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GLUCOCORTICOID RECEPTORS IN INTERSTITIAL CELLS OF THE RAT TESTIS

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SUMMARY

Specific binding sites for [^{3}H]-dexamethasone are present in testis cytosol ($K_D = 3 \pm 2 \times 10^{-9}$; number of binding sites: 0.2 ± 0.05 pmol/mg protein). After incubation of isolated interstitial cells and seminiferous tubules with [^{3}H]-dexamethasone it was demonstrated that both cell types contain a limited number of specific cytoplasmic and nuclear binding sites, which are saturated with low hormone concentration (5×10^{-8} M). The number of binding sites in both cytosol and nuclei is higher in interstitial cells, but the affinity is similar. In short term *in vitro* incubations, dexamethasone did not modify the stimulatory effect of human chorionic gonadotropin on testosterone production by isolated interstitial cells, but the *in vivo* administration of dexamethasone for three days partially blocked the stimulatory effect of human chorionic gonadotropin on testicular testosterone production.

INTRODUCTION

It is currently believed that the initial step in the mechanism of steroid hormone action involves specific binding of the hormone to the receptor proteins in target tissues [1]. In the case of the rat testis, such binding macromolecules have been demonstrated for androgens [2–4], estrogens [5, 6] and glucocorticoids [7]. The androgen receptors different than testicular androgen binding protein (ABP) are localized in the seminiferous tubules [2], while the estrogen receptors are mainly localized in the interstitial cells [5, 6]. On the other hand the testicular cytosol binding macromolecules of glucocorticoids has been only determined in the whole testis [7]. In this paper evidence is reported that the glucocorticoid receptors in both cytosol and nuclear fraction are mainly localized in the interstitial cells. In addition, some data are presented which suggest that glucocorticoids may modify the specific function of interstitial cells.

MATERIALS AND METHODS

Male Sprague–Dawley rats of different ages either intact or 2–5 days after adrenalectomy were used. After decapitation of the rats the testis were isolated and the tunica albuginea was removed. Whole testis were homogenized in 3 vol. 25 mM Tris–HCl buffer pH 7.4, containing 1 mM EDTA, 6 mM mercaptoethanol, 3 mM $MgCl_2$ and 10% glycerol (Buffer A). The cytosol was prepared by centrifugation of the homogenate at 105000 *g* for 90 min. Aliquots of the cytosol were incubated for 3 h at 4° with [^{3}H]-dexamethasone (27 Ci/mmol) alone or in combination with a 500-fold excess of non-labeled steroid to evaluate the non-specific binding. The unbound steroid was adsorbed by a charcoal activated method [8]. Interstitial cells and seminiferous tubules were obtained by incubation of decapsulated testis in MEM medium (Eurobio) pH 7.4 containing 20 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), BSA (0.5 mg/ml) (buffer B) and collagenase (1 mg/ml) at 37° for 7–15 min as described by Catt *et al.* [9]. Both isolated interstitial cells and seminiferous tubules were washed three times with the same medium without collagenase, followed each time by a centrifugation at 100 *g* for 20 min. Seminiferous tubules were taken in a vol. 5 times greater than interstitial cells. Aliquots of interstitial cells or seminiferous tubules in buffer B were incubated with [^{3}H]-dexamethasone either at 4° for 3 h or at 33° for 2 h. Parallel incubations with the same concentrations of [^{3}H]-dexamethasone plus a 500-fold excess of unlabeled dexamethasone were run to correct for the non-specific binding of the labeled hormone either to the cytosol or to the nuclei. At the end of the incubation period the samples were rapidly diluted in 10 vol. of ice-cold buffer B and centrifuged. The pellet was washed 3 times with ice-cold buffer A, homogenized in the same buffer and centrifuged at 800 *g* for 10 min. The supernatant was centrifigued at 105000 *g* for 90 min to obtain the cytosol. The 800 *g* pellet was washed twice in buffer A containing 0.25 M sucrose, then the nuclei were purified by homogenization in buffer A containing 1.8 M sucrose, followed by centrifugation at 50,000 g for 90 min. The nuclear pellet was resuspended and centrifuged once in buffer A containing 0.25 M sucrose, once in the same buffer containing 0.2% Triton X-100 and twice in buffer A. The purified nuclei were extracted with 25 mM Tris–HCl, 0.4 M KCl pH 7.4 buffer for 2 h at 4° and then with ethanol. The

radioactivity extracted by ethanol represented about one third of that extracted by 0.4 M KCl.

The sedimentation coefficient of [^{3}H]-dexamethasone receptor complexes either in the cytosol or in the nuclei was examined by centrifugation in 5–20% linear sucrose gradient prepared in buffer A.

In vitro testosterone production by interstitial cells or seminiferous tubules was studied by incubation of the corresponding fractions in buffer B for 2 h at 33° in the presence or absence of 100 ng of human chorionic gonadotropin (hCG) (biological activity 12,600 IU/mg). After incubation, the testosterone in the medium was estimated by a specific radioimmunoassay [10]. Protein was determined by the method of Lowry *et al.* [11] and DNA by the diphenylamine method [12].

RESULTS

(1) *Cytosol glucocorticoids receptor of whole testis*

Figure 1 shows that testis cytosol prepared from prepuberal rats contains a glucocorticoid-binding macromolecule. The dissociation constant is $3 \pm 2 \times 10^{-9}$ M (5 determinations) and the number of binding sites is 0.2 ± 0.05 pmol/mg protein. Analysis of cytoplasmic complex on sucrose density gradient reveals [^{3}H]-dexamethasone is bound to a macromolecule in the region of 7 to 8 S (Fig. 2). Since the unbound labeled steroid was removed by the charcoal treatment before layering the sample on sucrose gradient, there was some dissociation of the complex during the time of centrifugation. The specificity of dexamethasone binding to testis cytosol was inferred from the observation that an excess of unlabeled steroid completely inhibited the binding of [^{3}H]-dexamethasone to the 7–8 S macromolecule and the *in vivo* administration of dexamethasone inhibits partially *in vitro* the binding of [^{3}H]-dexamethasone to the macromolecule (Fig. 2). Table 1 shows that the inhibitory effects of several non-radioactive steroids on the binding of [^{3}H]-dexamethasone to testis cytosol is similar to that observed with the glucocorticoid receptor obtained from other tissues [Reviewed in Ref. 13].

(2) *Cytosol and nucleus receptors in interstitial cells and seminiferous tubules*

Since the heterogeneity of the testicular tissue, it was important to specify in which particular cell of the testis the glucocorticoid receptor was localized. Microscopic control of the interstitial cells preparations revealed only a small contamination (less than 10%) with spermatozoa. On the other hand, seminiferous tubules were contaminated by Leydig cells. Nevertheless, the number of Leydig cells present in an aliquot of seminiferous tubules was always less than 10% than that of a similar aliquot of interstitial cells. The production of testosterone by aliquots of interstitial cells and seminiferous tubules under basal

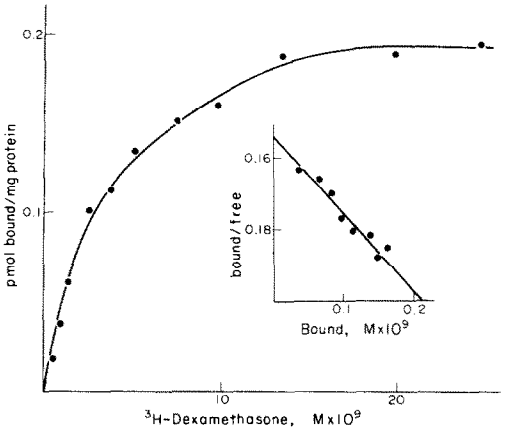

Fig. 1. Specific binding of [^{3}H]-dexamethasone by testis cytosol of 45 days intact rats. Aliquots of cytosol were incubated with various concentrations of [^{3}H]-dexamethasone with or without a 500-fold excess of non-radioactive steroid for 2 h at 4°. Bound dexamethasone was measured by charcoal assay. The inset presents the Scatchard plot of the binding data.

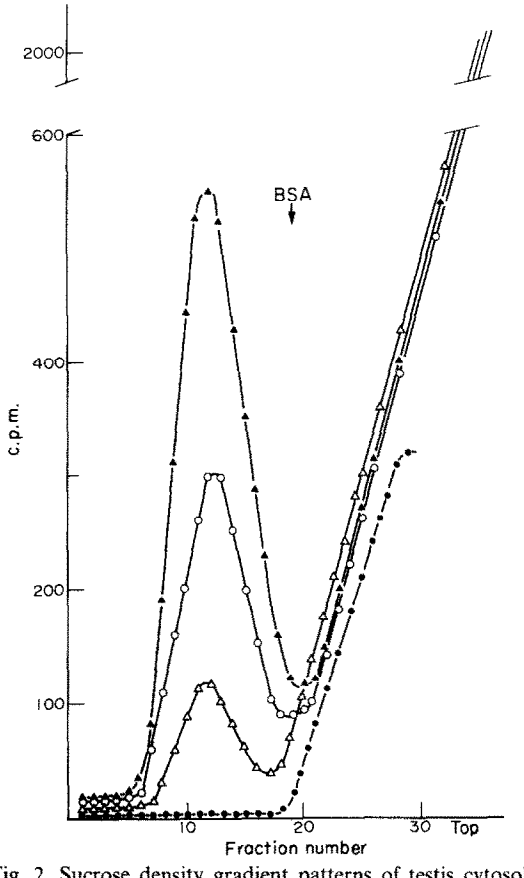

Fig. 2. Sucrose density gradient patterns of testis cytosol (3 mg of protein/ml) preincubated with [^{3}H]-dexamethasone (10^{-8} M) at 4° for 3 h. Following incubation the samples were mixed with charcoal and centrifuged at 2000 *g* for 20 min to remove the unbound steroid. Aliquots of the supernatant were layered on 5–20% sucrose gradient and centrifuged at 260000 *g* for 28 h. (○) intact rats, (▲) adrenalectomized rats, (△*) adrenalectomized rats injected 12 h before with 200 μg of dexamethasone, (●) cytosol of adrenalectomized rats in the presence of non radioactive dexamethasone (10^{-6} M).

Table 1. Effects of non-labelled steroids on specific binding of [^{3}H]-dexamethasone to cytosol of whole testis. Cytosol was incubated with 2×10^{-8} M [^{3}H]-dexamethasone alone with a 500-fold excess of non-labeled steroid. Each value is the mean of three determinations

Non labeled steroid	Binding %
None	100
Dexamethasone	5
Cortisol	14
Corticosterone	18
Progesterone	42
Estradiol	99
Testosterone	102

and maximal hCG stimulation confirmed the results of microscopic observations (Table 2).

When isolated interstitial cells or seminiferous tubules were incubated at 4° with increasing amounts of [^{3}H]-dexamethasone, the specific binding of this steroid to cytosol of both types of cells reached saturation with about 3×10^{-8} M [^{3}H]-dexamethasone (Fig. 3). The binding data obtained at saturation clearly show that the number of binding sites in the interstitial cells are about 5 times higher than those in the seminiferous tubules. Since the preparations of seminiferous tubules contain some interstitial cells, the real number of binding sites in the seminiferous tubules will be lower than that observed in Fig. 3. However the binding affinity of [^{3}H]-dexamethasone is similar for both preparations. Under 4° incubations there is little nuclear localization of [^{3}H]-dexamethasone (data not shown), a phenomenon known to occur in most steroid-receptor systems [1].

When [^{3}H]-dexamethasone is added to interstitial cells or seminiferous tubules and incubated at 33° for 2 h (equilibrium conditions) most of the radioactivity specific bound is localised in the nuclei (Fig. 4). The binding curve indicates that the nuclei of both interstitial cells and seminiferous tubules become saturated with [^{3}H]-dexamethasone concentrations near 5×10^{-8} M. However, the number of nuclear binding sites is about 4–5 times higher in the interstitial cells. Assuming 6 pg of DNA/cell nucleus [14], a homogenous cell population and one molecule of steroid per binding site, at saturation there is roughly 4000 binding sites per interstitial cell. Similar values were obtained when the calculations were done, taking into consideration the number of cells used in the assay.

Table 2. *In vitro* testosterone production by isolated interstitial cells and seminiferous tubules from 39-day old rats incubated at 33° for 2 h in the presence or absence of hCG (100 ng/ml)

	Control	hCG
Interstitial cells	29 ± 3*	107 ± 12
Seminiferous tubules	2 ± 1	7 ± 2

* pg testosterone/μg DNA/2 h (mean ± SD of triplicate).

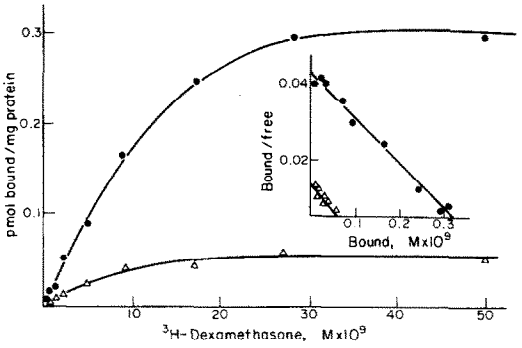

Fig. 3. Specific binding of [^{3}H]-dexamethasone by cytosol of isolated interstitial cells (●) or seminiferous tubules (△*) obtained from 50-days old rats, 3 days after adrenalectomy. Aliquots of interstitial cells or seminiferous tubules were incubated with various concentrations of [^{3}H]-dexamethasone for 3 h at 4°C. Following incubation cytosol was prepared and assayed for specific binding as described under Methods. The inset presents the Scatchard plot of the binding data.

(3) *Effects of dexamethasone on the interstitial cell function*

In general, the presence in a tissue of receptors for glucocorticoids has been found to correlate with some action of the hormone in that tissue [15]. Therefore we decided to investigate the possible action of dexamethasone in Leydig function. Table 3 shows that in short *in vitro* incubations dexamethasone does not modify the basal and the hCG stimulated testosterone production of isolated interstitial cells. However, *in vivo* dexamethasone administration to adrenalectomized rats partially blocks the stimulatory effects of hCG on plasma testosterone levels and on the testos-

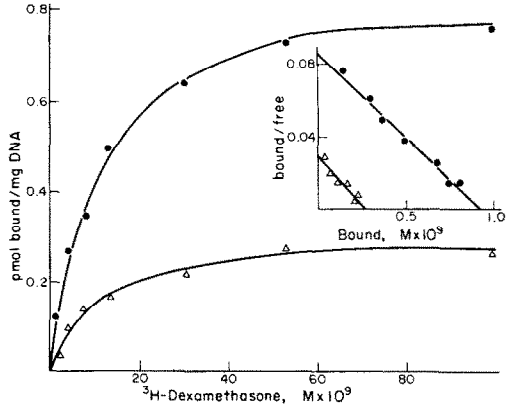

Fig. 4. Specific binding of [^{3}H]-dexamethasone by nuclei of isolated interstitial cells (●) or seminiferous tubules (△*) obtained from 49-day old rats, 3 days after adrenalectomy. Aliquots of interstitial cells or seminiferous tubules were incubated with various concentrations of [^{3}H]-dexamethasone for 2 h at 33°. At the end of the incubations, nuclei were prepared and assayed for specific binding as described under Methods. The bound steroid represents the addition of the radioactivity extract with 0.4 M KCl plus the extracts by ethanol. The inset presents the Scatchard plots of the binding data.

Table 3. *In vitro* testosterone production by isolated interstitial cells from 42 days old adrenalectomized rat. Identical aliquots of interstitial cells were incubated at 33° for 2 h without or with dexamethasone (10^{-6} M) and in the presence or in the absence of hCG (200 ng/ml)

Additions		Testosterone production
dexamethasone	hCG	
−	−	45 ± 10*
+	−	50 ± 8
−	+	180 ± 15
+	+	176 ± 18

* mean ± SD of three triplicates pg/μg DNA/2 h.

terone production by isolated interstitial cells (Table 4).

DISCUSSION

The data reported from testis cytosol fulfilled some of the criteria for specific glucocorticoid receptor identification: high binding affinity, saturability and hormone specificity. Ballard *et al.* [7] have already reported the existence of a specific glucocorticoid receptor in the cytosol prepared from the whole rat testis. The K_D and the number of binding sites found by these authors are similar to those observed in our study. The experiments reported here with isolated interstitial cells and seminiferous tubules have demonstrated that glucocorticoids receptors are localized mainly in the interstitial cell. Since the small contamination of seminiferous tubules by interstitial cells our results suggest but do not prove the presence also in the former of glucocorticoid receptor. Our studies also have shown that [^{3}H]-dexamethasone is taken up into nuclei of both interstitial and tubular cells, but again the number of "nuclear receptors" is higher in the interstitial cells. Therefore, the glucocorticoid receptor in the testis fulfilled the twostep hypothesis proposed for the mechanism of steroid action on target tissues [16].

Specific receptors for glucocorticoids have been found in many tissues [Reviewed *in* Ref. 13]. However, the delineation between target and non-target tissues for these steroids may not be absolute since glucocorticoids accumulated in most tissues of mice, including testis [17] and may enter into all cells to some extent [18]. Thus to assign a significance to glucocorticoid receptor in a tissue is critical to demonstrate a modification of a specific function of that tissue by the steroid. Administration of dexamethasone for three days to pubertal rats partially blocks the stimulatory effects of hCG on plasma testosterone levels (Table 4). This effect could be due either to an increase of the metabolic clearance rate of testosterone induced by unknown mechanism or to a decrease of the testicular testosterone production or both of these effects. The finding that the testosterone secretion by isolated interstitial cells of the dexamethasone treated rats was lower than that of control animals strongly suggests that the steroid partially inhibits the action of hCG on Leydig cells. However, in short term incubations, dexamethasone did not modify the steroidogenesis of isolated interstitial cells, neither under basal conditions, nor under maximal hCG stimulation. Therefore, the glucocorticoids have not any acute effect in the biochemical steps involved in steroidogenesis in Leydig cells. This finding is not surprising since most of the effects of glucocorticoids in target tissue are preceded by a characteristic lag period implying that the effects may be mediated through steroid interaction with the cell at the level of translation or transcription itself [19]. The exact mechanism by which dexamethasone inhibits the steroidogenic action of hCG on Leydig cells is not yet clear. Preliminary results in this laboratory suggest that this steorid inhibits the conversion of cholesterol to pregnenolone the limiting step which is stimulated by hCG [10]. Further studies are required to explain the inhibitory effects of dexamethasone on Leydig cell function and to find out whether or not this steroid has a direct regulatory effect on spermatogenesis.

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Table 4. Effects of various *in vivo* treatments on plasma testosterone levels and on the *in vitro* testosterone production by isolated interstitial cells. 47 days adrenalectomized rats were treated either with dexamethasone (200 μg/every 8 h), hCG (500 iu/every 12 h) or both for three days.

Treatment	Plasma testosterone	Testosterone production by isolated interstitial cells
	ng/100 ml	pg/μg DNA/2 h
None	36	16 ± 4*
hCG	600	107 ± 12
Dexamethasone	25	10 ± 2
Dexamethasone + hCG	380	77 ± 10

* mean ± SD of triplicate replications.

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