

Glucocorticoid Suppresses Steroidogenesis in Rat Progenitor Leydig Cells

Running title: Effects of DEX in Progenitor Leydig Cells

YE-CHEN XIAO^{a,b,d}, **YA-DONG HUANG**^{a,c,d}, **DIANNE O. HARDY**^a,

XIAO-KUN LI^b, **REN-SHAN GE**^{a*}

^a *Population Council, New York, NY 10065*

^b *Engineering Research Center of Bioreactor and Pharmaceutical Development, Ministry of Education, Jilin Agricultural University, Changchun, China, 130118*

^c *Biopharmaceutical research and Development Center, Institute of Life and Health Engineering, Jinan University, Guangzhou, China, 510642*

^d *contributed equally to this work.*

*Corresponding author

REN-SHAN GE, M.D,

Population Council, 1230 York Avenue, New York, NY 10065, USA.

Tel : 212-327-8761

Fax: 212-327-7678

E-mail address: rge@popcbr.rockefeller.edu

This study was supported by NIH grants RO1 HD050570.

ABSTRACT

Glucocorticoid (GC) inhibits testosterone production in adult Leydig cells by the glucocorticoid receptor (GR). However, whether GC affects the development of Leydig cells is unclear. The goal of the present study is to investigate the effects of GC on steroidogenesis of rat progenitor Leydig cells (PLCs) *in vitro*. Dexamethasone (DEX) inhibited androsterone (AO) production in PLCs. The GR antagonist, RU38486, reversed the DEX-induced inhibition of AO, while the MR antagonist, RU28318, did not. RU38486 also reversed DEX-induced reductions in steady-state mRNA levels of steroidogenic acute regulatory protein (*Star*) and 3 β -hydroxysteroid dehydrogenase 1 (*Hsd3b1*). StAR protein expression and 3 β HSD enzyme activity were affected similarly. These results show that GCs inhibit steroidogenesis of PLCs by suppression of StAR and 3 β HSD via GR-mediated mechanism.

Key words: dexamethasone, glucocorticoid receptor, steroidogenesis, progenitor Leydig cells

Introduction

Glucocorticoids (GCs) usually exert their effects through an intracellular receptor, the glucocorticoid receptor (GR), which is a member of the nuclear receptor family of ligand-dependent transcriptional factors (Schaaf and Cidlowski, 2002). The GC-activated GR translocates to the nucleus, homodimerizes and acts as a transcriptional factor by binding to the glucocorticoid response element within the promoter regions of GC responsive genes (Gupta, et al., 2007). Endogenous GCs can also bind to the mineralocorticoid receptor (MR) and exert mineralocorticoid actions like those of aldosterone (Frey, et al., 2004). Leydig cells contain GRs and MRs (Ge, et al., 1997; 2005). Many studies showed that GRs were the primary target of glucocorticoid action in the Leydig cells (Ortlip, et al., 1981, Schultz, et al., 1993, Stalker, et al., 1989), because GC-induced suppression of testosterone production was mediated by GR (Hales and Payne, 1989, Monder, et al., 1994).

To date, many studies have focused on GC action in immature and adult Leydig cells, the more mature cell types in the Leydig cell lineage. As endogenous GCs, cortisol or corticosterone, are required for some aspects of development, such as lung maturation in newborn mice (Noorlander, et al., 2006), we ask whether GCs might have a similar role in Leydig cell development. For the present study, we investigated the yet unresolved role of GCs in progenitor Leydig cells (PLCs). PLCs first become apparent by day 11 postpartum of rats (Ariyaratne, et al., 2000). These cells are different from immature and adult Leydig cells in many aspects, such as morphology,

size, androgen type, and expression levels of steroidogenic enzymes (Ge, et al., 2005, Ge and Hardy, 1998). For example, PLCs primarily produce androsterone (AO), while immature Leydig cells primarily produce 5 α -androstane-3 α , 17 β -diol (3 α -DIOL) and adult Leydig cells produce predominantly testosterone (Ge and Hardy, 1998).

The goal of the present study was to investigate the effect of dexamethasone (DEX), the potent synthetic GC, on androgen production, gene expression levels and activities of steroidogenic enzymes in PLCs from 21 days old rats. We used the GR antagonist RU38486 and the MR antagonist RU28318 in order to ask whether the effects of DEX were mediated through the GR or the MR. The results showed that GCs had an inhibitory role in steroidogenesis in PLCs.

Materials and Methods

Chemicals

22-Hydroxycholesterol, [7-N-³H] pregnenolone, [1, 2, 6, 7-³H] testosterone ([³H]T), [1,2-N-³H] dihydrotestosterone, 5 α -[9,11-N-³H] androstane-3 α ,17 β -diol, [1, 2, 6, 7-N-³H] androsterone (AO), and were purchased from DuPont-New England Nuclear (Boston, MA). [1, 2, 6, 7 - N-³H] Progesterone was purchased from Amersham International (Aylesbury, UK). Nonradioactive steroids were purchased from Sigma Chemical Co. (St. Louis, MO) or Steraloids (Newport, RI). Baker-Flex TLC plates (Polygram Silica Gel/UV254) were obtained from Krackeler Scientific

Inc.

Animals

Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). The male rats were 21 days of age on the day of cell isolation. The animals were killed by asphyxiation with CO₂. The animal protocol was approved by the institutional animal care and use committee of the Rockefeller University (Protocol 91200).

Primary PLC isolation and cell culture

PLCs were isolated from 21-day rats as described previously (Akingbemi, et al., 2000). Purities of Leydig cell fractions were evaluated by histochemical staining for 3 β -hydroxysteroid dehydrogenase (3 β HSD) activity, with 0.4 mM etiocholanolone as the steroid substrate (Payne, et al., 1980). More than 95% of Leydig cells isolated were intensely stained. PLCs were cultured for 24 h in buffered Dulbecco MEM: F12 medium alone before experiments, then they were cultured in buffered Dulbecco MEM:F12 medium containing 2.5% lipoprotein with 1-100 nM DEX with or without 1 μ M RU38486 or 1 μ M RU28318 for 24 h.

Radioimmunoassay (RIA)

The media were collected and stored at -20°C until the analysis of androgens by RIA. Medium AO concentrations were measured with a tritium-based RIA as

previously described (Cochran, et al., 1981).

RNA extraction and real-time RT-PCR

Total RNA was isolated using RNeasy mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. RNA pellets were resuspended in sterile ribonuclease-free water. Total RNA was used as the template for cDNA synthesis by MMLV reverse transcriptase (Promega, USA) and primed with random hexamers. The reaction mixture was incubated at 37°C for 60 minutes and then at 95°C for 15 min. PCR amplification took place in the presence of SYBR Green (Applied Biosystems, Foster City, CA, USA) in an ABI7700 Sequence Detector (Applied Biosystems). Primers were described previously (Lin, et al., 2008). The amplification of ribosomal protein S16 (*Rps16*) was used as an internal control.

Cell immunofluorescence

To examine intracellular steroidogenic acute regulatory protein (StAR) expression by immunofluorescence, we cultured PLCs on coverslips. Treatments with DEX (100 nM), RU38486 (1 µM) or RU28318 (1 µM) were for 24 h. The next day, the cells were fixed with 2% formaldehyde in PBS. Nonspecific binding sites were blocked with 10% Fetal bovine serum (FBS) in PBS and the cells were then incubated for 1 hr with the primary antibody rabbit-anti-StAR (diluted with PBS containing 0.1% saponin, 10% FBS) and washed for 5 min with PBS (containing 10% FBS) twice. The cells were exposed for 1 hr to secondary antibody (Alexa Fluor[®] 488

goat-anti-rabbit IgG (H + L), Invitrogen) , washed with PBS, and incubated with DAPI for 15 min. Coverslips were mounted on slides, using mounting medium and examined by fluorescence microscopy.

Enzyme assay

The activities of three androgen biosynthetic enzymes, P450 cholesterol side chain cleavage enzymes (P450scc), 3 β HSD and P450 17 α -hydroxylase/20-lyase (P450c17), and one androgen metabolizing enzyme, 5 α -reductase 1 (SRD5A1), were measured in the present study. Activity of P450scc was determined by measuring the conversion of 22-hydroxycholesterol to AO. The activities of 3 β HSD, P450c17 and SRD5A1 were determined by measuring conversion of pregnenolone to progesterone, progesterone to androstenedione and testosterone to dihydrotestosterone, respectively. The substrate concentrations used for each enzyme were maximal to ensure that the concentration of substrate was not rate limiting. Control samples of culture medium alone were run in parallel with each enzyme assay. Briefly, each reaction mixture (0.2 ml) was prepared in Leydig cell medium that contained 100 nM substrate (1 μ Ci).

Reactions were initiated by adding to the reaction medium an aliquot of 0.2×10^6 Leydig cells. After 45 min, reactions were terminated by adding ice-cold ethyl acetate, and steroids were rapidly extracted. The radioactivity was measured using a radiometric scanner (System 200/AC3000, Bioscan, Washington DC). The steroids

were separated on TLC plates in chloroform-methanol (97:3, v/v) for 3β HSD and SRD5A1 assays; chloroform-ether (7:1, v/v) for P450c17.

Statistical analysis

Values are expressed as means \pm S.E.M, and data were analyzed by the GraphPad Prism 4, (GraphPad Software Inc., San Diego, CA). Mean value comparisons between two groups were performed using the *t test*, and multiple groups were performed using One-way ANOVA with Dunnett's comparison of all columns vs control column, Differences were considered as significant at $P < 0.05$.

Results

Effects of DEX on steroidogenic functions of PLCs

The major androgen produced by PLCs is AO (Ge and Hardy, 1998). We exposed PLCs to 1-100 nM DEX for 24 h, and assayed AO levels. As shown in Figure 1A, DEX (1, 10, 100 nM) inhibited AO production compared to the control. When PLCs were cultured for 24 h in the presence of 1 or 10 nM DEX, AO production was decreased to 50% of the control. GR antagonist RU38486 (1 μ M) completely reversed the DEX-mediated inhibition of AO (Figure 1 B). The MR antagonist RU28318 (1 μ M), had no effects on the DEX-mediated inhibition of AO production. These results show that DEX exerts inhibitory effects on the steroidogenesis of PLCs via GR-mediated mechanisms.

Effects of DEX on steroidogenic protein expression in PLCs

PLCs express cholesterol transporter *Star* and three androgen synthetic enzymes, *Cyp11a1*, *HSD3b1*, *Cyp17a1*, and androgen metabolizing enzyme *Srd5a1* (Ge and Hardy, 1998). PLCs have almost undetectable 17 β -hydroxysteroid dehydrogenase 3, the last step androgen synthetic enzyme (Ge and Hardy, 1998). In order to dissect the inhibitory pathway of steroidogenesis by GCs, we measured, by real-time PCR, mRNA levels for *Star*, *Cyp11a1*, *HSD3b1*, *Cyp17a1* and *Srd5a1*. As shown in Figure 2, mRNA levels of *Star* and *HSD3b1* were significantly decreased by exposures to 100 nM DEX compared to the basal condition, but were significantly increased when RU38486 was present simultaneously. However, RU28318 did not reverse the DEX-mediated inhibition of the mRNA level of *HSD3b1* and *Star*. The mRNA levels of *Cyp11a1*, *Cyp17a1* and *Srd5a1* were not significantly different after any treatments compared to the control.

The activities of three androgen biosynthetic enzymes, P450_{scc}, 3 β HSD1 and P450_{c17} and an androgen metabolizing enzyme, SRD5A1, were assayed (Figure 3). In PLCs exposed to 100 nM DEX, 3 β HSD1 enzyme activity was 62% of the control. In PLCs exposed simultaneously to 100 nM DEX and 1 μ M RU38486, 3 β HSD1 activity was 100% of the control. In PLCs treated simultaneously with 100 nM DEX and 1 μ M RU28318, 3 β HSD1 was 56% of the control. These results are consistent with those of real-time PCR. P450_{scc}, P450_{c17} and SRD5A1 enzyme activities in any treatment group were not significantly different from control group levels.

Cellular levels of the StAR protein were detected by Alexa 488

immunofluorescence. The green Alexa 488 and blue nuclear DAPI staining were distinct (Figure 4A), therefore the StAR protein was present in the cytoplasm of PLCs. The statistical analysis showed DEX significantly decreased the StAR expression.

Discussion

The synthetic GC, DEX, is widely used as a drug to suppress symptoms of inflammation such as the development of local heat, redness, swelling and tenderness. DEX also influences gene transcription, through activation of the GR (Bhadhprasit, et al., 2007). In Leydig cells, glucocorticoid-induced inhibition of T biosynthesis occurs through suppression of *Cyp11a1*, *Hsd3b1*, and *Cyp17a1* gene expression in ALCs (Hales and Payne, 1989, Payne and Sha, 1991, Srivastava, et al., 1993, Welsh, et al., 1982). In the present study, we showed that DEX reduced the AO productions of PLCs. We also showed that DEX inhibited *Star* and *Hsd3b1* gene expressions. A GR antagonist, RU38486, but not a mineralocorticoid antagonist, RU28318, blocked the DEX suppression of *Star* and *Hsd3b1*. Therefore, we inferred that the DEX-induced suppressions were mediated by the GR. The lower levels of StAR protein and β HSD enzyme activity could have accounted for the decreased AO productions. GCs suppress Leydig cell steroidogenesis by decreasing gonadotropin stimulation of P450c17 activity and cAMP production which are correlated with StAR expression (Welsh, Bambino and Hsueh, 1982). In order to study the role of GCs in Leydig cell development, we examined PLCs. PLCs have the characteristics of unipotent stem cells, for example, *Hsd3b1* is highly expressed and *Cyp17a1* is weakly expressed. The

mRNA level of *Hsd17b3*, which encodes 17 β -hydroxysteroid dehydrogenase 3 for conversion of androstenedione to testosterone, was almost undetectable in the present study (data not shown), and it is consistent with our previous observation (Ge and Hardy, 1998). In the absence of 17 β HSD3 activity, SRD5A1 converts androstenedione to AO. In PLCs, AO is the most abundant androgen in PLCs as reported in our previous study (Ge and Hardy, 1998).

Our results showed that DEX treatment reduced levels of *Hsd3b1* mRNA, but not of *Cyp17a1* mRNA were consistent with the observations by Payne and Sha (Payne and Sha, 1991). Consistent with change of *Cyp17a1* mRNA level, CYP17A1 activity was not altered by DEX after progesterone was added as substrate for the measurement of CYP17A1. Thus addition of progesterone may reverse the DEX-induced reduction of aldosterone production. In the present study, we also found that DEX decreased the production of 3 β HSD protein in PLCs.

In this study, we used the GR antagonist RU38486 and MR antagonist RU28318 to identify the receptor through which DEX acts on PLCs. GRs are widely distributed in the brain and highly concentrated in regions involved in regulation of the stress response (De Kloet, et al., 1998). RU38486 is postulated to block the suppressive action of glucocorticoid on testosterone production (Baulieu, 1994).

RU28318 has been used widely to study MR function. In rat hippocampal tissue, receptor binding studies show that RU28318 treatment produces a selective 85% decrease in available MRs (Crochemore, et al., 2005). However, RU28318 did not

reverse the DEX-induced inhibition of AO production in PLCs. We infer that under basal conditions, AO synthesis is suppressed by a GR-mediated mechanism not a MR-mediated one.

It is true that some studies indicate that glucocorticoid may have a physiological role of Leydig cell development *in vivo*. For example, mammalian testes contain corticosterone-producing cells (Val, et al., 2006). We previously also demonstrated that 11 β -hydroxysteroid dehydrogenase 1, an enzyme responsible for the interconversion between biologically active corticosterone (in rats) and inactive 11-dehydrocorticosterone, was present in PLCs and behaved as a primary reductase to generate corticosterone (Ge, et al., 1997). However, the exact function of endogenous corticosterone to PLCs is unclear. Endogenous glucocorticoid may have different physiological role. For example, corticosterone has the same affinity for mineralocorticoid receptor and glucocorticoid receptor (Arriza, et al., 1987), while DEX has only high affinity to glucocorticoid receptor.

In summary, the present study show that DEX *in vitro* inhibits AO production of PLCs by lowering the expression of *Star* and *HSD3b1*. This action is mediated by GR.

Acknowledgements

We thank Chantal M. Sottas for technical assistance.

Reference

Akingbemi BT, Ge RS, Klinefelter GR, Gunsalus GL, Hardy MP. A metabolite of methoxychlor,

2,2-bis(p-hydroxyphenyl)-1,1, 1-trichloroethane, reduces testosterone biosynthesis in rat leydig cells through suppression of steady-state messenger ribonucleic acid levels of the cholesterol side-chain cleavage enzyme. *Biol Reprod.*2000;62:571-8.

Ariyaratne HB, Mills N, Mason JI, Mendis-Handagama SM. Effects of thyroid hormone on Leydig cell regeneration in the adult rat following ethane dimethane sulphonate treatment. *Biol Reprod.*2000;63:1115-23.

Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM. Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science.* 1987;237:268-75.

Baulieu EE. RU486: a compound that gets itself talked about. *Hum Reprod.*1994;9 Suppl 1:1-6.

Bhadhprasit W, Sakuma T, Hatakeyama N, Fuwa M, Kitajima K, Nemoto N. Involvement of glucocorticoid receptor and pregnane X receptor in the regulation of mouse CYP3A44 female-predominant expression by glucocorticoid hormone. *Drug Metab Dispos.*2007;35:1880-5.

Cochran RC, Ewing LL, Niswender GD. Serum levels of follicle stimulating hormone, luteinizing hormone, prolactin, testosterone, 5 alpha-dihydrotestosterone, 5 alpha-androstane-3 alpha, 17 beta-diol, 5 alpha-androstane-3 beta, 17 beta-diol, and 17 beta-estradiol from male beagles with spontaneous or induced benign prostatic hyperplasia. *Invest Urol.*1981;19:142-7.

Crochemore C, Lu J, Wu Y, Liposits Z, Sousa N, Holsboer F, Almeida OF. Direct targeting of hippocampal neurons for apoptosis by glucocorticoids is reversible by mineralocorticoid receptor activation. *Mol Psychiatry.*2005;10:790-8.

De Kloet ER, Vreugdenhil E, Oitzl MS, Joels M. Brain corticosteroid receptor balance in health and disease. *Endocr Rev.*1998;19:269-301.

Frey FJ, Odermatt A, Frey BM. Glucocorticoid-mediated mineralocorticoid receptor activation and hypertension. *Curr Opin Nephrol Hypertens.*2004;13:451-8.

Ge RS, Hardy DO, Catterall JF, Hardy MP. Developmental changes in glucocorticoid receptor and 11beta-hydroxysteroid dehydrogenase oxidative and reductive activities in rat Leydig cells. *Endocrinology.* 1997;138:5089-95.

Ge RS, Dong Q, Sottas CM, Chen H, Zirkin BR, Hardy MP. Gene expression in rat leydig cells during development from the progenitor to adult stage: a cluster analysis. *Biol Reprod.*2005;72:1405-15.

Ge RS, Dong Q, Sottas CM, Latif SA, Morris DJ, Hardy MP. Stimulation of testosterone production in rat Leydig cells by aldosterone is mineralocorticoid receptor mediated. *Mol Cell Endocrinol.*2005;243:35-42.

Ge RS, Hardy MP. Variation in the end products of androgen biosynthesis and metabolism during

postnatal differentiation of rat Leydig cells. *Endocrinology*.1998;139:3787-95.

Gupta S, Aslakson E, Gurbaxani BM, Vernon SD. Inclusion of the glucocorticoid receptor in a hypothalamic pituitary adrenal axis model reveals bistability. *Theor Biol Med Model*.2007;4:8.

Hales DB, Payne AH. Glucocorticoid-mediated repression of P450scc mRNA and de novo synthesis in cultured Leydig cells. *Endocrinology*.1989;124:2099-104.

Lin H, Ge RS, Chen GR, Hu GX, Dong L, Lian QQ, Hardy DO, Sottas CM, Li XK, Hardy MP. Involvement of testicular growth factors in fetal Leydig cell aggregation after exposure to phthalate in utero. *Proc Natl Acad Sci U S A*.2008;105:7218-22.

Monder C, Miroff Y, Marandici A, Hardy MP. 11 beta-Hydroxysteroid dehydrogenase alleviates glucocorticoid-mediated inhibition of steroidogenesis in rat Leydig cells. *Endocrinology*.1994;134:1199-204.

Noorlander CW, De Graan PN, Middeldorp J, Van Beers JJ, Visser GH. Ontogeny of hippocampal corticosteroid receptors: effects of antenatal glucocorticoids in human and mouse. *J Comp Neurol*.2006;499:924-32.

Ortlip SA, Li SA, Li JJ. Characterization of specific glucocorticoid receptor in the Syrian hamster testis. *Endocrinology*.1981;109:1331-8.

Payne AH, Downing JR, Wong KL. Luteinizing hormone receptors and testosterone synthesis in two distinct populations of Leydig cells. *Endocrinology*.1980;106:1424-9.

Payne AH, Sha LL. Multiple mechanisms for regulation of 3 beta-hydroxysteroid dehydrogenase/delta 5----delta 4-isomerase, 17 alpha-hydroxylase/C17-20 lyase cytochrome P450, and cholesterol side-chain cleavage cytochrome P450 messenger ribonucleic acid levels in primary cultures of mouse Leydig cells. *Endocrinology*.1991;129:1429-35.

Schaaf MJ, Cidlowski JA. Molecular mechanisms of glucocorticoid action and resistance. *J Steroid Biochem Mol Biol*.2002;83:37-48.

Schultz R, Isola J, Parvinen M, Honkaniemi J, Wikstrom AC, Gustafsson JA, Pelto-Huikko M. Localization of the glucocorticoid receptor in testis and accessory sexual organs of male rat. *Mol Cell Endocrinol*.1993;95:115-20.

Srivastava RK, Taylor MF, Mann DR. Effect of immobilization stress on plasma luteinizing hormone, testosterone, and corticosterone concentrations and on 3 beta-hydroxysteroid dehydrogenase activity in the testes of adult rats. *Proc Soc Exp Biol Med*.1993;204:231-5.

Stalker A, Hermo L, Antakly T. Covalent affinity labeling, radioautography, and immunocytochemistry localize the glucocorticoid receptor in rat testicular Leydig cells. *Am J Anat*.1989;186:369-77.

Val P, Jeays-Ward K, Swain A. Identification of a novel population of adrenal-like cells in the mammalian testis. *Dev Biol.* 2006; 299:250-256

Welsh TH, Jr., Bambino TH, Hsueh AJ. Mechanism of glucocorticoid-induced suppression of testicular androgen biosynthesis in vitro. *Biol Reprod.* 1982;27:1138-46.

Legends

Figure 1: Androgen production in progenitor Leydig cells (PLCs). All assays represent the results from 24 h incubations of 0.1×10^6 PLCs. The androsterone (AO) were measured as described in *Materials and Methods*. Data was presented as mean \pm SEM (n=6~8). **A** shows effects of increasing concentrations of DEX. The treatments were 0, 1, 10, and 100 nM DEX. A significant difference compared to control was shown at $^aP < 0.001$. **B** shows effects of GR and MR antagonists with and without DEX. The concentrations when used were DEX, 100 nM, RU38486, 1 μ M and RU28318, 1 μ M. Significant differences were shown at $^aP < 0.001$ vs control, $^bP < 0.01$ vs DEX, $^cP < 0.01$ vs DEX.

Figure 2: Real-time PCR analysis of mRNA levels in progenitor Leydig cells (PLCs). After the PLCs were cultured for 24 h in the presence of DEX (100 nM) and RU38486 (1 μ M) or RU28318 (1 μ M), RNA was isolated and templates were prepared as described in *Materials and Methods*. Data was presented as mean \pm SEM (n=6). A significant difference was shown at $^aP < 0.01$ vs control, $^bP < 0.01$ vs DEX, $^cP < 0.05$ vs DEX, $^dP < 0.05$ vs DEX.

Figure 3: Enzyme activities of P450_{scc}, P450_{c17}, 3 β HSD1 and SRD5A1 in progenitor Leydig cells (PLCs). After the PLCs were cultured in the presence of DEX (100 nM) and RU38486 (1 μ M) or RU28318 (1 μ M) for 24 h, the enzyme activities were measured as described in *Materials and Methods*. Data was presented as mean \pm SEM (n=6). A significant difference compared to control was shown at ^a $P < 0.01$ vs control, ^b $P < 0.01$ vs DEX, ^c $P < 0.05$ vs DEX.

Figure 4: Cell immunofluorescence of StAR. PLCs were cultured in the presence of DEX (100 nM) and either RU38486 (1 μ M) or RU28318 (1 μ M) for 24 h, and immunofluorescence was used to detect StAR as described in *Materials and methods*. StAR was present diffusely in the mitochondria of Leydig cells, and cell nuclei were stained blue by DAPI (Figure 4A, *Scale bar*, 10 μ m). Mean density was evaluated by Imagepro Plus software, and data presented as mean \pm SEM (n=5). A significant difference was shown at ^a $P < 0.01$ vs control, ^b $P < 0.05$ vs DEX, ^c $P < 0.05$ vs DEX (Figure 4B).

Figure 1

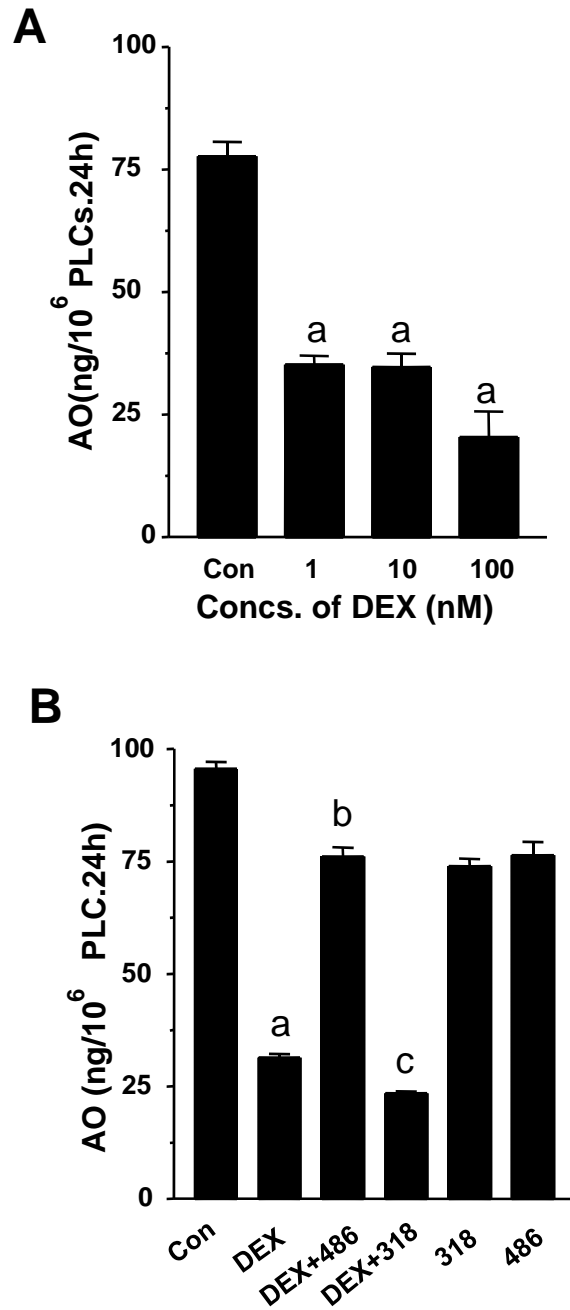


Figure 2

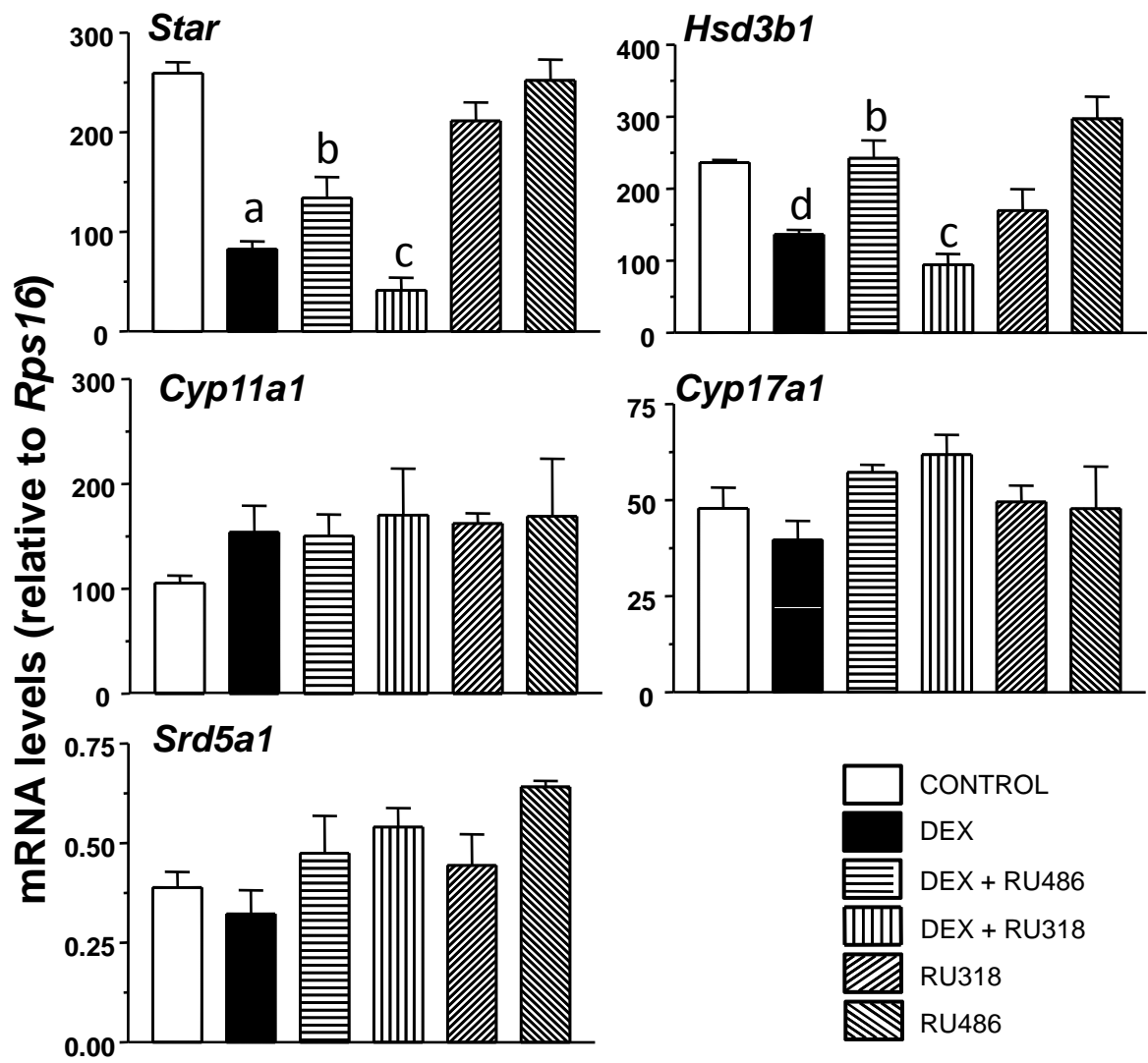


Figure 3

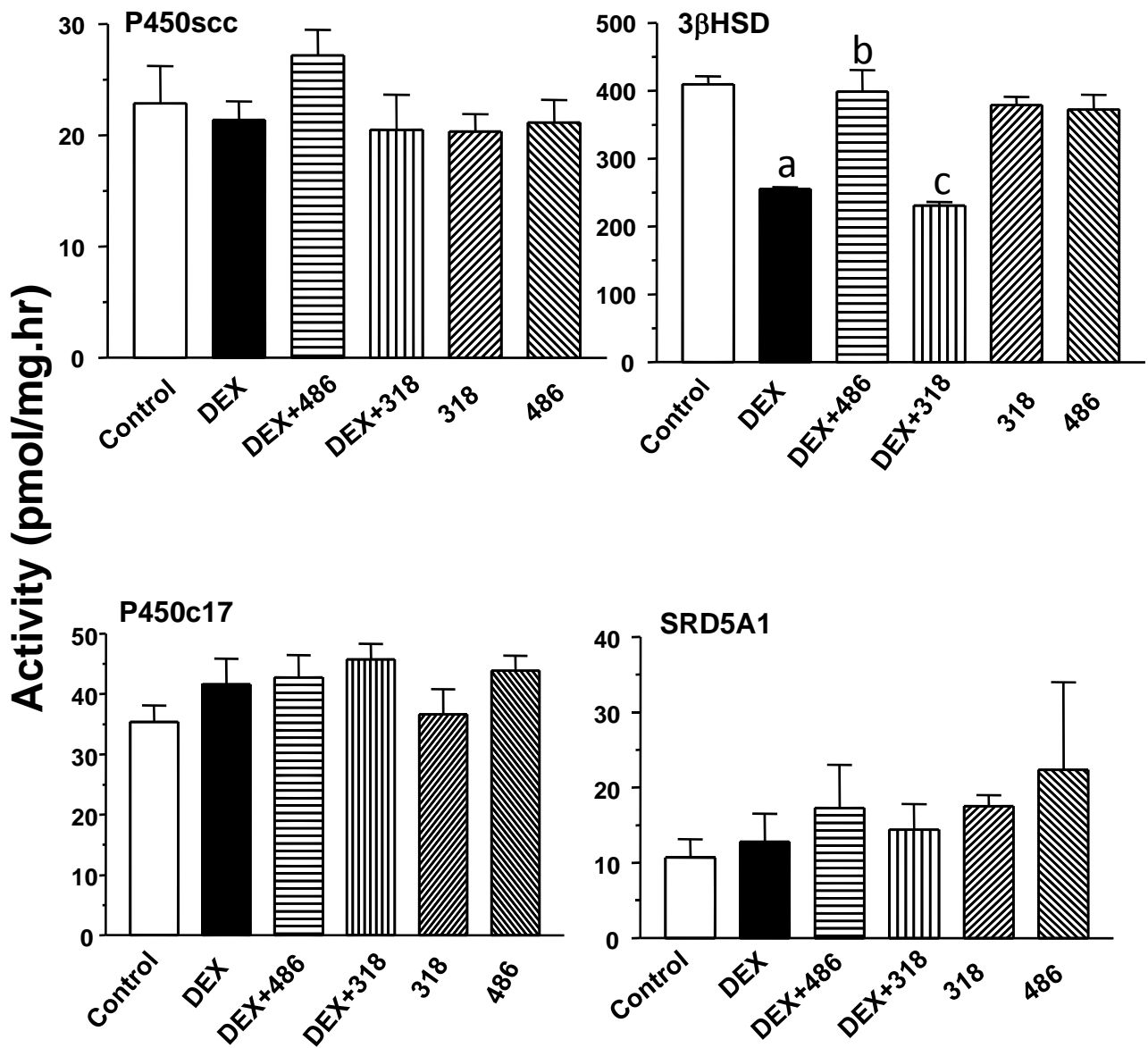


Figure 4

