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Chronic administration of corticosterone impairs LH signal transduction and steroidogenesis in rat Leydig cells

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Abstract

The mechanism involved in the inhibitory actions of chronic corticosterone treatment on Leydig cell steroidogenesis was studied in adult Wistar rats. Rats were treated with corticosterone-21-acetate (2 mg/100 g body weight, i.m., twice daily) for 15 days and another set of rats was treated with corticosterone plus ovine luteinizing hormone (oLH) (100 μg/kg body weight, s.c., daily) for 15 days. Chronic treatment with corticosterone increased serum corticosterone but decreased serum LH, testosterone, estradiol and testicular interstitial fluid (TIF) testosterone and estradiol concentrations. Administration of LH with corticosterone partially prevented the decrease in serum and TIF testosterone and estradiol. Leydig cell LH receptor number, basal and LH-stimulated cAMP production were diminished by corticosterone treatment which remained at control level in the corticosterone plus LH treated rats. Activities of steroidogenic enzymes, 3β- and 17β-hydroxysteroid dehydrogenase (3β-HSD and 17β-HSD) were significantly decreased in corticosterone treated rats. LH plus corticosterone treatment did not affect 3β-HSD activity but decreased 17β-HSD activity, indicating a direct inhibitory effect of excess corticosterone on Leydig cell testosterone synthesis. The indirect effect of corticosterone, thus, assume to be mediated through lower LH which regulates the activity of 3β-HSD. Basal, LH and cAMP-stimulated testosterone production by Leydig cells of corticosterone and corticosterone plus LH treated rats were decreased compared to control suggesting the deleterious effect of excess corticosterone on LH signal transduction and thus steroidogenesis.

1. Introduction

Clinical and experimental studies have shown that elevated levels of glucocorticoid due to adrenal dysfunction, stress or exogenous administration decrease plasma testosterone level [1–5]. Specific receptors for glucocorticoid in Leydig cells have been reported [6,7] and it is considered that the Leydig cell is a target for glucocorticoids. In addition, in vitro experiments show a direct effect of glucocorticoid on Leydig cell function [8–11]. Glucocorticoids potentially affect testicular function by acting at any level within the hypothalamo-pituitary-gonadal axis [12]. Although the mechanisms of inhibitory action of glucocorticoids have been studied in rats under stress [5,13,14], there have been conflicting reports on the inhibitory effects of stress on Leydig cell steroidogenesis [5,14]. Chronic stress decreases serum LH, but acute stress acts at the level of Leydig cells and inhibits testosterone production without any impact on serum LH level.

It has been claimed that the inhibitory actions of glucocorticoids on testicular testosterone production are mediated through decreased serum LH [15–17]. In contrast, a number of in vivo studies have shown that the decreased levels of serum testosterone are associated with normal levels of serum LH [3,5,18,19]. Despite these studies on inhibitory action of glucocorticoids, the principal pathways of excess glucocorticoid action on Leydig cell steroidogenesis are not clear. In the present study, the specific effects of chronic corticosterone treatment on basal and LH-induced testosterone production and LH signal transduction in

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Leydig cells were assessed by in vivo and in vitro studies.

2. Materials and methods

2.1. Chemicals

Dulbecco's Modified Eagle's Medium-Hams F12 Nutrient Mixture (1:1) (DMEM-F12), collagenase (type IV), Percoll, corticosterone-21-acetate, corticosterone antibody and pregnenolone were purchased from Sigma, USA. Rat LH (iodination grade), LH antibody, ovine LH (biological grade) and hCG (CR 127) were provided by NIDDK, USA as a gift. [1,2,6,7-^{3}H]-corticosterone and cAMP assay kits were purchased from Amersham–Pharmacia Biotech, UK. RIA kits for testosterone assay were purchased from Diagnostic Products Corporation (DPC), USA. Radioactive iodine [^{125}I] was obtained from Board of Radiation and Isotope Technology (BRIT), India. PD-10 G-25 Sephadex columns were purchased from Pharmacia LKB Biotechnology, Sweden. NAD, NADPH, NBT and other chemicals were from Sisco Research Laboratories (SRL), India.

2.2. Animals

Adult male Wistar rats (200–250 g) were maintained in a well ventilated, temperature controlled room with 12 h light and 12 h dark. Rats were fed with standard balanced rat pellet (Brooke Bond, Lipton India Ltd., India) and drinking water was available ad libitum.

Rats were divided into three groups each consisting of five rats. Group I — Control: rats were treated with equal volume of vehicle. Group II — Corticosterone treated: rats were treated with corticosterone-21-acetate (2.0 mg/100 g b.wt., i.m., twice daily at 9 am and 6 pm) for 15 days. Group III — Corticosterone plus LH: rats were treated concomitantly with corticosterone-21-acetate (dose as above) and oLH (100 μg/kg body weight, s.c., daily) for 15 days. At the end of the treatment period, animals were killed by decapitation and the trunk blood collected. Testes were removed and used for the collection of testicular interstitial fluid as described by Sharpe and Cooper [20] and isolation of Leydig cells.

2.3. Serum hormone assays

Serum LH and corticosterone were assayed by liquid-phase RIA. The cross-reactivity of LH antiserum with other hormones was $< 0.02\%$ with rFSH, $< 0.07\%$ with rTSH, and 0.01% with rPRL. The sensitivity of LH assay was 0.14 ng/ml. For the corticosterone assay, serum was extracted with diethyl ether and the extracts reconstituted with steroid assay buffer. Sensitivity of the assay was 40 pg/ml and the intra-and inter-assay co-efficient of variation 4.0–12.2% and 5–15%, respectively. Testosterone in the serum and TIF was assayed by solid-phase RIA kits obtained from DPC, USA. The sensitivity of the assay was 4 ng/dl, and the intra- and inter-assay co-efficient of variations 5% and 9.2%, respectively. The cross-reactivity of the testosterone antiserum with estradiol was 0.02%. Estradiol levels in serum and TIF were assayed by liquid-phase RIA after extraction with diethyl ether. The sensitivity of the assay was 0.1 pg/ml, the intra- and inter-assay co-efficient of variations 4.5% and 3.2–4.9%, and the cross-reactivity of estradiol antiserum with testosterone 0.001%.

2.4. Leydig cell separation

Leydig cells were isolated by the method of Rigaudiere et al. [21]. Testes were decapsulated and digested in collagenase containing medium (0.25 mg/ml) at 34°C for 15 min in a shaking water bath. The crude Leydig cell preparations so obtained were purified on discontinuous Percoll gradients. The purity of the Leydig cells was assessed by histochemical staining for 3β-HSD activity [22] and viability was determined by trypan blue exclusion. The purity of the Leydig cells was 80–85% and viability 90%.

2.5. ^{125}I-*hCG binding*

Highly purified hCG (CR 127) (NIDDK, USA) was iodinated with lactoperoxidase [23]. Leydig cells were pre-incubated under 5% CO_2 at 34°C for 12 h in culture medium (DMEM/F12 containing 1% BSA and 1% FCS). The cells were then incubated in FCS-free medium with 10,000 cpm of ^{125}I-hCG plus or minus unlabelled hormone for 16 h at 4°C. Non-specific binding (NSB) was determined by incubating the cells with ^{125}I-hCG in the presence of 1 μg of unlabelled hCG. At the end of incubation, cells were rinsed twice with PBS and lysed with 0.1 N sodium hydroxide. The cell-bound radioactivity was measured in the LKB gamma counter and the data subjected to Scatchard analysis.

2.6. Assay of steroidogenic enzymes

Purified Leydig cells were used for the assay of 3β- and 17β-hydroxysteroid dehydrogenases (3β- and 17β-HSDs) based on the spectrophotometric method of Bergmeyer [24].

2.7. In vitro studies

2.7.1. Testosterone production

Leydig cells were purified from control, corticoster-

one treated and corticosterone plus LH treated rats, plated in culture plates in DMEM/F12 containing 1% FCS and incubated under 5% CO_2 at 34°C for 12 h. At the end of incubation the medium was replaced with FCS-free fresh medium containing different concentrations of LH (50, 75, 100, 125, 150, 200 ng/ml) or cAMP (1, 2, 3, 4 and 5 mM), and incubated for another 24 h. The culture media were collected at the end of incubation period, centrifuged and used for testosterone assay. Testosterone in the Leydig cell culture media were extracted in dichloromethane and then assayed as per the kit procedure. The sensitivity of the assay was 1 pg/tube, cross reactivity of testosterone antiserum with other steroids was minimal, 0.34% for DHT, 0.021% for androsterone, 0.008% for 4-androstane-3,7-dione and 0.001% for corticosterone, estradiol and progesterone. Intra- and inter-assay co-efficient of variations were 3.4–8.0% and 5.9–12.1%, respectively.

2.8. LH stimulated cAMP production

Leydig cells isolated from control, corticosterone treated and corticosterone plus LH treated rats were plated and then stimulated with LH (as mentioned above). Cell pellets and media were used for cAMP assay. cAMP levels were assayed with kits purchased from Amersham–Pharmacia, UK. Cultured cells and media were extracted with 65% ice-cold ethanol and the extracts evaporated under nitrogen. The sensitivity of the assay was 1.0 fmole/tube.

2.9. Statistical analysis

Data were analysed by Analysis of Variance (ANOVA). When the F ratio was significant, multiple comparisons were done by Student–Newman–Keuls (SNK) tests to determine differences among various group means.

3. Results

3.1. Effects of 15-day corticosterone treatment on serum and TIF hormones

Exogenous administration of corticosterone significantly ($p < 0.05$) increased serum corticosterone levels but decreased serum LH concentrations compared with control. Administration of LH with corticosterone maintained serum LH concentrations at normal level (Fig. 1). As shown in Fig. 2, both serum and TIF testosterone and estradiol were significantly decreased in corticosterone and corticosterone plus LH treated rats compared with control. Nevertheless, serum and TIF concentrations of testosterone and estradiol in the corticosterone plus LH treated group were higher than

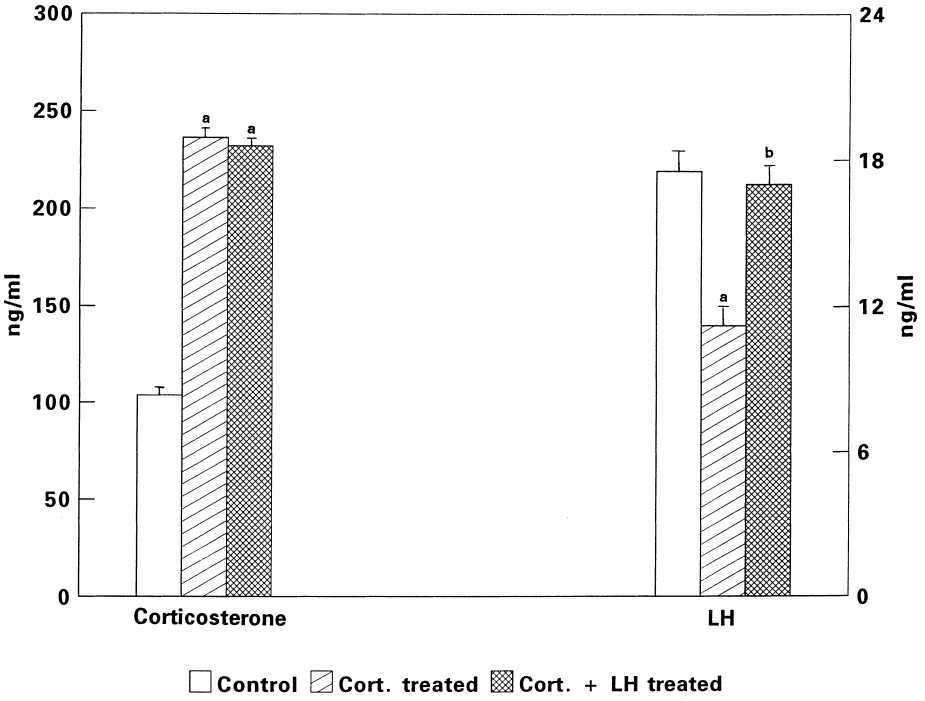

Fig. 1. Effects of corticosterone treatment on serum LH and corticosterone. Each bar represents the mean ± SEM of five estimations. Significance at $P < 0.05$; a — compared with control; b — compared with corticosterone treated.

corticosterone treated rats (the three groups were significantly different from each other at $p < 0.05$).

3.2. Effects of corticosterone treatment on Leydig cell LH/hCG receptor number and cAMP generation

In an attempt to determine the causes of the diminished testosterone production due to corticosterone treatment, we examined the binding of ^{125}I-hCG to Leydig cells and LH-stimulated cAMP production by Leydig cells. Corticosterone treatment significantly decreased Leydig cell LH/hCG receptor number and both basal and LH-stimulated cAMP production, changes which were not seen in corticosterone plus LH treated rats (Table 1).

3.3. Effects of corticosterone treatment on Leydig cell 3β- and 17β-hydroxysteroid dehydrogenases activities

To assess whether the inhibitory actions of excess corticosterone on Leydig cell steroidogenesis was at the level of steroidogenic enzymes, activities of 3β- and

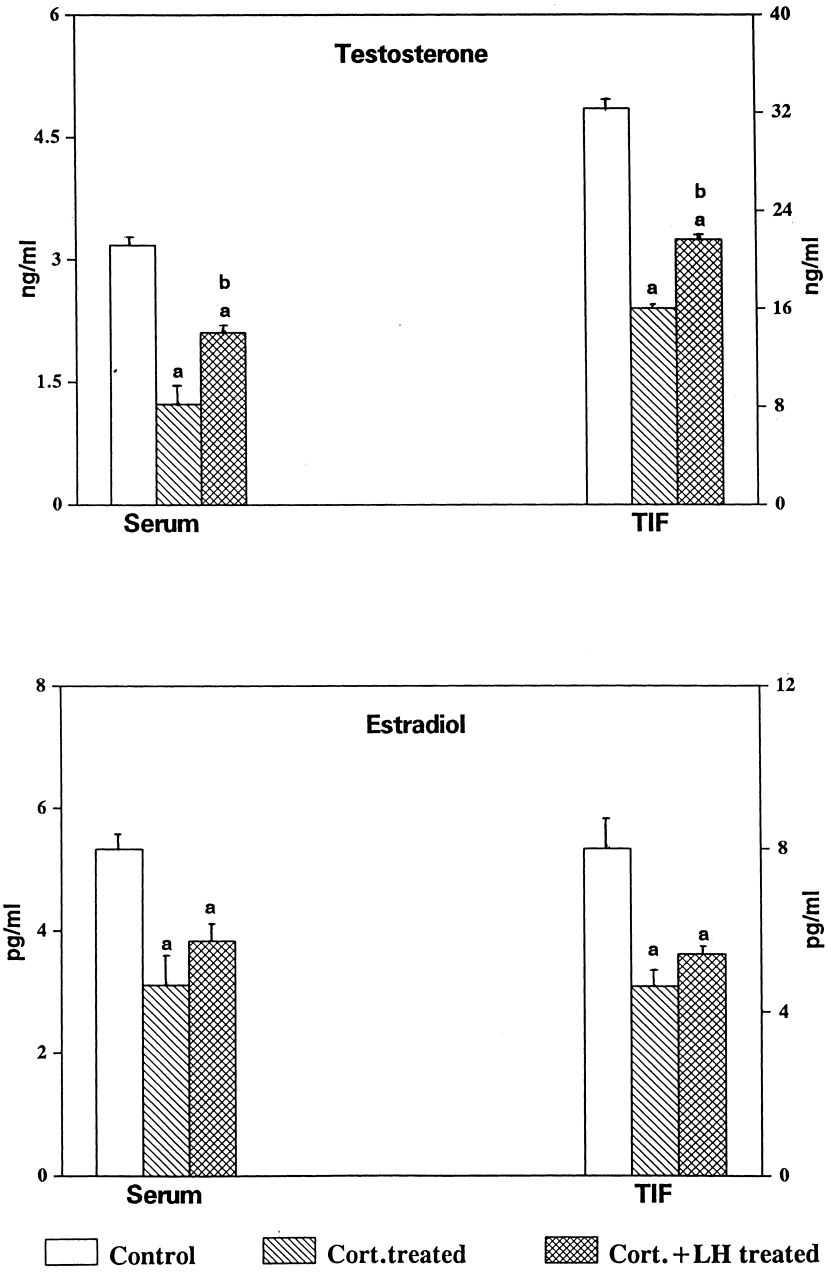

Fig. 2. Effects of corticosterone treatment on serum and testicular interstitial fluid (TIF) testosterone and estradiol. Each bar represents the mean ± SEM of five estimations. Significance at $P < 0.05$; a — compared with control; b — compared with corticosterone treated.

Table 1
Effects of corticosterone treatment on Leydig cell LH/hCG receptor and cAMP production[a]

	hCG receptors (fmole/10^6 cells)	cAMP (pmole/mg protein)	
		Basal	LH-stimulated
Control	15.8 ± 0.7	3.4 ± 0.2	32.9 ± 2.0
Corticosterone treated	9.1 ± 0.8[b]	1.9 ± 0.2[b]	23.1 ± 0.8[b]
Corticosterone + LH treated	15.7 ± 1.2[c]	3.0 ± 0.2[c]	31.5 ± 1.1[c]

[a] Values are mean ± SEM of six observations. Significance at $P < 0.05$ (SNK test).
[b] Compared with control.
[c] Compared with corticosterone treated.

17β-HSD were assayed. Corticosterone treatment effectively decreased Leydig cell 3β- and 17β-HSD activities. In corticosterone plus LH treated rats, the activity of 3β-HSD was restored to control level, but 17β-HSD activity remained significantly decreased (Fig. 3).

3.4. Effects of corticosterone treatment on LH and cAMP-stimulated leydig cellular testosterone production in vitro

The steroidogenic capacity of the Leydig cells of rats treated with corticosterone alone and in combination with LH was studied. Since decreased levels of serum LH and Leydig cell cAMP production were seen in corticosterone treated rats, Leydig cells were stimulated with oLH and 8-bromo cAMP in vitro. We used different concentrations of LH and cAMP to establish maximally effective doses for testosterone production. The maximum testosterone stimulation was achieved at 100 ng/ml for LH and 2 mM for cAMP (dose response curve not shown).

Basal (without hormone stimulation), LH (100 ng/ml) and cAMP (2 mM) stimulated testosterone production was decreased in Leydig cells of both corticosterone and corticosterone plus LH treated rats compared with control rats (Fig. 4).

4. Discussion

The present study clearly demonstrates that elevated levels of corticosterone resulting from exogenous administration of corticosterone decreased testicular testosterone production. The observed reduction in serum and TIF testosterone was associated with low levels of serum LH indicating that chronic treatment of corticosterone impaired pituitary LH secretion. These data are consistent with the observations of others [13,14],

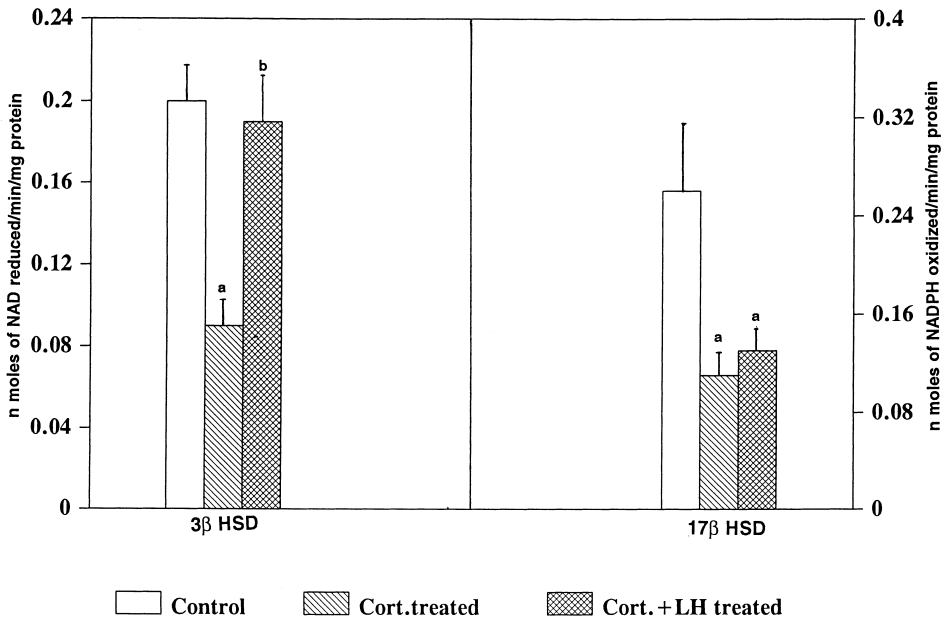

Fig. 3. Effects of corticosterone treatment on Leydig cellular 3β- and 17β- HSD activities. Each bar represents the mean ± SEM of five estimations. Significance at $P < 0.05$; a — compared with control; b — compared with corticosterone treated.

who have shown that chronic immobilization stress-induced increases in corticosterone decreased serum LH and testosterone in rats. The fall in serum testosterone associated with low levels of serum LH of the present study implies defective operation of negative feed back regulation in corticosterone treated rats. Rosen et al. [25] reported that dexamethasone suppresses hypothalamic GnRH and pituitary gonadotropin secretion. It has been shown [17] that restraint stress decreased hypothalamic luteinizing hormone-releasing hormone (LHRH) content, and that LHRH administration increased the pituitary gonadotropin secretion. These findings together with our results suggest that the inhibitory effects of chronic glucocorticoid treatment on LH secretion may be mediated by impaired hypothalamic secretion of GnRH and/or a direct pituitary effect on LH synthesis/release.

In the present study, chronic treatment of corticosterone decreased LH levels as well as Leydig cell LH receptor number; the expected up-regulation of LH receptors was not observed with subnormal circulating LH levels in corticosterone treated rats, whereas, LH receptor number was maintained at normal levels in corticosterone plus LH treated rats. This latter findings suggests that normal levels of circulating LH may be responsible for the maintenance of LH receptor number in Leydig cells of corticosterone plus LH treated rats. In agreement with this interpretation, immobilization stress (for 3 h) resulting in elevated corticosterone was also found to alter neither the serum LH levels nor Leydig cell LH receptors [5,10]. The decreased LH receptor number observed in this study might, thus, contribute to the inhibitory actions of corticosterone on Leydig cell testosterone production. In this regard, studies on the specific effect of corticosterone on LH receptor mRNA would be of great interest to assess the molecular mechanism by which excess glucocorticoid decreases Leydig cell LH receptor and thus, steroidogenesis.

To identify the post-receptor defect in LH action, basal as well as LH-stimulated cAMP production was assessed in Leydig cells. The decreased cAMP production by Leydig cells from corticosterone treated rats might be attributed to the decreased number of LH/hCG receptors. Since cAMP is the principal second messenger for LH in Leydig cells, generation of cAMP is dependent on LH and its receptor interaction and activation of adenylate cyclase [26]. This, therefore, suggests that the decreased basal and LH-stimulated cAMP generation and testosterone production by Leydig cells of corticosterone treated rats may be the consequence of defective transmembrane signaling and activation of adenylate cyclase activity. Administration of LH along with corticosterone maintained the basal and LH-stimulated cAMP production on a par with control rats. These data also support the existence of a direct relationship between LH receptor number and cAMP generation, because LH receptor levels and cAMP generation in corticosterone plus LH treated rats were both similar to that of control rats.

We also examined the effects of corticosterone on Leydig cell steroidogenic enzymes. 3β-HSD is involved in the conversion of Δ^5 steroids to Δ^4 steroids, which form the precursors for testosterone and thereby estrogens. 17β-HSD is specifically involved in the terminal step of testosterone synthesis i.e. conversion of andros-

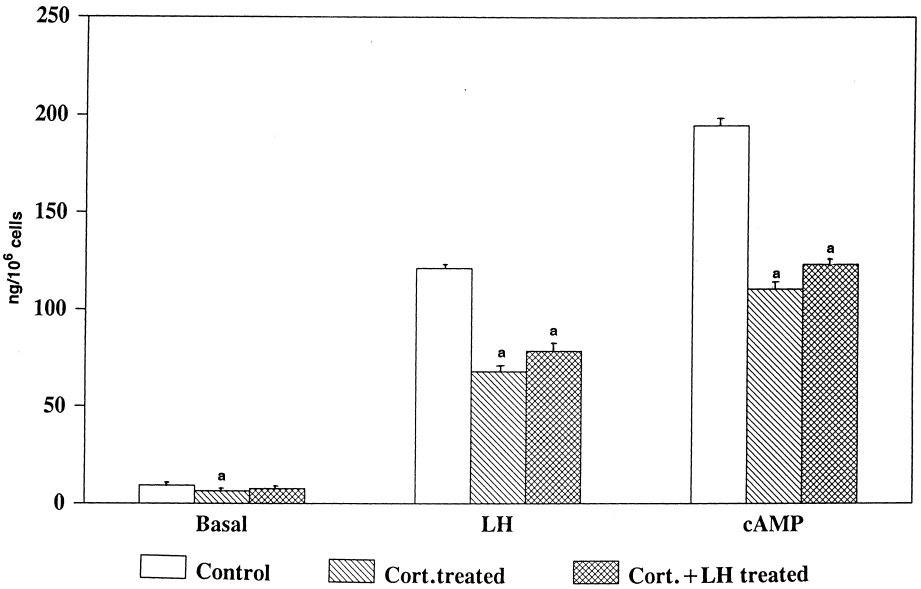

Fig. 4. Effects of corticosterone treatment on Leydig cell basal, LH (100 ng/ml) and cAMP (2 mM) stimulated testosterone production in vitro. Each bar represents the mean ± SEM of five estimations. Significance at $P < 0.05$; a — compared with control.

tenedione to testosterone. Decreased activity of steroidogenic enzymes viz., 3β-and 17β-HSD observed in the present study is consistent with the observations of Agular et al. [27,28], who showed a dose dependent inhibitory effects of dexamethasone on 3β-HSD in adult rat Leydig cell culture. Acute immobilisation stress decreased 17α-hydroxylase and 17,20-lyase activities [5]. Glucocorticoids also inhibit the constitutive and cAMP-stimulated $P450_{scc}$ enzyme activity, as well as mRNA of this enzyme [9]. Nevertheless, the present study is first of its kind to identify the inhibitory effects of corticosterone excess on 17β-HSD which is involved in the final step of testosterone synthesis. Addition of LH with corticosterone treatment prevented the decline in Leydig cell 3β-HSD activity but 17β-HSD activity remained similar to that in rats receiving corticosterone alone, suggesting that the 3β-HSD activity is directly regulated by LH. This is consistent with previous studies [27,29] which recorded the hCG-stimulated increase in 3β-HSD activity in purified Leydig cells.

The second messenger cAMP is also shown to be an important factor to regulate 3β-HSD activity in Leydig cells. Payne et al. [11] demonstrated that cAMP stimulates 3β-HSD mRNA expression. Corticosterone plus LH treated rats did not maintain 17β-HSD activity at control levels, indicating that corticosterone may directly regulate 17β-HSD activity in Leydig cells. It is deduced from the present study that the suppressive effect of corticosterone excess on Leydig cell steroidogenesis is the consequence of its direct inhibitory effect on 17β-HSD activity and that its indirect effect on 3β-HSD is through subnormal LH. It is also implied that corticosterone affects testosterone production distal to cAMP formation by inhibiting steroidogenic enzymes.

In vitro experiments were performed to explore the capacity of Leydig cells to produce testosterone in corticosterone treated rats. Testosterone production by Leydig cells of corticosterone treated and corticosterone plus LH treated rats under LH and cAMP stimulation was lower than control. Bambino and Hsueh [30] and Welsh et al. [8] have reported that glucocorticoids inhibit hCG-stimulated testosterone production. These authors added glucocorticoid to the Leydig cell culture medium along with hCG. In the present study, we assessed testosterone production by adding LH or cAMP to the culture media of Leydig cells isolated from the control and corticosterone treated rats. Both basal and LH or cAMP-stimulated testosterone production were decreased in the Leydig cells of corticosterone treated and corticosterone plus LH treated rats, emphasising that corticosterone treatment decreased the steroidogenic capacity of the Leydig cells through defective LH signal transduction. Circulating levels of testosterone declined in both corticosterone alone and corticosterone plus LH treated groups compared with control, with the decrease more pronounced in the corticosterone alone treated group. This suggests that LH replacement to corticosterone treated rats ameliorates steroidogenesis in Leydig cells, and also decreases the magnitude of the inhibitory effects of corticosterone on testosterone production. The direct inhibitory effect of corticosterone on 17β-HSD activity may thus underlie, the low levels of testosterone in culture media of Leydig cells of corticosterone and corticosterone plus LH treated rats compared with control.

The direct effect of corticosterone is mediated through specific receptors in the Leydig cells [6,7]. It has been reported that Leydig cells possess 11β-hydroxysteroid dehydrogenase (11β-HSD) enzyme activity, which metabolizes the active glucocorticoid to inactive form and protects Leydig cells from the receptor mediated inhibitory effects of glucocorticoids [31,32]. Though the protective role of 11β-HSD has been recognised in physiological conditions, studies on its role under excess corticosterone would be interesting.

We conclude from the present study that the inhibitory effect of chronic corticosterone treatment on Leydig cell steroidogenesis is mediated through impaired LH secretion, lowered LH receptor numbers and impaired signal transduction at pre-and post-cAMP levels. Administration of LH along with corticosterone unveils the direct inhibitory effect of corticosterone on Leydig cell 17β-HSD which is involved in the conversion of androstenedione to testosterone.

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