Journal of Cellular Biochemistry 104:1671–1683 (2008)

Effects of Corticosterone Deficiency and its Replacement on Leydig Cell Steroidogenesis

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Abstract Clinical and experimental studies have shown the adverse effects of glucocorticoid deficiency/metyrapone treatment on testicular Leydig cell testosterone production. However, molecular mechanisms that underlie the effects of glucocorticoid deficiency on Leydig cell steroidogenesis are not yet determined. Therefore, the present study was designed to assess the mechanism of this phenomenon. Following metyrapone-induced corticosterone deficiency, serum testosterone, and Leydig cell ^{14}C-glucose oxidation were decreased. StAR mRNA and protein levels were significantly increased in Leydig cells of corticosterone-deficient animals. mRNA levels and the specific activities of P_{450}scc and 17β-HSD were decreased by corticosterone deficiency, whereas the activity and mRNA of 3β-HSD were increased. Simultaneous administration of corticosterone prevented its deficiency-induced changes in Leydig cells. Our results show that metyrapone-induced corticosterone deficiency impairs Leydig cell testosterone production by decreasing the activities of steroidogenic enzymes and their mRNA expression and glucose oxidation. J. Cell. Biochem. 104: 1671–1683, 2008. © 2008 Wiley-Liss, Inc.

Key words: corticosterone deficiency; Leydig cell; StAR; steroidogeneisis; mRNA expression; testosterone

Leydig cells populate the interstitial compartment and produce testosterone, which is involved in the regulation of spermatogenesis and manifestation of secondary sexual characteristics. Any defect in Leydig cell function may have a significant impact on the quality-of-life manifested by sexual and psychosocial problems [Fossa et al., 1999]. Although Leydig cell steroidogenic function is primarily controlled by the gonadotropin luteinizing hormone (LH), emerging evidence suggests a vital role of glucocorticoid in control of Leydig cell function [Haider, 2004; Hardy et al., 2005]. Cortisol in men and corticosterone in rats are the main circulating glucocorticoid involved in different physiological functions such as metabolism, immunity, learning and reproduction [Schimmer and Parker, 1996; Conrad, 2005; Hardy et al., 2005; Kajantie and Phillips, 2006]. Elevated glucocorticoid as a result of Cushing's disease, stress or therapeutic exposure to excess glucocorticoid is associated with reduced circulating testosterone level and reproductive dysfunction [Vierhapper et al., 2000; Hardy et al., 2002; Breen and Karsch, 2006]. Previous studies from our laboratory showed that exogenous administration of corticosterone impairs LH signal transduction and steroidogenesis in Leydig cells of adult male rats [Sankar et al., 2000]. Recently our laboratory has reported that excess corticosterone significantly reduces the activities and mRNA expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) in adult rat Leydig cells in vivo and in vitro [Badrinarayanan et al., 2006]. Apart from elevated glucocorticoid, adrenal insufficiency as a result of Addison's disease in men and adrenalectomy in experimental animals showed testicular deficiency [Lescoat et al., 1982; Betterle et al., 2002; Sugino et al., 2006]. Testicular dysfunction in both primary and

Grant sponsor: Council of Scientific and Industrial Research; Grant number: 9/115 (613)/2004-EMR-I dt. 16-06-2004 and 11-09-2006.

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Received 27 August 2007; Accepted 23 January 2008
DOI 10.1002/jcb.21733

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secondary adrenal insufficiency was also recorded [Seminara et al., 1999; Betterle et al., 2002; Sugino et al., 2006]. Studies on the effect of adrenalectomy on testicular function showed a reduction in Leydig cell testosterone production and sperm density [Lescoat et al., 1982; Nair and Bedwal, 2004]. Since adrenalectomy depresses both adrenocortical and medullary hormones, the exact effect of glucocorticoid deficiency on Leydig cell steroidogenesis is not easy to ascertain. Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone), a specific inhibitor of 11β-hydroxylase, inhibits glucocorticoid production and is used in the diagnosis/treatment of Cushing's syndrome/disease, depression and also to test the function of hypothalamo-pituitary-adrenal axis [Berneis et al., 2002; Calis et al., 2004; Annane et al., 2006]. An in vitro study to test the direct effect of metyrapone on Leydig cell steroidogenesis showed that steroidogenic potency is not altered [Parthasarathy and Balasubramanian, 2008a]. Our previous study showed a depressed testicular function in metyrapone-induced corticosterone-deficient rats [Parthasarathy et al., 2002]. Thus, studies including our own have shown the adverse effects of metyrapone treatment/glucocorticoid deficiency on Leydig cell testosterone production. However, the molecular mechanisms that underlie such effects are yet to be recognized. Based on the available information it is hypothesized that metyrapone-induced corticosterone deficiency may impair Leydig cell testosterone production by decreasing StAR protein and steroidogenic enzymes in adult male albino rats. The present study was designed to test this hypothesis.

MATERIALS AND METHODS

After obtaining clearance from the local animal ethical committee, adult male albino rats of the Wistar strain (*Rattus norvegicus*) (180–200 g) were used for the present study. Group-I: Control (treated with equal volume of vehicle, 5% dimethyl sulfoxide), Group-II: Metyrapone treated (10 mg/100 g body weight, s.c., twice daily for 10 days), Group-III: Metyrapone (10 mg/100 g body weight, s.c., twice daily for 10 days) + Corticosterone (1 mg/100 g body weight, s.c., daily for 10 days). After treatment, rats were anesthetized by sodium pentobarbital injection and decapitated. Testes were decapsulated under aseptic conditions and Leydig cells were isolated by enzymatic digestion and purified on discontinuous Percoll gradient [Rigaudiere et al., 1988]. In brief, testes were decapsulated and digested in collagenase-containing medium (0.25 mg/ml) at 37°C for 15 min in a shaking water bath. The resulting crude Leydig cell preparations were further purified on discontinuous Percoll gradients. The purity of Leydig cells were assessed by histochemical staining of 3β-HSD activity and viability was determined by trypan blue exclusion [Aldred and Cooke, 1983]. The purity was 80–90% and viability was 90–95%. Serum testosterone was assayed using RIA kit obtained from Diasorin (Saluggia, Italy). Corticosterone in serum samples were assayed by liquid-phase RIA using corticosterone antiserum (Sigma Chemical), following the method of Etches [1976]. Leydig cells were used to assess the StAR gene expression and activities of cytochrome P_{450}scc, 3β-HSD and 17β-HSD and their mRNA expression, and glucose oxidation.

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

P_{450}scc activity in Leydig cells was determined by the method of Georgiou et al. [1987]. Briefly, after treatment, cells were isolated and incubated in extraction tubes for 1 h at 34°C with a saturating concentration of [26,27-^{3}H]-5-hydroxycholesterol (5 μM/0.5 μCi) (NEN Life) dissolved in 100 nM dimethyl sulfoxide. The reaction was stopped by addition of 0.1 ml of 1 N NaOH. To estimate recovery, [^{14}C]-isocaproic acid (Amersham Biosciences, Kwai Chung, Hong Kong) was processed separately. The medium was removed from the extraction tube, then the tubes were 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 vial and the radioactivity was quantified by liquid scintillation counter (Wallac OY, Turiku, Finland). Activity of the enzyme is expressed as fmol of pregnenolone formed/5×10^5 cells.

Assay of 3β-HSD Enzyme Activity

The activity of 3β-HSD in Leydig cells was determined by an established colorimetric method of Shivanandappa and Venkatesh

[1997]. Leydig cells (5×10^5) were sonicated in ice-cold Tris–HCl buffer (pH 7.2) and centrifuged at 10,000*g* for 15 min at 4°C. The supernatant was then used as an enzyme extract for the assay of 3β-HSD. The reaction mixture contained 500 μM of NAD, 0.08% of iodonitrotetrazolium chloride (w/v), 2% of Tween-20 and 100 μM of pregnenolone. The reaction was started by adding the enzyme and incubated at 37°C for 60 min. The reaction was stopped by addition of 2 ml of phthalate buffer (pH 3.0). Turbidity was removed by centrifugation at 1,500*g* for 20 min and the supernatant was read at 490 nM in a spectrophotometer. Enzyme activity was calculated from the standard curve of NADH and expressed as pmol of NADH formed/5×10^5 cells.

Assay of 17β-HSD Enzyme Activity

17β-HSD activity was determined based on the radiometric method described by Murono [1990]. Briefly, the cells were incubated for 1 h at 34°C in a humidified atmosphere of 95% air and 5% CO_2 with [^{3}H]-androstenedione (10 μM/0.5 μCi) (Amersham Biosciences) dissolved in culture medium containing 0.1% dimethyl sulfoxide (final concentration). 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, [^{3}H]-testosterone (Amersham Biosciences) was processed separately. Samples were extracted with 5 volume of diethyl ether and the residue of ether extracts was chromatographed by TLC using chloroform/methanol (99.5:0.5, v/v) as solvent system. The product testosterone was localized using anisaldehyde spray, which was cut out and counted using liquid scintillation counter (Wallac OY, Turuku, Finland). Enzyme activity is expressed as fmol of testosterone formed/5×10^5 cells.

Determination of ^{14}C-Glucose Oxidation

Leydig cell ^{14}C-glucose oxidation was estimated by the method of Kraft and Johnson [1972]. Twenty microliters of cell suspension containing 5×10^5 cells were pipetted and placed in a 2 ml ampule containing 170 μl DMEM (pH 7.4), 10 U pencillin in 10 μl DMEM and 0.5 μCi [^{14}C]-glucose (BRIT, Mumbai, India). After aeration with gas mixture (5% CO_2 and 95% air) for 30 s, the ampule was tightly closed with a rubber cork containing CO_2 trap and incubated at 34°C. CO_2 traps were replaced every 2 h and 0.01 ml of 1 N H_2SO_4 was added to the ampule upon the removal of second trap to halt further metabolism. The ampule was again closed for 1 h before the third and final trap was removed and all the CO_2 traps were placed in the scintillation vials containing 10 ml of scintillation fluid and the samples were counted using liquid scintillation beta counter (Wallac OY, Turku, Finland). Results are expressed as cpm of $^{14}CO_2$ released/5×10^5 cells.

Western Blot Analysis of StAR Protein

Purified Leydig cells were washed once with ice-cold phosphate buffered saline (PBS) to remove the excess culture medium and 500 μl of ice-cold lysis buffer with protease inhibitor cocktail (Sigma Chemical) was added. The cells were lysed by keeping them on ice in a rocker for 30 min. The cell lysate was transferred into a 1.5 ml tube and centrifuged for 15 min at 12,000*g* at 4°C. The supernatants were transferred to new microcentrifuge tubes and protein concentration was determined [Lowry et al., 1951]. Total protein (100 μg) with 6× sample buffer was boiled for 5 min. The sample mixture was run on 12% SDS–PAGE [Laemmli, 1970] and electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham Pharmacia Biotech, Inc., UK) for immuno blot analysis [Clark et al., 1994]. The membrane was blocked with blocking buffer containing 10% non-fat dry milk for overnight. The membrane was then incubated with primary polyclonal antibody for StAR for 3 h (1:500; StAR antibody was a generous gift from Douglas Stocco, Texas Tech University, Lubbock, TX), after washing with the blocking buffer the membrane was again incubated with the horseradish peroxidase-conjugated secondary antibody (1:10,000) (Amersham) for 1 h and finally the membrane was washed using washing buffers tris-based saline (TBS) and 0.2% Tween-20 in TBS (TTBS). ECL (Amersham) was added to detect the protein bands and the membrane was exposed to X-ray film. The exposed membrane was then stripped and used to identify the loading control (β-actin) expression using the same procedure with specific antibody (Sigma Chemical). After washing with TBS and TTBS, ECL was added to detect the protein bands and the membrane was exposed to X-ray film. The films were developed and the intensity of the bands was quantified using

Quantity One software in a Bio-Rad Gel documentation unit against the internal control β-actin.

Quantification of StAR Protein, Cytochrome P_{450}scc, 3β-HSD and 17β-HSD mRNA Expression

Total RNA was isolated from Leydig cells using TRIR (Total RNA Isolation Reagent, AB gene, Surrey, UK) and RNA with $A_{260/280}$ ratio of 1.8 and above was used. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to quantify the expression of StAR protein, P_{450}scc, 3β-HSD, and 17β-HSD mRNAs against the internal control β-actin and RPS16. The primer sequences for StAR protein, P_{450}scc, 3β-HSD, 17β-HSD, β-actin, and RPS16 are gene-specific and are presented in Table I. To find out saturation point, different concentrations (0.25, 0.5, 0.75, 1, 1.5, and 2 μg) of total RNA from normal cells (basal) were used for RT-PCR and it was found that band intensity was saturated at 1.5 μg level after optimizing various parameters including tuning the temperature and duration of various steps. Therefore, 1 μg of total RNA was taken for each reaction. Total RNA (1 μg) was reverse transcribed and PCR was performed using Qiagen one-step RT-PCR kit (Germany) with gene-specific primers along with the housekeeping gene as an internal control. The reverse transcriptase (RT) reaction was performed at 50°C for 30 min followed by initial PCR activation at 95°C for 15 min. The three-step PCR cycles include (i) denaturation at 95°C for 1.5 min, (ii) annealing at 57°C for 1.5 min, and (iii) extension at 72°C for 1.5 min. The product was amplified 30 times and then finally extended at 72°C for 10 min. PCR mixture (5 μl) was taken from each reaction tube and resolved on a standard 2% agarose gel containing ethidium bromide (0.5 μg/ml). Molecular weight marker (100 bp ladder) was simultaneously resolved in the first lane. Then the gels were subjected to densitometric scanning (Bio-Rad) to determine the OD of each band and then normalized against β-actin/RPS16 using Quantity One software.

Statistics

Data were presented as mean ± SEM of three or more independent experiments. For group comparison (Group-I Control, Group-II Metyrapone treated, and Group-III Metyrapone + Corticosterone), one-way analysis of variance (ANOVA) followed by Student–Newman–Kuel's test was performed to assess the significance of individual variations among the treatment groups. Differences were considered as significant at $P < 0.05$.

RESULTS

Serum Corticosterone

Metyrapone a specific inhibitor of 11β-hydroxylase, inhibits the conversion of 11-deoxycorticosterone into active corticosterone and thus reduces the circulating level of corticosterone. In the present study serum corticosterone was significantly decreased up to fivefold by metyrapone treatment compared to control and simultaneous corticosterone replacement completely maintained serum corticosterone at control level (Fig. 1).

Serum Testosterone

Circulating level of testosterone reflects the steroidogenic activity of Leydig cells. Serum

TABLE I. Oligonucleotide Primers Used for RT-PCR

Gene product	Oligonucleotide	Gene bank accession No.	Amplicon size (bp)	References
StAR protein	Sense primer: 5′-TTGGGCATACTCAACAACCA-3′ Anti-sense primer: 5′-ATGACACCGCTTTGCTCA-3′	AB001349	389	Akingbemi et al. [2004]
P_{450}scc	Sense primer: 5′-CGCTCAGTGCTGGTCAAAA-3′ Anti-sense primer: 5′-TCTGGTAGACGGCGTCGAT-3′	NM017286	688	Sakaue et al. [2002]
3β-HSD type-I	Sense primer: 5′-TTGGTGCAGGAGAAAGAAC-3′ Anti-sense primer: 5′-CCGCAAGTATCATGACAGA-3′	M38178	547	Sakaue et al. [2002]
17β-HSD type-III	Sense primer: 5′-TTCTGCAAGGCTTTACCAGG-3′ Anti-sense primer: 5′-ACAAACTCATCGGCGGTCTT-3′	NM054007	653	Sakaue et al. [2002]
β-actin	Sense primer: 5′-GCCATGTACGTAGCCATCCA-3′ Anti-sense primer: 5′-GAACCGCTCATTGCCGATAG-3′	NM012969	374	Oaks and Raff [1995]
RPS16	Sense primer: 5′-AAGTCTTCGGACGCAAGAAA-3′ Anti-sense primer: 5′-TTGCCCAGAAGCAGAACAG-3′	XM341815	148	Shan et al. [1995]

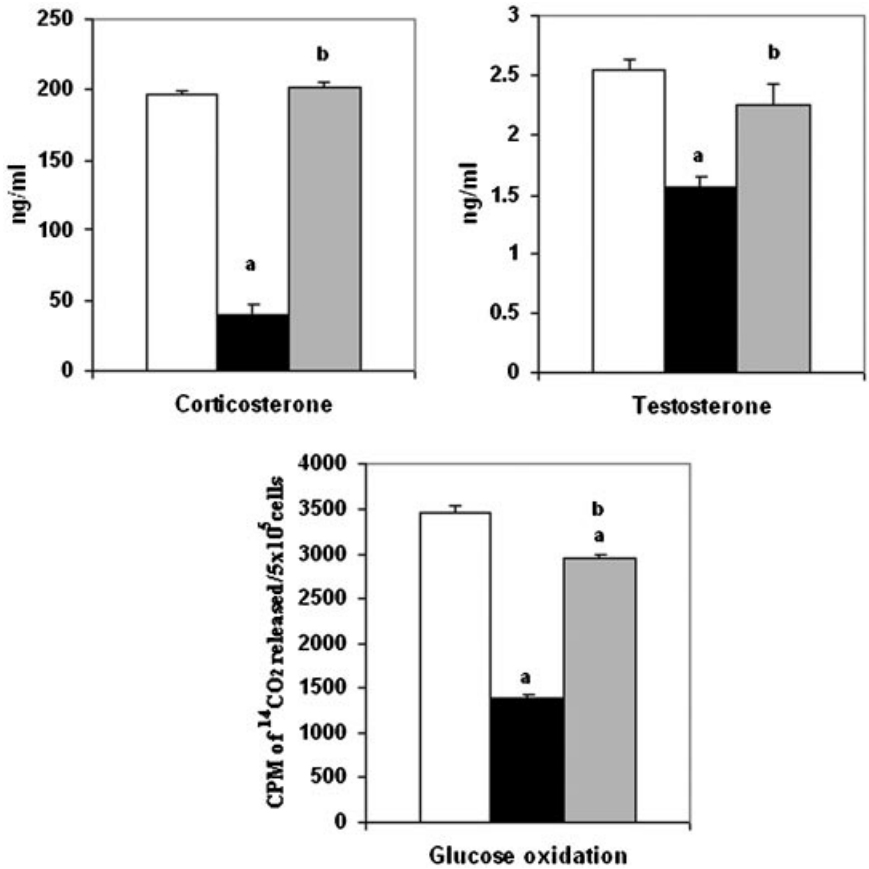

Fig. 1. Effect of metyrapone-induced corticosterone deficiency and corticosterone replacement on serum corticosterone, testosterone and Leydig cell ^{14}C-glucose oxidation. Serum collected from control, corticosterone deficiency and corticosterone replacement rats was processed for the assay of corticosterone and testosterone by liquid and solid phase RIA respectively. Leydig cells isolated from the above said groups were used for ^{14}C-glucose oxidation; CPM of ^{14}C released during oxidation of glucose was trapped and counted using liquid scintillation beta counter and results are expressed as cpm of $^{14}CO_2$ released/5×10^5 cells. Each bar represents the mean $\pm$ SEM of five animals. Significance at $P < 0.05$. **a**: Compared with control; (**b**) compared with metyrapone-induced corticosterone deficiency. (□) Control, (■), Metyrapone treated, (▩) Metyrapone, and (+) Corticosterone treated.

testosterone was significantly reduced in metyrapone-induced corticosterone-deficient rats compared to control; simultaneous corticosterone treatment prevented the effect of its deficiency and maintained serum testosterone at normal level (Fig. 1).

Effect of Metyrapone-Induced Corticosterone Deficiency and Simultaneous Corticosterone Treatment on Leydig Cell ^{14}C-Glucose Oxidation

Compared to control Leydig cells, glucose oxidation was significantly decreased in Leydig cells of metyrapone-induced corticosterone-deficient rats, whereas simultaneous corticosterone treatment partially reversed the effects of corticosterone deficiency and maintained glucose oxidation at near normal levels (Fig. 1).

Western Blot and RT-PCR Analysis of Steroidogenic Acute Regulatory (StAR) Protein in Leydig Cells of Corticosterone-Deficient and Replaced Rats

StAR protein expression was significantly increased in Leydig cells of corticosterone-deficient rats and simultaneous corticosterone treatment maintained the protein expression at near normal level (Fig. 2). StAR protein mRNA expression was significantly increased in corticosterone-deficient rats; simultaneous corticosterone treatment maintained expression of StAR mRNA at near normal levels (Fig. 3).

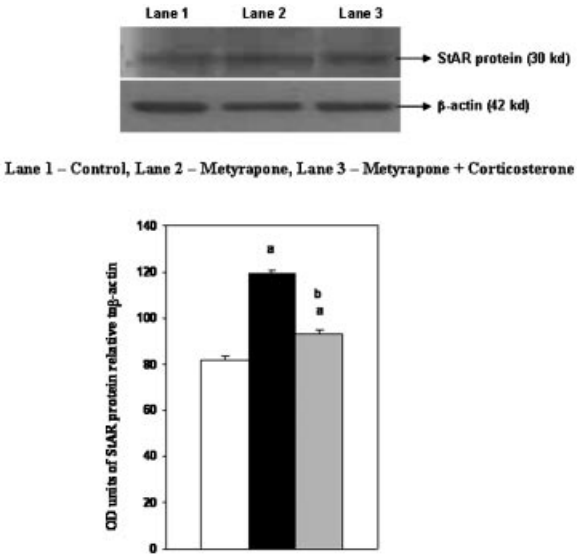

Fig. 2. Expression of StAR protein in Leydig cells of control, corticosterone-deficient and simultaneous corticosterone treated rats. Western blot analysis for StAR protein and β-actin (internal control) was performed as described in Materials and Methods Section and a representative autoradiogram illustrates the expression of the 30-kDa StAR protein. Intensity of the bands was quantified in a Bio-Rad Gel documentation unit using Quantity One software. Each bar represents the mean ± SEM of three observations. Significance at $P < 0.05$. **a**: Compared with control; (**b**) compared with metyrapone-induced corticosterone deficiency. (□) Control, (■) Metyrapone treated, (▩) Metyrapone, and (+) Corticosterone treated.

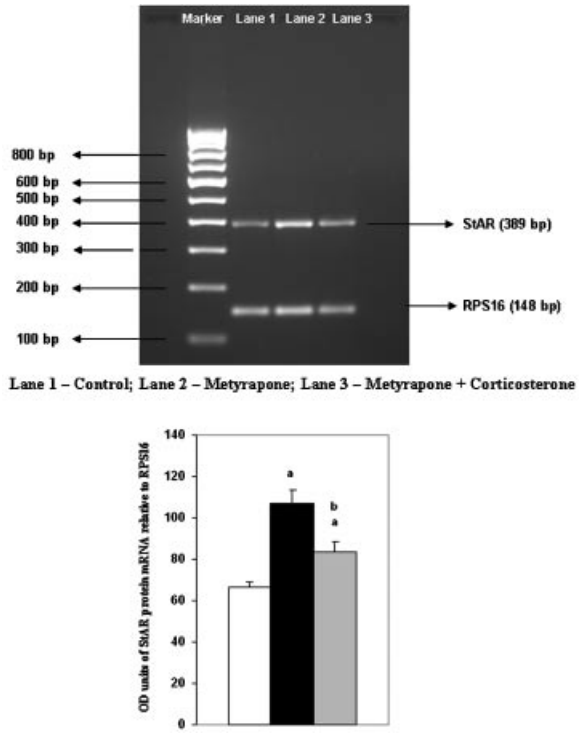

Fig. 3. Effect of metyrapone-induced corticosterone deficiency and simultaneous corticosterone treatment on Leydig cell StAR protein mRNA expression. Agarose gel electrophoretic pattern shows RT-PCR analysis of StAR mRNA expression from the Leydig cells of control, corticosterone deficiency and corticosterone replacement rats. RT-PCR was performed as described in the Materials and Methods Section and a representative graph illustrates the mRNA level of StAR relative to RPS16 (internal control). Intensity of the bands was quantified using Quantity One software in a Bio-Rad Gel documentation unit against RPS16. Each bar represents the mean ± SEM of three observations. Significance at $P < 0.05$. **a**: Compared with control; (**b**) compared with metyrapone-induced corticosterone deficiency. (□) Control, (■) Metyrapone treated, (▩) Metyrapone, and (+) Corticosterone treated.

Metyrapone-Induced Corticosterone Deficiency and Corticosterone Treatment on Leydig Cell P_{450}scc, 3β-HSD, and 17β-HSD

Corticosterone deficiency significantly decreased the activity of P_{450}scc, but activity was maintained completely in corticosterone-treated rats compared to control (Fig. 4). Activity of 3β-HSD was significantly increased in metyrapone-induced corticosterone-deficient rat Leydig cells, whereas simultaneous corticosterone treatment maintained the activity completely at control level (Fig. 4). Activity of 17β-HSD was significantly decreased in metyrapone-induced corticosterone-deficient rat Leydig cells compared to control, whereas simultaneous administration of corticosterone partially prevented this and maintained the activity at near normal levels (Fig. 4).

Effect of Metyrapone-Induced Corticosterone Deficiency and Simultaneous Corticosterone Treatment on Leydig Cell P_{450}scc, 3β-HSD, and 17β-HSD mRNA Expression in Corticosterone-Deficient and Replaced Rats

A significant decrease in levels of P_{450}scc mRNA was observed in Leydig cells of corticosterone-deficient rats, but simultaneous corticosterone treatment completely maintained P_{450}scc mRNA at control levels (Fig. 5). Similar to 3β-HSD activity, its mRNA level also significantly increased in Leydig cells of corticosterone-deficient rats and simultaneous corticosterone treatment completely maintained its expression at control levels (Fig. 6). Similar to the activity of 17β-HSD in Leydig cells, its mRNA levels also significantly decreased in the Leydig cells of corticosterone-deficient rats compared to control and simultaneous administration of corticosterone partially reversed the effect of corticosterone deficiency and maintained 17β-HSD mRNA at near normal levels (Fig. 7).

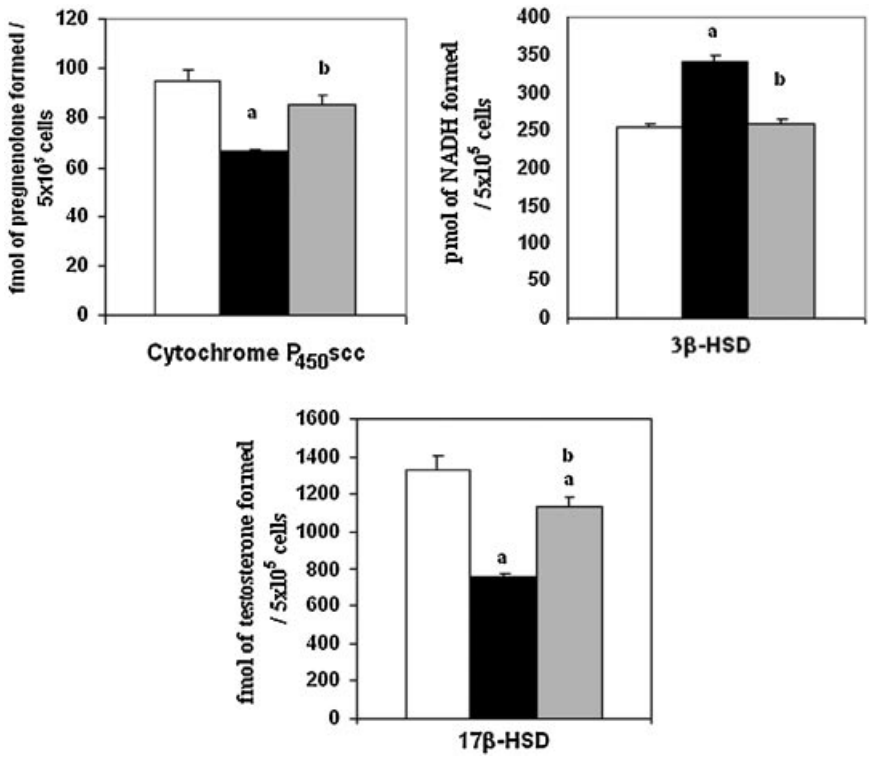

Fig. 4. Activities of P_{450}scc, 3β-HSD and 17β-HSD in the Leydig cells isolated from control, corticosterone deficiency and corticosterone replacement rats. 3β-HSD was assayed calorimetrically as described under Materials and Methods Section and the enzyme activity was calculated from the standard curve of NADH and expressed as pmol of NADH formed/5×10^5 cells. P_{450}scc and 17β-HSD were assayed radiometrically as described under Materials and Methods Section and the results are expressed as fmol of pregnenolone formed/5×10^5 cells and fmol of testosterone formed/5×10^5 cells respectively. Each bar represents the mean ± SEM of five observations. Significance at $P < 0.05$. **a**: Compared with control; (**b**) compared with metyrapone-induced corticosterone deficiency. (□) Control, (■) Metyrapone treated, (▩) Metyrapone, and (+) Corticosterone treated.

DISCUSSION

In the present study, serum corticosterone was significantly decreased in metyrapone treated rats compared to control and simultaneous corticosterone treatment maintained the same at control level. In accordance with the present study, metyrapone at a dose of 10 mg/100 g body weight was shown to decrease serum corticosterone [Baram and Schultz, 1990; Valli et al., 2000]. Reduced serum corticosterone level confirmed the inhibitory effect of metyrapone on glucocorticoid synthesis.

The levels of testosterone in the circulation are primarily set by the steroidogenic capacity of testicular Leydig cells. The present study clearly demonstrates that the metyrapone-induced corticosterone deficiency has adverse effect on serum testosterone. Insufficiency of adrenal glucocorticoid as a result of Addison's disease in men and adrenalectomy in experimental animals showed a decrease in serum testosterone [Betterle et al., 2002; Nair and Bedwal, 2004; Sugino et al., 2006]. Androgen production by the Leydig cell is dependent upon the activity of rate limiting steroidogenic enzymes [Saez, 1994]. In the present study, a correlation exists between changes in serum testosterone and Leydig cellular P_{450}scc and 17β-HSD activities. Therefore, it is suggested that the decreased activity of P_{450}scc and 17β-HSD may be responsible for the decreased serum testosterone. Simultaneous corticosterone replacement maintained the serum testosterone at control level suggesting that it is one of the essential factors to maintain normal circulating level of testosterone.

Leydig cells depend upon the availability of glucose and its metabolism for its functional activity [Amrolia et al., 1988; Ferguson et al., 1999; Dufau et al., 2001]. It has been reported that glucocorticoids play a critical role in energy balance [King, 1988; Drazen et al., 2003; Smart et al., 2006]. Leydig cell glucose oxidation was

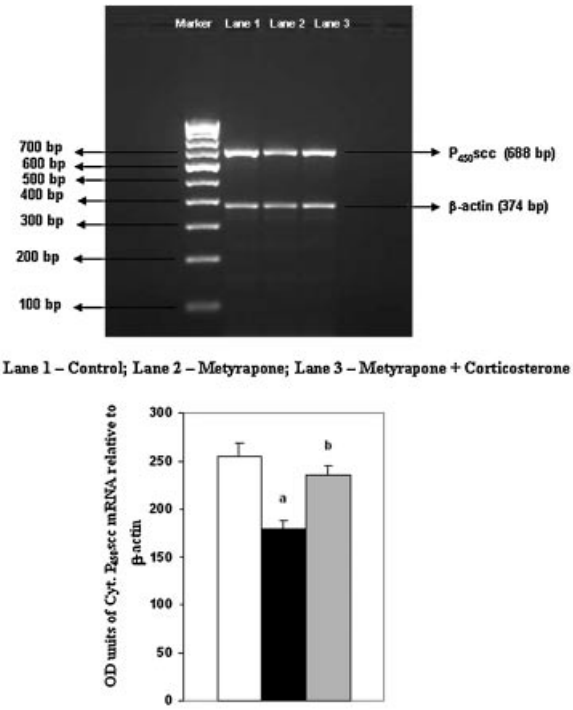

Fig. 5. Leydig cell P_{450}scc mRNA expression in control, corticosterone-deficient and simultaneous corticosterone treated rats. Agarose gel electrophoretic pattern shows RT-PCR analysis of P_{450}scc mRNA expression from the Leydig cells of control, corticosterone deficiency and corticosterone replacement rats. RT-PCR was performed as described in the Materials and Methods Section and a representative graph illustrates the mRNA level of P_{450}scc relative to β-actin (internal control). Intensity of the bands was quantified using Quantity One software in a Bio-Rad Gel documentation unit against β-actin. Each bar represents the mean ± SEM of three observations. Significance at $P < 0.05$. **a**: Compared with control; (**b**) compared with metyrapone-induced corticosterone deficiency. (□) Control, (■) Metyrapone treated, (▩) Metyrapone, and (+) Corticosterone treated.

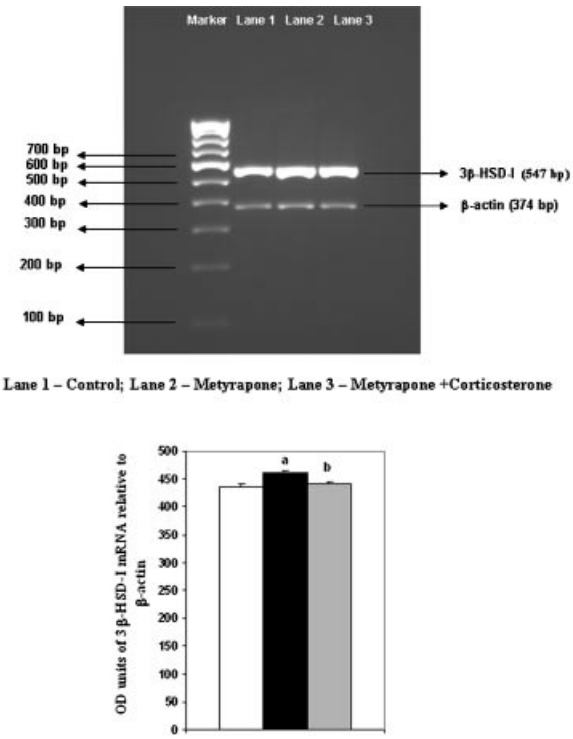

Fig. 6. Expression of 3β-HSD mRNA in Leydig cells of control, corticosterone-deficient and simultaneous corticosterone treated rats. Agarose gel electrophoretic pattern shows RT-PCR analysis of 3β-HSD mRNA expression from the Leydig cells of control, corticosterone deficiency and corticosterone replacement rats. RT-PCR was performed as described in the Materials and Methods Section and a representative graph illustrates the mRNA level of 3β-HSD relative to β-actin (internal control). Intensity of the bands was quantified using Quantity One software in a Bio-Rad Gel documentation unit against β-actin. Each bar represents the mean ± SEM of three observations. Significance at $P < 0.05$. **a**: Compared with control; (**b**) compared with metyrapone-induced corticosterone deficiency. (□) Control, (■) Metyrapone treated, (▩) Metyrapone, and (+) Corticosterone treated.

severely impaired in metyrapone-induced corticosterone-deficient rats compared to control but simultaneous corticosterone treatment prevented the effect of corticosterone deficiency. Borelli et al. [1982] have found that adrenalectomy attenuates glucose-induced insulin secretion and glucose oxidation in pancreatic islets of rat. When supplemented with dexamethasone an increase in glucose-induced insulin secretion and glucose oxidation was observed. It is therefore suggested that the decreased glucose oxidation and maintenance of the same may be due to corresponding changes in the level of corticosterone. Low level of testosterone in corticosterone deficient rats may be the consequence of impaired glucose oxidation. It has been shown that LH favors glucose uptake by Leydig cells [Amrolia et al., 1988; Chen et al., 2003]. Both basal and LH/hCG-stimulated Leydig cell testosterone synthesis depends on ambient glucose concentrations [Murono et al., 1982]. In addition, inhibition of glucose uptake is associated with decreased testosterone formation. Although serum LH was unaltered in corticosterone deficient and replaced rats, the sensitivity of Leydig cells to LH was severely impaired by corticosterone deficiency [Parthasarathy and Balasubramanian, 2008b]. It is therefore suggested that the reduced sensitivity of Leydig cells to LH resulted in depressed glucose oxidation and thus testosterone production.

Although StAR protein is primarily regulated at the transcriptional level via cAMP-protein

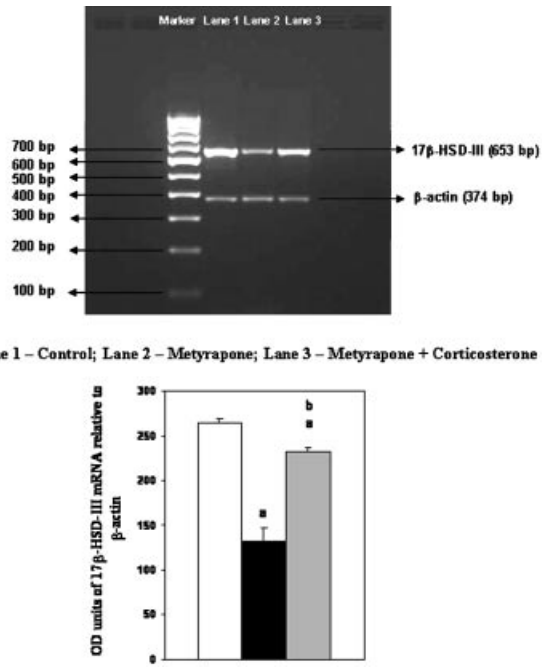

Fig. 7. Expression of 17β-HSD mRNA in Leydig cells of control, corticosterone-deficient and simultaneous corticosterone treated rats. Agarose gel electrophoretic pattern shows RT-PCR analysis of 17β-HSD mRNA expression from the Leydig cells of control, corticosterone deficiency and corticosterone replacement rats. RT-PCR was performed as described in the Materials and Methods Section and a representative graph illustrates the mRNA level of 17β-HSD relative to β-actin (internal control). Intensity of the bands was quantified using Quantity One software in a Bio-Rad Gel documentation unit against β-actin. Each bar represents the mean ± SEM of three observations. Significance at $P < 0.05$. **a**: Compared with control; (**b**) compared with metyrapone-induced corticosterone deficiency. (□) Control, (■) Metyrapone treated, (▨) Metyrapone, and (+) Corticosterone treated.

kinase A (PKA) signaling cascade within the gonads, it lacks a canonical cAMP responsive element (CRE) in its promoter [Caron et al., 1997; Stocco et al., 2005; Manna et al., 2006]. Clem et al. [2005] have reported that even in the absence of a canonical CRE, the classical cAMP-PKA signaling cascade activates cAMP-responsive element binding protein (CREB) phosphorylation and CREB binding protein (CBP) binding to the CCAAT/enhancer binding protein-β/nonconsensus activating protein-1/ nuclear receptor half-site (CAN) region and up-regulates transcription of the StAR gene. Interestingly, Houk et al. [2004] have reported that cAMP-stimulated StAR promoter activity was inhibited by dihydrotestosterone and they concluded that androgens inhibit StAR mRNA expression at the transcriptional level. In addition to the above, they also identified three possible ARE half-sites in the proximal StAR promoter by sequence analysis, thus testosterone may repress the transcription of StAR through this consensus sequences. It is suggested that testosterone through an autocrine mechanism can regulate its own production at the crucial, rate-limiting step of cholesterol transfer to the inner mitochondrial membrane. Results of the present study suggest that the increased StAR gene expression may be due to corticosterone deficiency-induced low levels of testosterone.

The first step in steroid hormone biosynthetic pathway is the conversion of cholesterol to pregnenolone by the cholesterol side-chain cleavage enzyme cytochrome P_{450}scc and auxiliary electron-transferring proteins, localized on inner mitochondrial membrane [Black et al., 1994]. The activity of P_{450}scc in the Leydig cells of metyrapone-induced corticosterone-deficient rats was significantly decreased compared to control but simultaneous corticosterone treatment maintained the activity at control level reinforcing the importance of corticosterone. As in the case of P_{450}scc enzyme activity, its mRNA level also showed a similar trend of results due to corticosterone deficiency and replacement suggesting the possible defective transcription of the gene and translation of mRNA. In rat, the P_{450}scc gene has been shown to have the fragment between −73 and −38 that confers cAMP inducibility to the promoter. Importantly, this fragment contains two previously characterized and highly conserved binding sites for SF-1 [Morohashi et al., 1993; Chau et al., 1997]. SF-1 is the pivotal factor that mediates hormone-induced expression of P_{450}scc at the transcriptional level in the gonads [Morohashi et al., 1992]. The proximal SF-1 binding site is situated in the basal promoter region and the upstream SF-1 binding site is in the response to hormonal stimulation located in the cAMP-responsive region [Hu et al., 2001]. This region binds proteins in the cAMP response element binding protein/AP1 families in addition to SF-1 [Wantanabe et al., 1994; Chen and Guo, 2000]. The orphan receptor SF-1 interacts with shared promoter elements to increase the expression of cytochrome P_{450}scc in vitro [Chau et al., 1997]. Recently, it has been shown that AP1 cooperates with SF-1 to regulate cAMP dependent transcription of human Leydig cell P_{450}scc in vivo and in vitro [Guo et al., 2007]. Studies in cultured rat Leydig cells demonstrated that treatment with hCG or

dibutyryl cAMP increased synthesis of P_{450}scc [Anderson and Mendelson, 1985; Payne and Youngblood, 1995]. These studies clearly show the importance of LH and cAMP on P_{450}scc expression. Although serum LH was unaltered, the mRNA level of P_{450}scc was significantly decreased in corticosterone deficient rats and this may be due to reduced sensitivity of Leydig cells to LH [Parthasarathy and Balasubramanian, 2008b]. Hence, the decreased P_{450}scc mRNA in the present study may be due to impaired sensitivity of Leydig cells to LH as a result of glucocorticoid insufficiency.

The enzyme 3β-hydroxysteroid dehydrogenase/$\Delta^5-\Delta^4$ isomerase, plays a central role in the biosynthesis of testosterone [Dufau et al., 2001]. In the rat, 3β-HSD type I and II are expressed in Leydig cells [Simard et al., 2005]. In vitro studies demonstrated that the activity of type I enzyme is higher than that of type II enzyme, due to lower affinity to substrates [Simard et al., 2005]. In adult rat testis, the control of 3β-HSD gene expression occurs primarily through the action of LH [Keeney and Mason, 1992]. Nevertheless, it has been suggested that androgen can inhibit its own production by the testis and this repression could occur at the level of 3β-HSD [Simard et al., 2005]. Endogenously produced testosterone negatively regulates the expression of cAMP-induced 3β-HSD mRNA in mouse Leydig cell [Payne and Sha, 1991; Heggland et al., 1997]. Rats treated with hCG showed an increase in 3β-HSD activity, but androgen agonist (R1881) decreased the hCG induction, whereas cyproterone acetate (androgen receptor antagonist) increased the activity [Ruiz de Galarreta et al., 1983]. It is evident from these studies that testosterone negatively regulates the activity and mRNA expression of 3β-HSD in Leydig cells. The rat 3β-HSD promoters have not yet been characterized, but the human 3β-HSD type-I promoter has the half-site of specific androgen response element (ARE) [Claessens et al., 1996; Zhou et al., 1997]. These findings tempt to propose that testosterone inhibits 3β-HSD gene transcription at normal or in excess conditions through nuclear actions or by activating the trans-repressors. Therefore, depressed testosterone recorded in the present study may be responsible for increased 3β-HSD mRNA and activity in corticosterone-deficient condition.

17β-HSD, which converts androstenedione to testosterone, is one of the rate-limiting enzymes involved in the regulation of steroid hormone production in Leydig cells [Dufau et al., 2001]. In the present study, both activity and the mRNA levels of 17β-HSD showed similar trend due to corticosterone deficiency. Therefore, decreased and the partial maintenance of 17β-HSD activity due to corticosterone deficiency and replacement, respectively may be attributed to corresponding changes in their mRNA level suggesting the importance of optimal level of corticosterone. Among the different forms of 17β-HSDs, three forms (type I, III and VII) participate in the final step of biosynthesis of active steroid hormones in gonads [Adamski and Jakob, 2001]. 17β-HSD type III is exclusively expressed in testes and, its expression is restricted to the adult Leydig cell population and thus serves as a specific marker for Leydig cell development [Andersson, 1995]. Baker et al. [1997] have compared the expression pattern of 17β-HSD type III mRNA between androgen receptor deficient and hypogonadal mice testes and they concluded that the androgen may be a major regulator than gonadotropin in the regulation of transactivation of 17β-HSD type III gene. Tsai-Morris et al. [1999] reported that the control of 17β-HSD type III gene expression could be exerted directly or indirectly by the actions of steroid products in rat Leydig cells. Therefore, the decreased expression of 17β-HSD type III mRNA due to corticosterone deficiency may be the result of corresponding changes in serum testosterone. Simultaneous corticosterone replacement maintained the expression of 17β-HSD type-III mRNA at near normal level suggesting that corticosterone is also an essential factor involved in the regulation of expression of this rate limiting steroidogenic enzyme.

In conclusion, corticosterone deficiency impairs Leydig cell glucose oxidation and steroidogenesis by inhibiting expression of rate-liming steroidogenic enzymes and the physiological level of corticosterone is one of the essential factors for Leydig cell steroid hormone production.

ACKNOWLEDGMENTS

Financial assistance through CSIR-SRF, UGC-SAP-DRS (Phase II), UGC-ASSIST, and DST-FIST Programmes are gratefully acknowledged.

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