leydig cells and StAR protein expression in other cell types, the direct dose-dependent effect of corticosterone on StAR protein and steroidogenic enzymes' activities and gene expression in adult rat Leydig cells remains largely unknown. Therefore, the present study was designed to assess the dose-dependent direct effect of corticosterone on activities and mRNA expression of steroidogenic enzymes, StAR protein gene expression and testosterone production in cultured adult rat Leydig cells.

MATERIALS AND METHODS

Animals

Healthy adult male albino rats (110–120 days old) weighing 200–225 g were maintained in clean polypropylene cages in a well ventilated animal house under 12 h light:12 h dark schedule and fed with standard rat chow and drinking water ad libitum. The Institutional Animal Ethical Committee (IAEC) approved the animal handling procedures of the present study.

Chemicals

Dulbecco's modified eagle's medium/Ham F-12 (1:1) nutrient mixture (DMEM/F-12), Percoll, collagenase type IV, pregnenolone, testosterone, estradiol, penicillin–streptomycin solution, amphotericin B solution, bovine serum albumin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H-25,26]-hydroxycholesterol was obtained from NEN Life Sciences Products. ^{3}H-testosterone and ^{3}H-androstenedione were procured from Amersham Biosciences Ltd. (Birmingham, UK). StAR protein antibody (mouse polyclonal) was a generous gift from Prof. D.M. Stocco, Texas Tech University, USA and antibody (mouse monoclonal) for β-actin was purchased from Sigma Chemical Co. Enhanced chemiluminescence (ECL) solution was purchased from Pierce Biotechnology, Inc. (Rockford, IL). Total RNA isolation reagent (TRIR) was purchased from ABgene (Surrey, UK) and one-step reverse-transcriptase polymerase chain reaction kit was procured from QIAGEN (Hilden, Germany). Solid-phase

radioimmunoassay kits for testosterone and estradiol were procured from DiaSorin (Saluggia, Italy). Gene specific oligonucleotide primers for steroidogenic enzymes, StAR protein, β-actin and RPS-16 were obtained from Integrated DNA Technologies (Coralville, IA). Other chemicals and reagents used are of analytical grade and were purchased from SISCO Research Laboratories (Mumbai, India).

Isolation and Purification of Leydig Cells

Leydig cells were isolated as previously described [Rigaudiere et al., 1988]. Briefly, the rats were anaesthetized, and killed by decapitation, testes were removed, decapsulated aseptically, and digested in collagenase containing medium (0.25 mg/ml) at 37°C for 30 min in a shaking water bath. The resulting crude Leydig cell preparations were further purified on discontinuous Percoll gradients. The purity of Leydig cells was assessed by histochemical staining for 3β-HSD activity [Aldred and Cooke, 1983] and viability was determined by trypan blue dye exclusion. The purity of Leydig cells was 80–90% and viability was 85–90%.

Leydig Cell Culture and Corticosterone Exposure

Purified Leydig cells were plated in culture plates/flasks overnight at 34°C in a CO_2 incubator under 95% air and 5% CO_2 using DME/F12 medium containing 1% fetal bovine serum. After the attachment of cells, serum-containing medium was removed and cells were exposed to different doses (0, 50, 100, 200, 400, and 800 nM) of corticosterone using serum-free fresh medium for 24 h at 34°C. At the end of exposure period, cells were utilized for assessment of the activities and mRNA expression of steroidogenic enzymes (cytochrome P_{450} side chain cleavage enzyme, 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and cytochrome P_{450} aromatase) and steroidogenic acute regulatory protein gene expression.

Hormone Assays

Testosterone and estradiol in cell pellet and culture media were assayed using solid-phase RIA kits from DiaSorin (Saluggia, Italy). The testosterone assay had the sensitivity of 0.04 ng/ml with inter- and intra-assay coefficients of variation at 4–11% and 7.3–11%, respectively. Cross-reactivity of the testosterone antiserum with estradiol was 0.02%. Estradiol assay had sensitivity of 5 pg/ml with inter- and intra-assay coefficients of variation at 4.2–7.4% and 6.8–9.7%, respectively. Cross-reactivity of estradiol antiserum with testosterone was 0.001%.

Assay of Cytochrome P_{450} Side Chain Cleavage Enzyme Activity

P_{450}scc activity in Leydig cells was determined by the method previously described [Georgiou et al., 1987]. Briefly, after corticosterone exposure, the cells were incubated with 5 μM (0.5 μCi) of [26,27-^{3}H]-hydroxycholesterol dissolved in 100 nM dimethyl sulfoxide for 1 h at 34°C. The reaction was stopped by the addition of 0.1 ml of 1 N NaOH and [^{14}C]-isocaproic acid was added as a recovery standard. The medium was removed from the extraction tube and the culture plate was washed with 1 ml alkalinized medium, which was combined with the original 1 ml and extracted aqueous phase was vortexed with 0.8 g neutral alumina for 1 min followed by centrifugation at 1,200*g* for 25 min. The supernatant aqueous phase (0.4 ml) was transferred to scintillation vials and the radioactivity was measured using a beta counter.

Assay of 3β-Hydroxysteroid Dehydrogenase Enzyme Activity

Leydig cells exposed to corticosterone were immediately processed for the assay of 3β-HSD enzymatic activity based on an established spectrophotometric method [Bergmeyer, 1974]. Briefly, Leydig cells (2×10^6 cells) were homogenized, sonicated in ice-cold Tris-HCl buffer (0.1 μM; pH 7.2) and centrifuged at 16,000*g* for 5 min at 4°C. The supernatant was taken as the enzyme extract. The reaction mixture contained 0.6 ml pyrophosphate buffer (100 μM), 0.2 ml of NAD (0.5 μM), 2 ml distilled water, and 0.1 ml of pregnenolone (0.1 μM). After the addition of the enzyme extract, the absorbance at 340 nm was measured at 20 s intervals for 5 min in a spectrophotometer against blank, which contains all the components except the enzyme extract.

Assay of 17β-Hydroxysteroid Dehydrogenase Enzyme Activity

17β-HSD activity was assessed based on the radiometric method described previously [Murono, 1990]. Briefly, the cells were incubated with [^{3}H]-androstenedione (10 μM/0.5 μCi) for 1 h at 34°C. The reaction was terminated by

the addition of 0.1 ml of 1 N NaOH. An aliquot of 100 μg of each androstenedione and testosterone was added as carriers. To estimate recovery, 4,000 cpm. of [^{3}H]-testosterone was processed separately. Samples were extracted with five volumes of diethyl ether and the residue of ether extracts was subjected to thin layer chromatography using chloroform/methanol (99.5:0.5, v/v) as the solvent system. The product testosterone was localized by anisaldehyde spray, the steroid spot was scraped off, transferred to scintillation vials, and counted using a beta counter.

Assay of Cytochrome P_{450} Aromatase Enzyme Activity

Aromatase activity was assessed by radiometric method described previously [Genti-Raimondi et al., 1993]. In brief, after corticosterone exposure, cells were incubated with a saturating concentration of [1,2,6,7-^{3}H]-testosterone (1 μM/0.5 μCi) for 1 h at 34°C. At the end of incubation, steroids were extracted with five volumes of diethylether, 40 μg unlabeled testosterone and estradiol were added as carriers to the organic phase and evaporated under nitrogen. The residues were dissolved in 100 μl chloroform–methanol (1:1, v/v), and subjected to thin layer chromatography, using cyclohexane:ethyl acetate (1:1, v/v) as the solvent system. The product testosterone was localized using anisaldehyde spray, the steroid spots were scraped off, transferred to scintillation vials and counted using a beta counter. The loss of tritium from 1β- and 2β-atoms as tritiated water during aromatization reaction was also determined and this value was included for the calculation of aromatase activity.

Western Blot Analysis of StAR Protein Expression

Corticosterone exposed Leydig cells were washed once with ice-cold phosphate buffered saline (PBS) to remove excess culture medium and 500 μl of ice-cold lysis buffer with protease inhibitor cocktail was added. The cells were lysed on ice by keeping them in a rocker for 30 min. The cell lysates were transferred into a 1.5 ml microcentrifuge tube and centrifuged at 12,000*g* for 15 min at 4°C. The supernatants were transferred to new microcentrifuge tubes and total protein in the cell lysates were estimated by the method described previously [Lowry et al., 1951]. Total protein (100 μg) with 6× sample buffer was boiled for 5 min and the sample mixture was run on 10% SDS–PAGE and electrotransferred onto a nitrocellulose membrane. The membrane was incubated overnight in blocking buffer containing 10% non-fat dry milk. The membrane was then incubated with primary antibody (mouse polyclonal) for StAR (1:500 dilution) for 5 h. After the incubation with primary antibody, the membrane was washed with blocking buffer and again incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000 dilution) for 1.5 h and finally the membrane was washed thrice with tween-20 containing tris buffered saline and tris buffered saline alternatively. At the end of final wash with tris buffered saline, the membrane was exposed to ECL solution. β-Actin was used as the loading control and this protein was detected using primary antibody (mouse monoclonal) for β-actin at 1:1,000 dilution for 3 h and HRP-conjugated secondary antibody at 1:15,000 dilution for 1 h. The chemiluminescence from the protein bands was recorded in a Kodak X-Mat film using a hypercasette. The film was developed and the band intensity of StAR protein was normalized and quantified using quantity one software in a Bio-Rad Gel documentation unit against the band intensity of the internal control, β-actin.

mRNA Expression Analysis

In order to assess the mRNA expression of StAR protein, cytochrome P_{450}scc, 3β- and 17β-HSDs, and cytochrome P_{450} aromatase, total RNA was isolated from Leydig cells exposed to corticosterone using total RNA isolation reagent. The concentration and purity of total RNA was determined by absorbance at 260/280 nm in a UV-Spectrophotometer. If the ratio of $A_{260/280}$ is 1.8–2.0, then 2 μg of total RNA was used for reverse-transcriptase polymerase chain reaction (RT-PCR) analyses. RT-PCR was carried out using one-step RT-PCR kit in a total reaction volume of 50 μl. The first strand synthesis was carried out at 50°C for 30 min using gene specific oligonucleotide primers for each steroidogenic enzyme and StAR protein followed by the initial PCR activation at 95°C for 15 min. The selection of primer sequences was based on previous publications (Table I). The three step PCR cycles consisted of denaturation at 94°C for 1.5 min, annealing at 57°C for StAR protein, P_{450}scc, 3β- and 17β-HSDs, and 60°C for P_{450} aromatase for 1.5 min and extension at

TABLE I. Oligonucleotide Primers Used for RT-PCR

Name of the gene	Primer sequence	Product size (bp)	References
StAR Protein	Sense 5′-TTG GGC ATA CTC AAC AAC CA-3′ Anti-sense 5′-ATG ACA CCG CTT TGC TCA G-3′	389	Akingbemi et al. [2004]
Cytochrome P_{450} scc	Sense 5′-GAC CAA GGG AAA GGC GT-3′ Anti-sense 5′-GCA TCC ACG ATA CCC TC-3′	688	Sakaue et al. [2002]
3β-HSD	Sense 5′-TTG GTG CAG GAG AAA GAA C-3′ Anti-sense 5′-CCG CAA GTA TCA TGA CAG A-3′	547	Sakaue et al. [2002]
17β-HSD	Sense 5′-TTC TGC AAG GCT TTA CCA GG-3′ Anti-sense 5′-ACA AAC TCA TCG GCG GTC TT-3′	653	Sakaue et al. [2002]
Cytochrome P_{450} aromatase	Sense 5′-GCT TCT CAT CGC AGA GTA TCC CG-3′ Anti-sense 5′-CAA GGG TAA ATT CAT TGG GCT TGG-3′	290	Geniuses and Carreau, [2001]
β-Actin	Sense 5′-GCC ATG TAC GTA GCC ATC CA-3′ Anti-sense 5′-GAA CCG CTC ATT GCC GAT AG-3′	374	Oaks and Raff, [1995]
RPS-16	Sense 5′-AAG TCT TCG GAC GCA AGA AA-3′ Anti-sense 5′-TTG CCC AGA AGC AGA ACA G-3′	148	Shan et al. [1995]

72°C for 3 min. The PCR amplification was carried out up to 30 cycles and to ensure that the products are extended completely, a final extension at 72°C for 10 min was carried out. Gene specific oligonucleotide primers for the house-keeping gene, β-actin/ribosomal protein S-16 (RPS-16) was added to the same PCR reaction vial and co-amplified simultaneously. RT-PCR product (5 μl) was taken from each reaction tube, mixed with gel loading dye and resolved in a standard 2% agarose gel containing ethidium bromide (0.5 μg/ml) under an electrical field (60 mA and 80 V) for 2.5 h. Molecular weight DNA marker (100 bp ladder) was simultaneously resolved in the first lane. After electrophoresis, the gel was subjected to densitometric scanning and the band intensity of cDNA fragment of each gene of interest was normalized against the band intensity of cDNA fragment of the house keeping gene β-actin or RPS-16 using quantity one software (Bio-Rad).

Statistical Analysis

The data were analyzed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test to assess the significance between the control and experimental groups. Statistical significance was considered at the level of $P < 0.05$.

RESULTS

Dose-Dependent Effect of Corticosterone on StAR Protein mRNA Expression in Adult Rat Leydig Cells In Vitro

When compared with basal, 50 and 100 nM doses of corticosterone did not induce any change in the mRNA levels of StAR protein whereas at 200–800 nM doses, it significantly reduced the same. Nevertheless, no dose-dependent change in StAR protein mRNA level was noticed between 400 and 800 nM doses (Fig. 1a,b).

Dose-Dependent Effect of Corticosterone on StAR Protein Expression in Adult Rat Leydig Cells In Vitro

The level of StAR protein was significantly decreased by corticosterone at 100–800 nM concentrations when compared to basal with a maximum reduction at 800 nM dose while 50 nM dose did not induce any change (Fig. 2a,b).

Dose-Dependent Effect of Corticosterone on Cytochrome P_{450} Side Chain Cleavage mRNA Expression in Adult Rat Leydig Cells In Vitro

Corticosterone did not induce any change in the P_{450}scc mRNA level at 50 nM dose whereas at 100–800 nM concentrations, it significantly decreased the same in a dose-related fashion (Fig. 3a,b).

Dose-Dependent Effect of Corticosterone on Cytochrome P_{450} Side Chain Cleavage Enzyme Activity in Adult Rat Leydig Cells In Vitro

Corticosterone, at all doses employed, significantly decreased the activity of cytochrome P_{450}scc in a dose-dependent manner and maximum reduction in the activity was recorded at 800 nM (Fig. 4).

Dose-Dependent Effect of Corticosterone on 3β- and 17β-Hydrxoysteroid Dehydrogenases mRNA Expression in Adult Rat Leydig Cells In Vitro

When compared with basal, corticosterone reduced the levels of 3β-HSD mRNA significantly

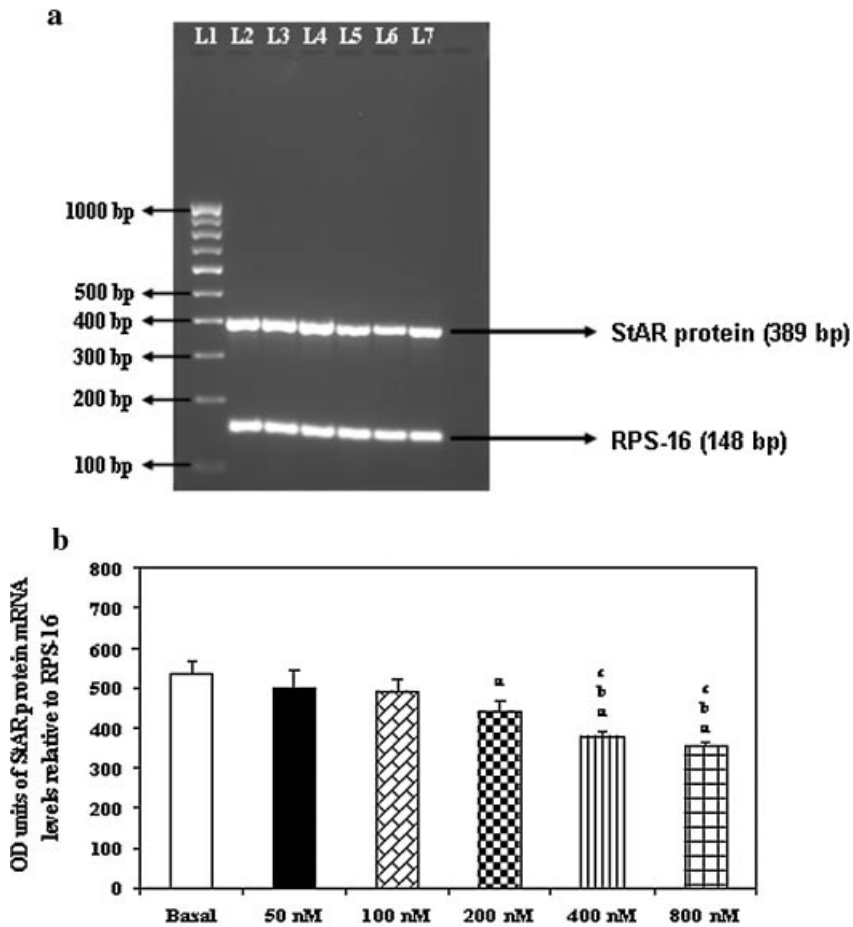

Fig. 1. **a**,**b**: Dose-dependent effect of corticosterone on StAR protein mRNA expression in adult rat Leydig cells in vitro, L1: 100 bp DNA marker; L2: Basal; L3: 50 nM corticosterone; L4: 100 nM corticosterone; L5: 200 nM corticosterone; L6: 400 nM corticosterone; L7: 800 nM corticosterone. Each bar represents mean ± SEM of three separate experiments. Significance at $P < 0.05$. a: compared with basal; b: compared with 50 nM; c: compared with 100 nM.

at all doses employed and maximum reduction in mRNA level was seen at 800 nM dose. When compared with basal, 50 nM dose did not induce any change whereas other doses (100–800 nM) employed significantly decreased 17β-HSD mRNA levels (Fig. 5a,b).

Dose-Dependent Effect of Corticosterone on 3β- and 17β-Hydrxoysteroid Dehydrogenases Activity in Adult Rat Leydig Cells In Vitro

When compared to basal, corticosterone exposure significantly diminished the activity of 3β-HSD at all doses employed. Nevertheless, the magnitude of decrease in the activity remains same between 400 and 800 nM doses. 17β-HSD activity declined in a dose-dependent manner after corticosterone exposure. Nevertheless, the reduction in the activity did not vary between 400 and 800 nM doses (Fig. 6a,b).

Dose-Dependent Effect of Corticosterone on Cytochrome P_{450} Aromatase mRNA Expression in Adult Rat Leydig Cells In Vitro

When compared with basal, 50 nM dose did not induce any change in the level of aromatase mRNA whereas 100 nM dose significantly increased the same. Corticosterone at 200–800 nM concentrations significantly reduced the aromatase mRNA levels (Fig. 7a,b).

Dose-Dependent Effect of Corticosterone on Cytochrome P_{450} Aromatase Activity in Adult Rat Leydig Cells In Vitro

Corticosterone, at 50 and 100 nM doses significantly increased the activity of P_{450} aromatase when compared with basal whereas at 200–800 nM doses, it markedly decreased the activity even below the basal level (Fig. 8).

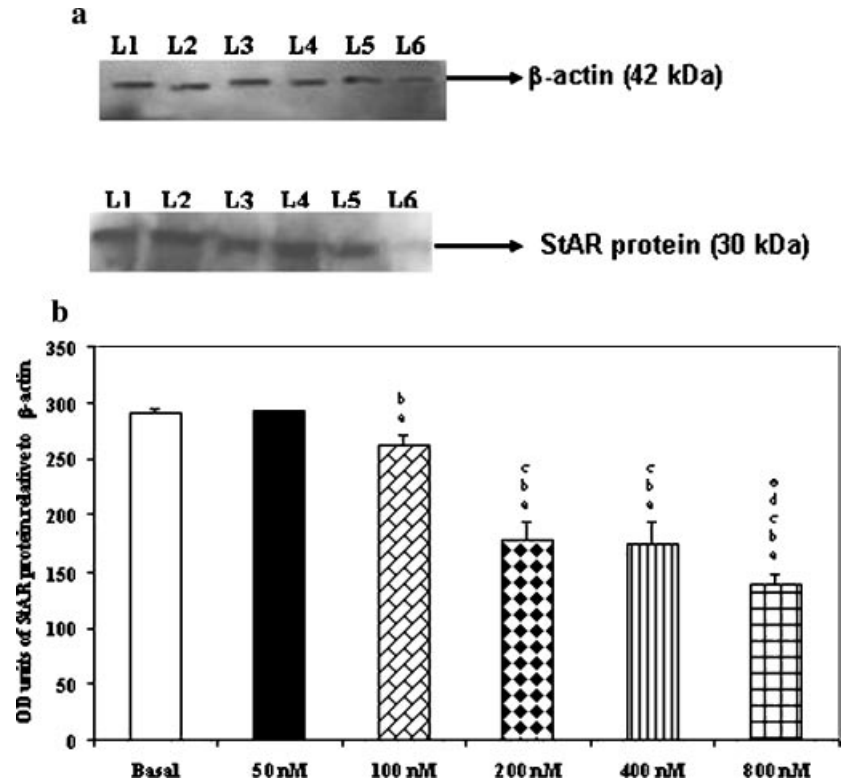

Fig. 2. **a**,**b**: Dose-dependent effect of corticosterone on StAR protein expression in adult rat Leydig cells in vitro, L1: Basal; L2: 50 nM corticosterone; L3: 100 nM corticosterone; L4: 200 nM corticosterone; L5: 400 nM corticosterone; L6: 800 nM corticosterone. Each bar represents mean ± SEM of three separate experiments. Significance at $P < 0.05$. a: compared with basal; b: compared with 50 nM; c: compared with 100 nM; d: compared with 200 nM; e: compared with 400 nM.

Dose-Dependent Effect of Corticosterone on Testosterone and Estradiol Production by Adult Rat Leydig Cells In Vitro

At all doses of corticosterone, testosterone levels registered a significant reduction when compared with the basal. Nevertheless, reduction in the levels of testosterone between 400 and 800 nM doses did not vary significantly. Unlike testosterone, estradiol levels exhibited a significant increase in both 50 and 100 nM doses and thereafter it declined significantly below the basal level (Fig. 9a,b).

DISCUSSION

The data on testosterone and estradiol suggest that corticosterone has a direct dose-dependent effect on the synthesis and secretion of testosterone and estradiol in Leydig cell. The decrease in testosterone may be attributed to reduction in the binding of LH to its cell surface receptors recorded in the present study (data not shown), since it is the major stimulator of Leydig cell steroidogenesis [Dufau, 1988; Saez, 1994]. Corticosterone exhibits a biphasic effect on estradiol production. Low doses (50 and 100 nM) increased the secretion of estradiol whereas higher doses (200, 400, and 800 nM) decreased the same. The increase in estradiol at low doses may be due to enhanced conversion of testosterone and androstenedione to estradiol and the decrease in estradiol titer at high doses of corticosterone may be the result of a decrease in the availability of steroid precursors, testosterone/androstenedione due to diminution in steroidogenic enzymes' activities. In addition to this, it may also be contributed by impaired activity of P_{450} aromatase in Leydig cells, as higher doses of glucocorticoids were shown to decrease the activity of aromatase in human adipocytes [McTernan et al., 2002]. In the present study also both the activity and mRNA expression of aromatase were increased at 50 and 100 nM doses of corticosterone, whereas at 200–400 nM concentrations both the parameters were significantly decreased below the

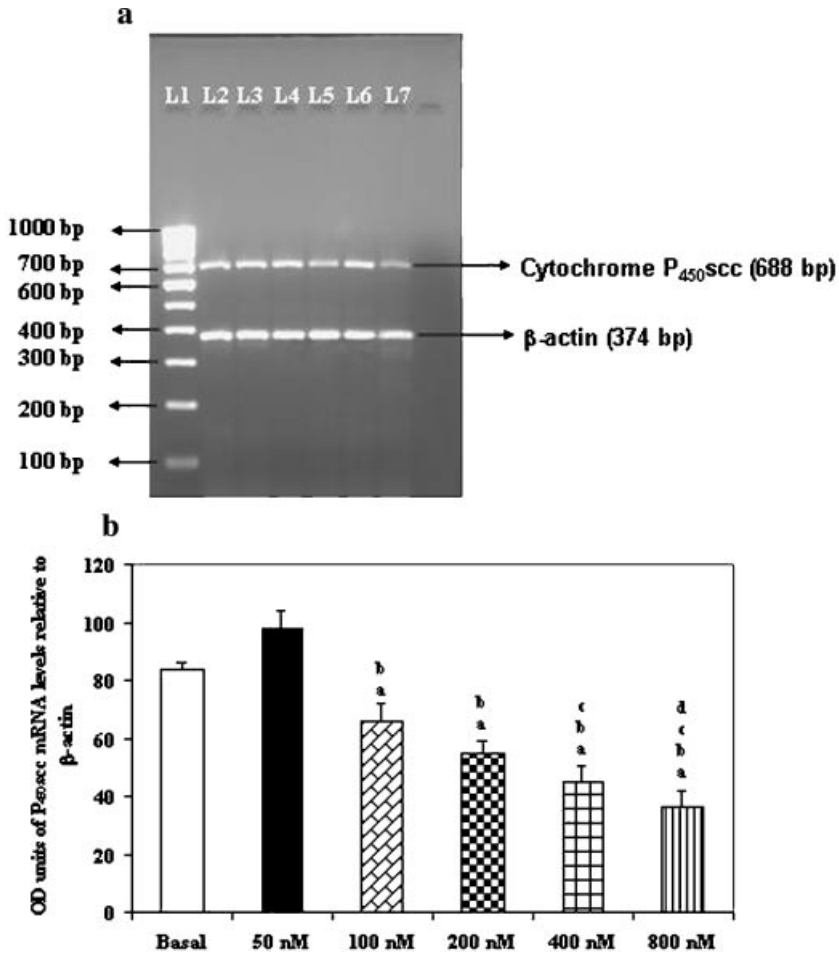

Fig. 3. **a**,**b**: Dose-dependent effect of corticosterone on cytochrome P_{450} side chain cleavage mRNA expression in adult rat Leydig cells in vitro, L1: 100 bp DNA marker; L2: Basal; L3: 50 nM corticosterone; L4: 100 nM corticosterone; L5: 200 nM corticosterone; L6: 400 nM corticosterone; L7: 800 nM corticosterone. Each bar represents mean ± SEM of three separate experiments. Significance at $P < 0.05$. a: compared with basal; b: compared with 50 nM; c: compared with 100 nM; d: compared with 200 nM.

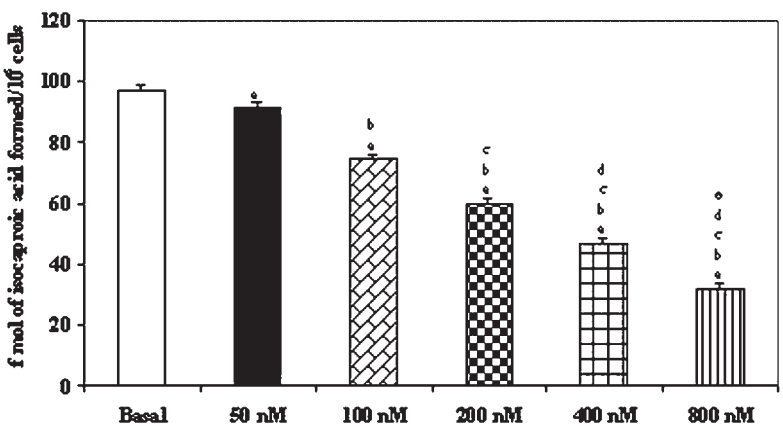

Fig. 4. Dose-dependent effect of corticosterone on cytochrome P_{450} side chain cleavage enzyme activity in adult rat Leydig cells in vitro. Each bar represents mean ± SEM of six observations. Significance at $P < 0.05$. a: compared with basal; b: compared with 50 nM; c: compared with 100 nM; d: compared with 200 nM; e: compared with 400 nM.

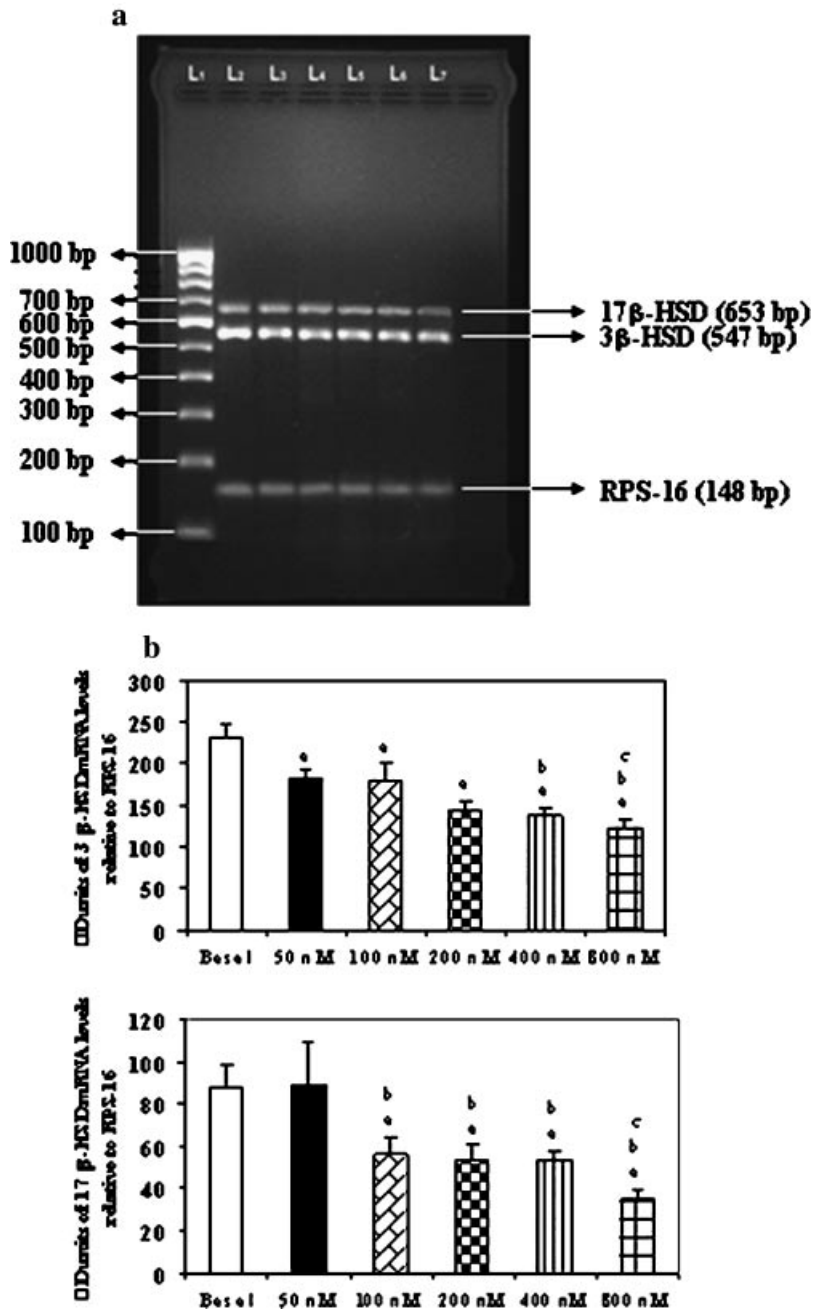

Fig. 5. **a**,**b**: Dose-dependent effect of corticosterone on 3β- and 17β-hydroxysteroid dehydrogenases mRNA expression in adult rat Leydig cells in vitro, L1: 100 bp DNA marker; L2: Basal; L3: 50 nM corticosterone; L4: 100 nM corticosterone; L5: 200 nM corticosterone; L6: 400 nM corticosterone; L7: 800 nM corticosterone. Each bar represents mean ± SEM of three separate experiments. Significance at $P < 0.05$. a: compared with basal; b: compared with 50 nM; c: compared with 100 nM; d: compared with 200 nM.

basal level. Furthermore, the decrease in testosterone output may also be attributed to the reduction in StAR protein gene expression and decreased mRNA expression and activities of steroidogenic enzymes in Leydig cells.

The data on StAR protein gene expression suggest that corticosterone has an adverse effect on the same. StAR protein mRNA as well as the protein expression were significantly decreased by corticosterone exposure barring no change in StAR protein mRNA expression at 50 nM dose. The function of StAR protein is one of the rate-limiting steps in Leydig cell steroidogenesis as it is involved in the transfer of

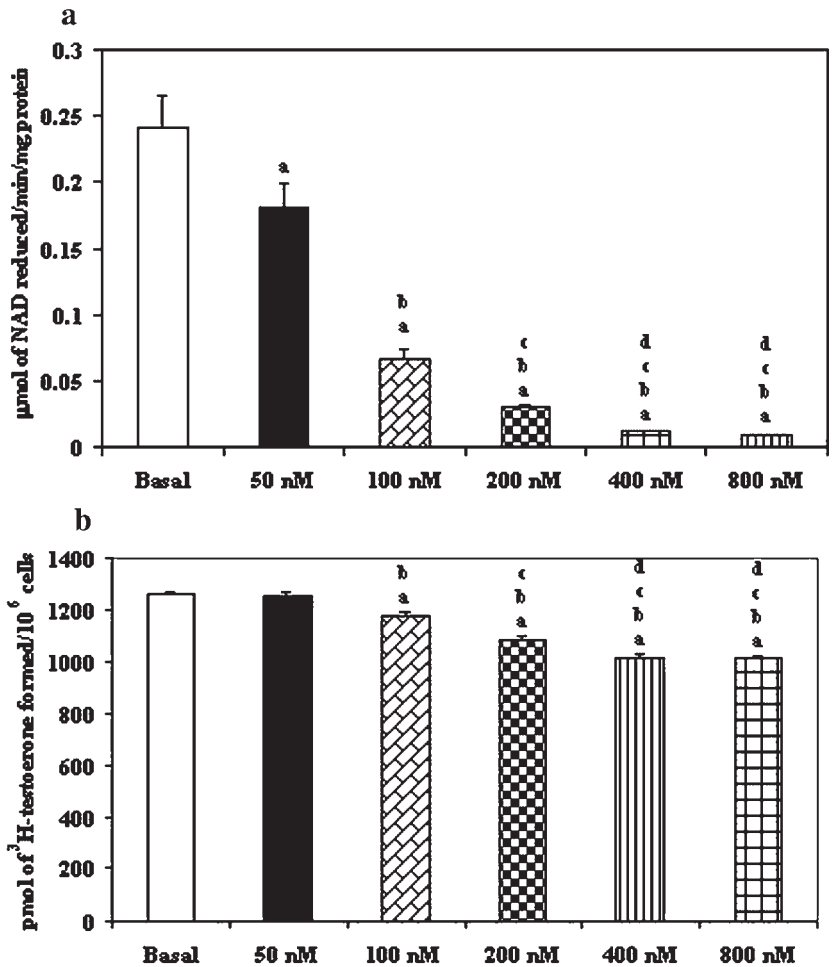

Fig. 6. **a**,**b**: Dose-dependent effect of corticosterone on 3β- and 17β-hydroxysteroid dehydrogenases activity in adult rat Leydig cells in vitro. Each bar represents mean ± SEM of six observations. Significance at $P < 0.05$. a: compared with basal; b: compared with 50 nM; c: compared with 100 nM; d: compared with 200 nM.

cholesterol from outer to the inner mitochondrial membrane for the production of pregnenolone by a mitochondrial enzyme cytochrome P_{450}scc [Miller, 1988, 2007]. Dexamethasone (100 nM), a synthetic glucocorticoid has been shown to reduce the mRNA expression of StAR protein in human H295R adrenocortical cell line as well as in primary cells of mouse adrenal and this reduction seems to be mediated through an increase in the transcription factor, DAX-1, which is a negative regulator of StAR protein gene transcription [Gummow et al., 2006]. This mechanism might hold good for the changes recorded in the present study on StAR protein mRNA expression. Moreover, glucocorticoid has been shown to reduce the translation of mRNAs by inhibiting the activity of eukaryotic translation initiation factor eIF-4 [Shah et al., 2000]. Dexamethasone (100 nM) also inhibits the translation of various ribosomal protein mRNAs such as 18s, 28s and L32 in P1798 murine lymphosarcoma cells [Meyuhas et al., 1987]. In view of these findings, it is suggested that the reduction in StAR protein gene expression in Leydig cells after corticosterone exposure may be due to the negative influence of corticosterone on the StAR gene transcription and translation.

Cytochrome P_{450}scc is involved in the conversion of cholesterol (C-27 steroid hormone precursor) to pregnenolone (C-21 steroid hormone precursor) inside the mitochondria and it is one of the rate limiting enzymes in Leydig cellular steroidogenic pathway [Dufau, 1988; Saez, 1994]. In the present study, corticosterone significantly reduced the levels of P_{450}scc mRNA in Leydig cells. In accordance with this, dexamethasone was shown to decrease the

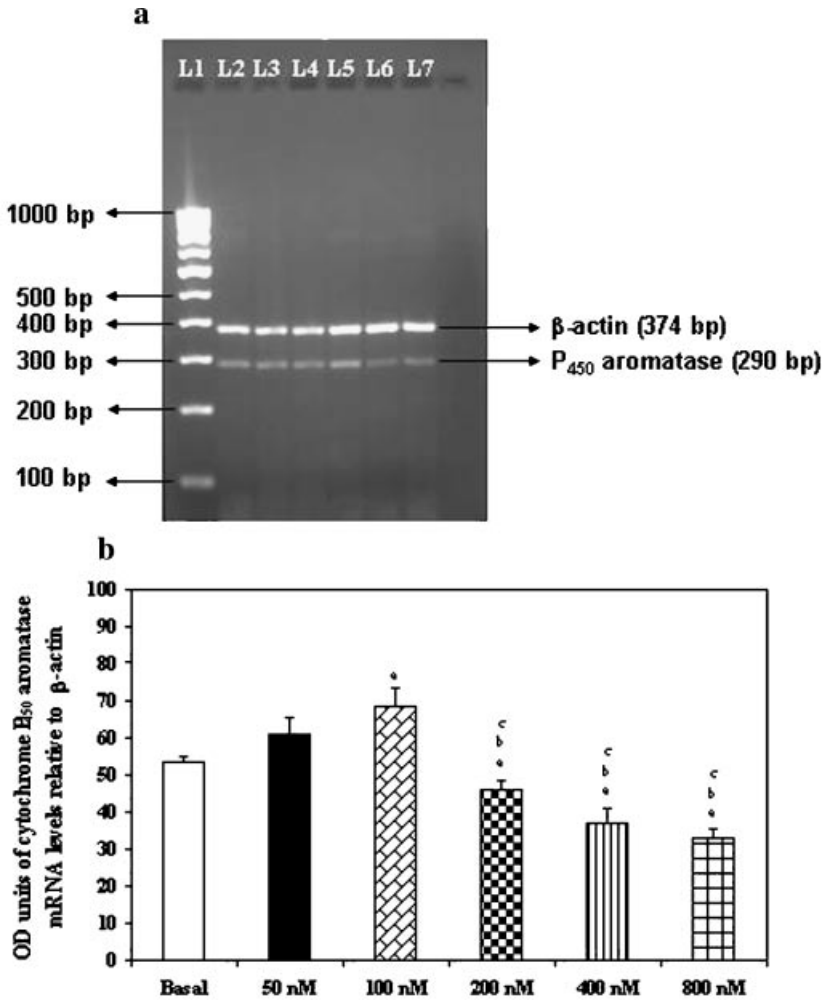

Fig. 7. **a**,**b**: Dose-dependent effect of corticosterone on cytochrome P_{450} aromatase mRNA expression in adult rat Leydig cells in vitro, L1: 100 bp DNA marker; L2: Basal; L3: 50 nM corticosterone; L4: 100 nM corticosterone; L5: 200 nM corticosterone; L6: 400 nM corticosterone; L7: 800 nM corticosterone. Each bar represents mean ± SEM of three separate experiments. Significance at $P < 0.05$. a: compared with basal; b: compared with 50 nM; c: compared with 100 nM.

levels of P_{450}scc mRNA in mouse Leydig cell cultures [Hales and Payne, 1989]. Reduction in the activity of this enzyme may be due to decreased synthesis of the same as its mRNA was markedly reduced after corticosterone exposure. In this regard, dexamethasone was also shown to reduce P_{450}scc protein content in mouse Leydig cell cultures [Hales and Payne, 1989]. A previous study from our laboratory has shown that chronic corticosterone treatment to adult rats decreased the production of NADPH, which is essential for the optimal activity of P_{450}scc enzyme by inhibiting the activities of NADPH-generating enzymes in Leydig cells [Kavitha et al., 2006] and the same may be responsible for the dose-dependent reduction in the activity of this enzyme observed in the present study eventhough the mRNA levels of this enzyme does not show a dose-dependent decrease.

This in vitro study clearly indicates that exposure of Leydig cells to corticosterone suppresses the activities as well as the mRNA expression of 3β- and 17β-HSDs. This is consistent with the previous in vivo study from our laboratory on the activities of 3β- and 17β-HSDs in Leydig cells of adult rats treated with corticosterone [Sankar et al., 2000a]. The conversion of pregnenolone to progesterone catalyzed by 3β-HSD is an important step in Leydig cell steroidogenesis [Tang et al., 1998] and it results in the formation of a precursor for

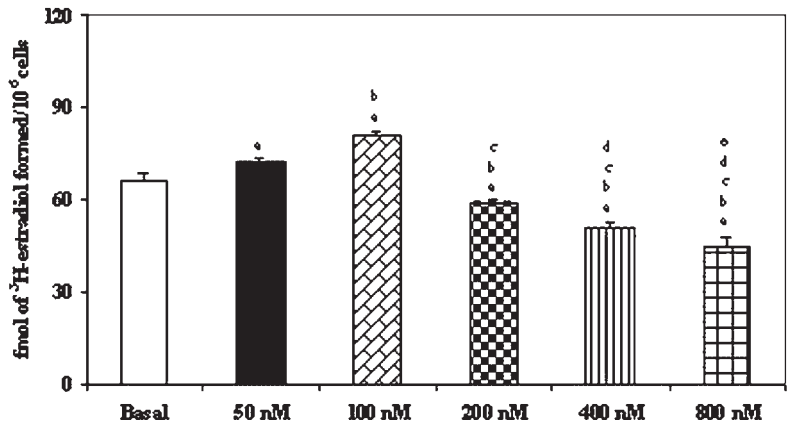

Fig. 8. Dose-dependent effect of corticosterone on cytochrome P_{450} aromatase activity in adult rat Leydig cells in vitro. Each bar represents mean ± SEM of six observations. Significance at $P < 0.05$. a: compared with basal; b: compared with 50 nM; c: compared with 100 nM; d: compared with 200 nM; e: compared with 800 nM.

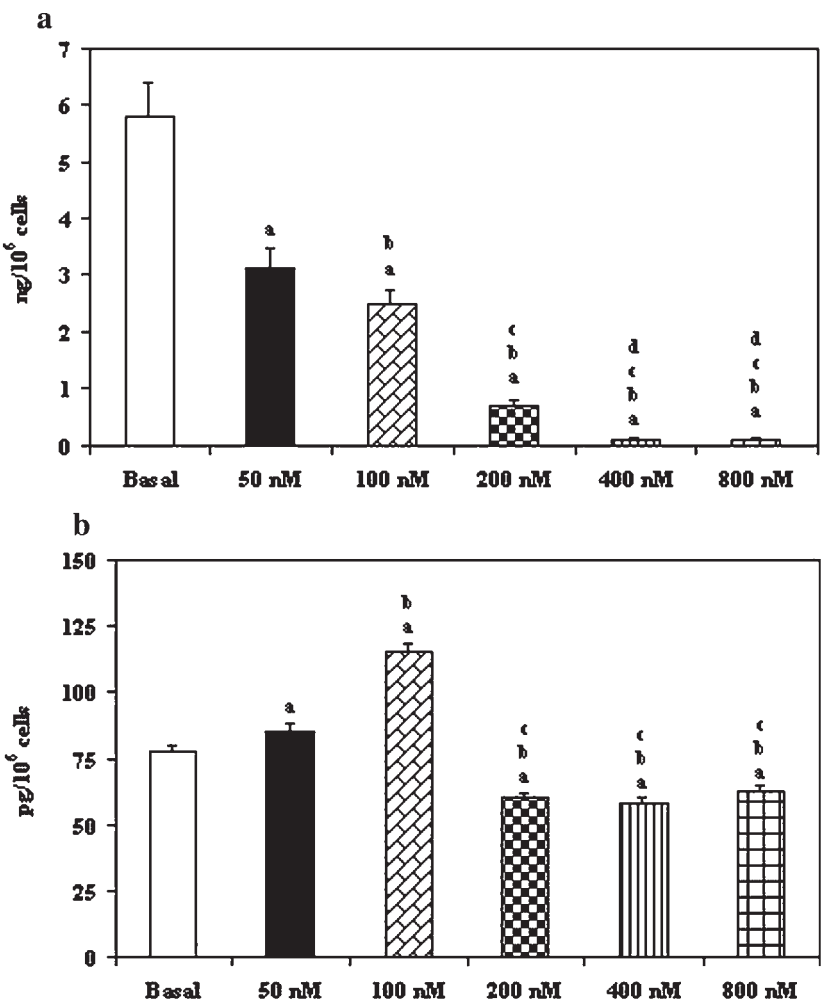

Fig. 9. **a,b**: Dose-dependent effect of corticosterone on testosterone and estradiol production by adult rat Leydig cells in vitro. Each bar represents mean ± SEM of six observations. Significance at $P < 0.05$. a: compared with basal; b: compared with 50 nM; c: compared with 100 nM; d: compared with 200 nM.

testosterone. In support of the present study, evidence can be drawn from the report of Agular and Vind [1995] who have shown that dexamethasone diminished the activity of 3β-HSD in Leydig cells from 3-, 5-, 7-, and 10-week-old rats. The dose-dependent reduction in the activity of 3β-HSD recorded in the present study may be attributed to the direct genomic (reduction in mRNA expression) and non-genomic (reduction in the availability of co-factor) actions of corticosterone.

17β-HSD is involved in the conversion of androstenedione to testosterone and requires ATP, which is produced through glucose oxidation for its optimal activity [Khanum et al., 1997]. A recent in vitro study from our laboratory showed a significant dose-dependent reduction in glucose oxidation in Leydig cells exposed to various doses of corticosterone (unpublished observation). It is therefore suggested that the reduced glucose oxidation would have resulted in impaired ATP production, which in turn might have affected the activity of 17β-HSD. In addition to this, since corticosterone was shown to decrease the availability of NADPH [Kavitha et al., 2006], the co-factor essential for optimal activity of 17β-HSD, the same may be responsible for the decreased activity of 17β-HSD.

The dose-dependent effects of corticosterone on 3β- and 17β-HSDs mRNA expression are not quite comparable with that of the activities observed because the activities of both the enzymes showed significant reduction even among higher doses of corticosterone unlike the mRNA expression. The plausible reason for the increased adverse effect of corticosterone on 3β-HSD activity at higher doses may be due to reduction in the availability of NAD^+, as 3β-HSD is NAD^+-dependent for its activity [Mason et al., 1998]. In addition to this, defective translation of its mRNA could be an additional causative factor. Nonetheless, further studies on the rate of transcription and mRNA stability would be interesting. The possible differential influence of excess corticosterone on levels of mRNA expression and enzyme activity is also evident from the study of Akinbami et al. [1999] who have shown a reduction in the levels of cytochrome P_{450}scc and 3β-HSD proteins in the testis of rats subjected to immobilization stress but the expression of their mRNA remain unchanged. In the present in vitro study, the adverse effect of corticosterone on Leydig cell 3β- and 17β-HSDs mRNA expression was much pronounced at higher doses. In this regard, it is worth to recall the work of Lim et al. [1996] who showed a marked decrease in the mRNA expression of androgen binding protein in Sertoli cells exposed to higher doses of dexamethasone (1 and 10 μM).

Since the promoter regions of rat 3β- and 17β-HSD genes have not yet been fully characterized, the molecular mechanism by which corticosterone would have brought about its inhibitory effect on 3β- and 17β-HSDs gene transcription appears intriguing. There are several mechanisms by which glucocorticoid receptor (GR) can down-regulate transcription have been documented, and different types of negative glucocorticoid response elements (nGREs) within the promoter of target genes have been identified [Newton, 2000]. One of the mechanisms is the binding of ligand-bound, homodimers of GR to GREs consisting palindromic half sites, which result in activation or repression of the target genes [Tsai and O'Malley, 1994]. Another mechanism by which the GR can affect transcription is through protein–protein interactions with heterologous transcription factors independent of this type. In this regard, GR has been shown to modulate AP-1 action by physical association with c-jun [Yang-Yen et al., 1990]. In addition, GR interacts with the CREB [Imai et al., 1993] and GATA-1 [Chang et al., 1993].

In this regard, Olswang et al. [2003] have demonstrated that in 3T3-F442A adipocyte cell lines, glucocorticoid (100 nM) repressed the transcription of phosphoenol pyruvate carboxykinase (PEPCK) gene by inhibiting the CCAAT/enhancer binding protein (C/EBP)-mediated activation. Members of the C/EBP family have been shown to bind directly to the ligand-binding domain of a number of nuclear receptors, including GR [Hu et al., 2001]. These interactions resulted in either induction or inhibition of the target genes and did not necessarily involve binding of GR to the DNA [Boruk et al., 1998]. Furthermore, it has been reported that glucocorticoid-repressible transcription of the gonadotropin-releasing hormone gene is mediated through a multiprotein complex in which glucocorticoid receptor does not directly bind to the negative regulatory region, but rather tethered to DNA bound octamer binding protein (oct-1) [Chandran et al., 1999]. In view of these findings, it is

suggested that excess corticosterone might have impaired the expression of 3β- and 17β-HSDs mRNA by any one of the above said mechanisms.

In the present study, the activity and mRNA expression of cytochrome P_{450} aromatase were significantly increased following 50 and 100 nM doses of corticosterone exposure whereas at 200–800 nM doses, both mRNA expression and activity of the enzyme were significantly reduced. The promoter region of aromatase gene has been reported to possess glucocorticoid response element and glucocorticoid has been shown to regulate the expression of aromatase through its response element [Carreau et al., 2004]. Testosterone and 5α-dihydrotestosterone enhance the expression of aromatase gene in Leydig cells and germ cells of adult rats [Bourguiba et al., 2003] and in addition, androgens positively modulated the epididymal P_{450} aromatase mRNA levels [Shayu and Rao, 2006]. In the present study, Leydig cell testosterone production was found to be significantly low after corticosterone exposure and this implies that the increase in aromatase mRNA levels may be due to the direct genomic effect of corticosterone since dexamethasone has been shown to induce the mRNA expression of aromatase in pachytene spermatocytes of adult rats [Bourguiba et al., 2003]. The activity of P_{450} aromatase also registered a similar trend as that of its mRNA. The increase in the activity of this enzyme after 50 and 100 nM corticosterone exposure may be attributed to the increased synthesis as evidenced by elevated mRNA levels at these dose levels of corticosterone whereas the decrease in the activity at 200–800 nM doses may be due to an opposite effect as seen in the levels of its mRNA. In accordance with the present study, cortisol (100 nM) was shown to increase the activity of aromatase enzyme in human adipocytes [McTernan et al., 2002].

Normal level of serum testosterone is indispensable for various physiological functions, as testosterone deficiency is shown to be associated with disorders such as reduced muscle and bone mass, fatigue, sexual dysfunction (erectile dysfunction and loss of libido), decreased lean body mass, skin alterations, osteoporosis as well as increased visceral fat and neuropsychiatric problems such as depression, irritability, insomnia, and memory impairment [Villareal and Morley, 1994; Morales et al., 2000; Vermeulen, 2003]. The present in vitro study is the first of its kind to reveal the molecular mechanism underlying the dose-dependent adverse effects of corticosterone on testosterone production. This study also provides a clue to physicians for the optimization of dose and to develop a suitable glucocorticoid therapy to the young patients suffering from asthma, arthritis, systemic lupus erythematosus etc in order to avoid the adverse side effects of glucocorticoid on reproductive function.

It is concluded from this study that the dose-dependent direct inhibitory effect of corticosterone on Leydig cell steroidogenesis is mediated through defective expression of StAR protein and steroidogenic enzymes.

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