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In Vivo Inhibition of Steroidogenic Acute Regulatory Protein Expression by Dexamethasone Parallels Induction of the Negative Transcription Factor DAX-1

Chi-Chuan Yu and Pi-Hsueh S. Li

Department of Physiology, National Cheng Kung University College of Medicine, Tainan 70101, Taiwan, Republic of China

In this study, we investigated the effect of dexamethasone on the synthesis of steroidogenic acute regulatory protein (StAR) and the expression of DAX-1 (dosage sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome, gene 1) and SF-1 (steroidogenic factor-1) in vivo. Male rats were treated with dexamethasone (0.4 and 4 mg/kg body wt per day) by intraperitoneal injections using phosphate-buffered saline as the vehicle for 7 d. At the end of 7 d, serum testosterone levels were decreased. Response to luteinizing hormone (LH) and 8-bromo-cyclic-AMP (8-Br-cAMP) in vitro was reduced in testicular cells isolated from dexamethasone-treated rat testes. Dexamethasone decreased LH-stimulated cAMP production. The conversion of 22(R)-hydroxycholesterol, pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone, and androstenedione to testosterone was not affected by dexamethasone. Dexamethasone increased DAX-1 expression and concordantly decreased StAR protein and mRNA in testicular cells. The increase in DAX-1 protein corresponded to a 57% reduction in StAR mRNA levels concomitant with a 79% reduction in serum testosterone levels. Dexamethasone had no effect on the level of SF-1, but increased the amount of complexed DAX-1–SF-1. Dexamethasone in vitro suppressed StAR promoter activity when an increasing amount of DAX-1 cDNA was transfected. These results demonstrate that dexamethasone increases expression of DAX-1, which results in increased amounts of complexed DAX-1–SF-1, in the absence of any change in the expression of SF-1. These observations strongly support the concept that dexamethasone suppresses rat testicular testosterone production, at least in part, by increasing the amount of complexed DAX-1–SF-1 in these cells, which leads directly to decreased availability of free SF-1 and, therefore, decreased activation of transcription of the rat StAR gene.

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Author to whom all correspondence and reprint requests should be addressed: Dr. Pi-Hsueh Shirley Li, Department of Physiology, National Cheng Kung University Medical College, 1 University Road, Tainan 70101, Taiwan, Republic of China. E-mail: lifupi@mail.ncku.edu.tw

Key Words: Rat testicular cells; dexamethasone; steroidogenic acute regulatory protein; testosterone; DAX-1.

Introduction

Stress and other conditions that elevate the plasma concentrations of glucocorticoids reduce testosterone concentrations in the plasma in all known animal species *(1)*. This glucocorticoid-mediated suppression of testosterone can have profound physiological consequences, including muscular atrophy and sexual dysfunction *(2–4)*. The effects of glucocorticoids on testosterone production and male reproductive function may occur at the hypothalamus (to decrease the synthesis and release of GnRH), the anterior pituitary gland (to inhibit the synthesis and release of gonadotropins), or the testis (to modulate steroidogenesis). Glucocorticoids have been demonstrated to decrease LH secretion in vivo *(5)* and may influence either pituitary responsiveness to GnRH *(6)* or GnRH release from the hypothalamus *(7,8)*. The presence of glucocorticoid receptors in interstitial cells of the rat testis was first demonstrated by Evain et al. *(9)*, whose data suggested that glucocorticoids may influence steroidogenesis in the Leydig cell. Since then, reports of several investigators have shown that glucocorticoids can directly inhibit testicular steroidogenesis in vitro *(10–12)*. In accordance with these results, in vivo investigations also demonstrated a direct influence of glucocorticoids on Leydig cells *(13–15)*. On the other hand, a significant increase of steroidogenic capacity of purified Leydig cells was reported if endogenous plasma levels were reduced by adrenalectomy *(16)*. The intracellular mechanism of the decreased steroidogenesis has not been definitely established, but a number of in vitro studies have shown that glucocorticoid directly inhibits processes in the Leydig cell critical to the biosynthesis of testosterone, including cholesterol side-chain cleavage cytochrome (P450scc) enzyme, 3β-hydroxysteroid dehydrogenase/$\Delta^{5\text{-}4}$-isomerase (3β-HSD), and 17α-hydroxylase/17-20-lyase *(10–12,17)*. In most cases, glucocorticoids have no effects on basal enzyme activity, but inhibit the stimulation induced by LH or FSH. One mechanism underlying such effects might be the inhibition of the cAMP-mediated induction of steroidogenic enzyme expression by LH and FSH.

The biosynthesis of testosterone begins from cholesterol. The acute regulated and rate-limiting step for the synthesis of testosterone is the delivery of cholesterol from cellular stores to the mitochondrial inner membrane where the cholesterol is then converted to pregnenolone by P450scc enzyme *(18)*. The transport of cholesterol in steroidogenic cells is thought to be mediated by steroidogenic acute regulatory (StAR) protein *(19)*. The StAR protein has been identified as a phosphoprotein *(20)* that is rapidly induced by tropic hormones, such as gonadotropins and corticotropin *(21,22)*. StAR protein expression and regulation by tropic hormones has been examined in great detail *(22)*. StAR gene expression is regulated at the transcriptional level by steroidogenic factor-1 (SF-1) *(23–25)*. This transcription factor is highly expressed in steroidogenic tissues and is thought to be involved in mediating transcriptional activation of the rat StAR promoter in response to cAMP stimulation *(25)*. On the other hand, SF-1 activity is blocked by DAX-1 (dosage-sensitive sex reversal adrenal hypoplasia congenita, critical region on the X chromosome, gene-1), another transcription factor whose expression is mostly restricted to steroidogenic tissues, such as adrenal cortex, ovary, and Leydig cells *(26)*. DAX-1 represses SF-1–mediated transactivation of StAR gene *(27,28)*. Overexpression of DAX-1 in Y-1 mouse adrenal tumor cells represses both basal and cAMP-induced StAR promoter activity by binding to DNA hairpin secondary structures on the StAR gene promoter or to the SF-1 protein itself *(27)*. The co-localization of SF-1 and DAX-1 in steroidogenic tissues led to the suggestion of a functional interaction between these two nuclear proteins *(26)*. The complex endocrine phenotypes with the SF-1 and DAX-1 mutations also point strongly to their interactions in a hierarchial pathway, and they may activate the target genes in a cooperative fashion *(29)*. It is apparent that understanding the involvement of glucocorticoids in the regulation of steroidogenesis requires exploration of the two transcription factors SF-1 and DAX-1.

In light of these aforementioned observations in the involvement of glucocorticoids in steroidogenesis, their precise mode of action remains to be determined. The important role of StAR in steroidogenesis prompted us to examine its involvement in glucocorticoid-induced inhibition of testosterone production, and, consequently, in the mechanism of action by employing the orphan nuclear receptors SF-1 and DAX-1.

Results

Effect of Treatment with Dexamethasone on Serum Testosterone Concentration

Injection of dexamethasone (4 mg/kg body wt) caused a significant ($p < 0.05$) reduction in serum testosterone levels (Fig. 1). When animals were injected with 0.4 mg/kg body wt of dexamethasone, the decrease in serum testosterone was not significant (Fig. 1).

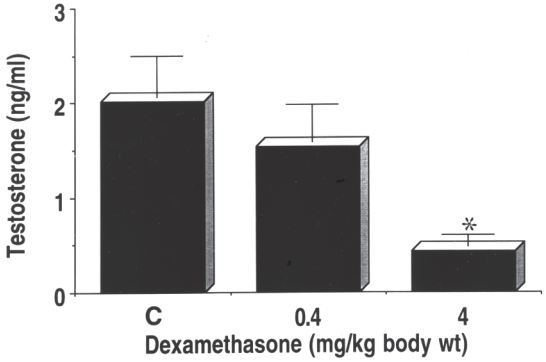

Fig. 1. Serum testosterone levels in PBS- and dexamethasone-injected rats. Rats were injected daily with PBS (C, control) or dexamethasone (0.4 and 4 mg/kg body wt) for 7 d. At approx 18 h after the last injection, rats were killed and blood was collected from the trunk. Serum testosterone levels were measured by RIA. Data are expressed as the mean ± SEM; $n = 5$. *$p < 0.05$ between control and concentration of 4 mg/kg body wt.

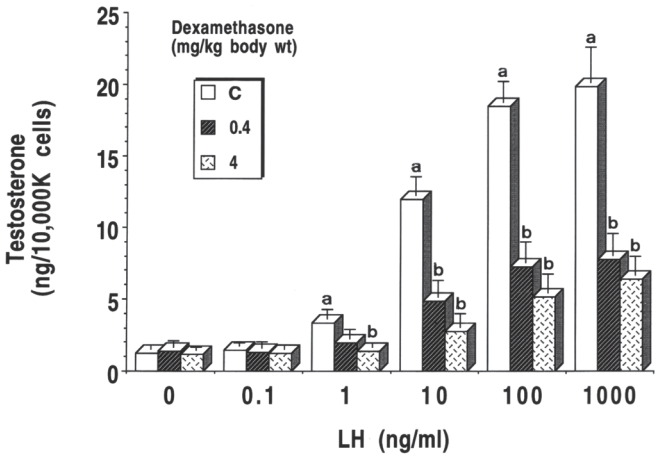

Fig. 2. Effect of in vivo treatment with dexamethasone on in vitro testosterone production in response to LH. Rats were injected daily with PBS (C, control) or dexamethasone (0.4 and 4 mg/kg body wt) for 7 d. At approx 18 h after the last injection, rats were killed and testicular cells were incubated in 0.5 mL F12/DMEM-0.1% BSA with or without porcine LH at 34°C for 3 h. The medium content of testosterone was measured by RIA. Data are expressed as the mean ± SEM; $n = 6$. a, $p < 0.05$ when compared with LH = 0 in the control group; b, $p < 0.05$ when compared with control value at each dose of LH.

Effect of Treatment with Dexamethasone on Testicular Steroidogenic Responsiveness

To investigate the effect of dexamethasone on testosterone production in response to LH, rats were treated with dexamethasone (0.4 and 4 mg/kg body wt) for 7 d. Testicular steroidogenic capacity was measured in vitro. As shown in Fig. 2, LH stimulated testosterone production in a dose-related manner. LH at a dose above 0.1 ng/mL significantly ($p < 0.05$) increased testosterone production over basal in control group. Treatment with dexamethasone inhibited testosterone production in response to LH at concentrations of 1 ng/mL or above.

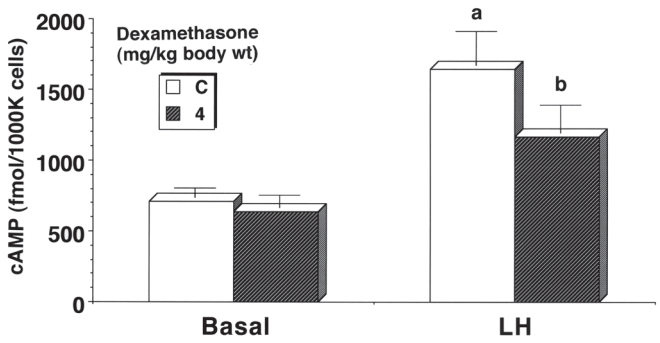

Fig. 3. Effect of in vivo treatment with dexamethasone on in vitro cAMP production in response to LH. Rats were treated with PBS (C, control) or dexamethasone (4 mg/kg body wt) as described in Fig. 2. Testicular cells were incubated with or without (Basal) LH (10 ng/mL) at 34°C for 3 h. The cellular content of cAMP was measured by RIA. Data are expressed as the mean ± SEM; $n = 6$. a, $p < 0.05$ when compared with basal value in the control group; b, $p < 0.05$ when compared with control value in LH-treated group.

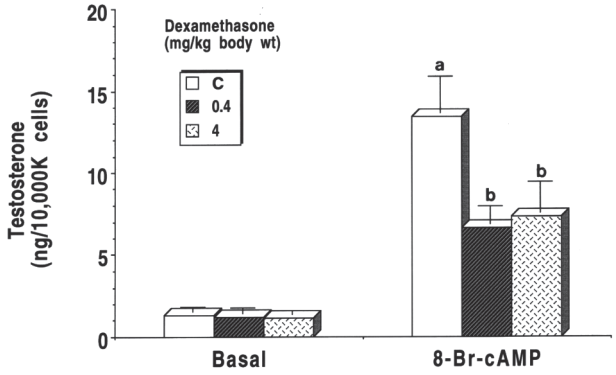

Fig. 4. Effect of in vivo treatment with dexamethasone on in vitro testosterone production in response to 8-Br-cAMP. Rats were treated with PBS (C, control) or dexamethasone (0.4 and 4 mg/kg body wt) as described in Fig. 2. Testicular cells were incubated with or without (Basal) 8-Br-cAMP (0.5 m*M*) at 34°C for 3 h. The medium content of testosterone was measured by RIA. Data are expressed as the mean ± SEM; $n = 6$. a, $p < 0.05$ when compared with basal value in the control group; b, $p < 0.05$ when compared with control value in 8-Br-cAMP-treated group.

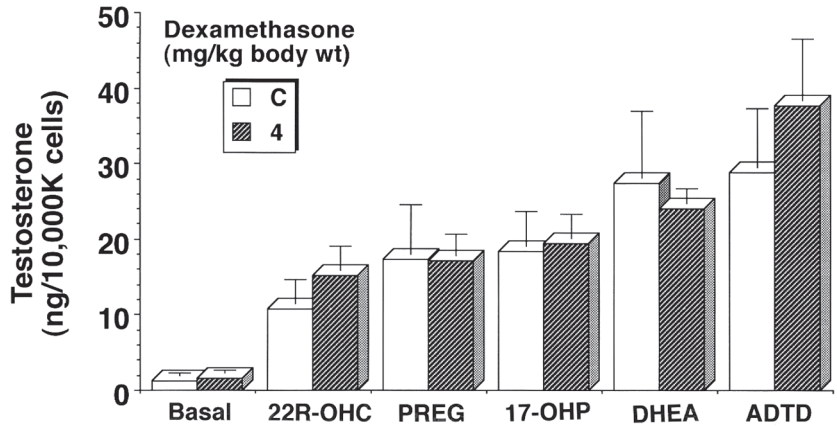

Fig. 5. Effect of in vivo treatment with dexamethasone on the conversions of steroid precursors to testosterone. Rats were treated with PBS (C, control) or dexamethasone (4 mg/kg body wt) as described in Fig. 2. Testicular cells were incubated with or without (Basal) 22(R)-hydroxycholesterol (22R-OHC), pregnenolone (PREG), 17-hydroxypregnenolone (17-OHP), dehydroepiandrosterone (DHEA) and androstenedione (ADTD) of 1 μ*M* at 34°C for 3 h. Testosterone levels were measured in the supernatants by RIA. Results are the mean ± SEM; $n = 7$.

Because the LH-stimulated testosterone production is mediated via increases in cAMP production, the influence of dexamethasone (4 mg/kg body wt) treatment on LH-stimulated cAMP production was examined. As shown in Fig. 3, the cellular content of cAMP was low in both control and dexamethasone-treated testicular cells, while LH (10 ng/mL) administration significantly ($p < 0.05$) increased cAMP production as compared to basal. Dexamethasone reduced LH-stimulated cAMP production by 30% ($p < 0.05$).

The influence of dexamethasone upon the ability of 8-Br-cAMP to stimulate testosterone production was also investigated (Fig. 4). Treatment with 8-Br-cAMP (0.5 m*M*) significantly ($p < 0.05$) increased testosterone production over basal in the three treatment groups. However, dexamethasone reduced the stimulatory effect for 8-Br-cAMP ($p < 0.05$).

Effect of Treatment with Dexamethasone on the Conversion of Steroid Precursors to Testosterone

To examine the site(s) in the steroidogenic pathway affected by dexamethasone, the effects on the conversion of steroid precursors in concentrations of 1 μ*M* to testosterone by testicular cells isolated from control and dexamethasone (4 mg/kg body wt)-treated rats were characterized. As shown in Fig. 5, addition of either 22(R)-hydroxycholesterol, pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone, or androstenedione produced significant ($p < 0.05$) increases in testosterone production. The conversion

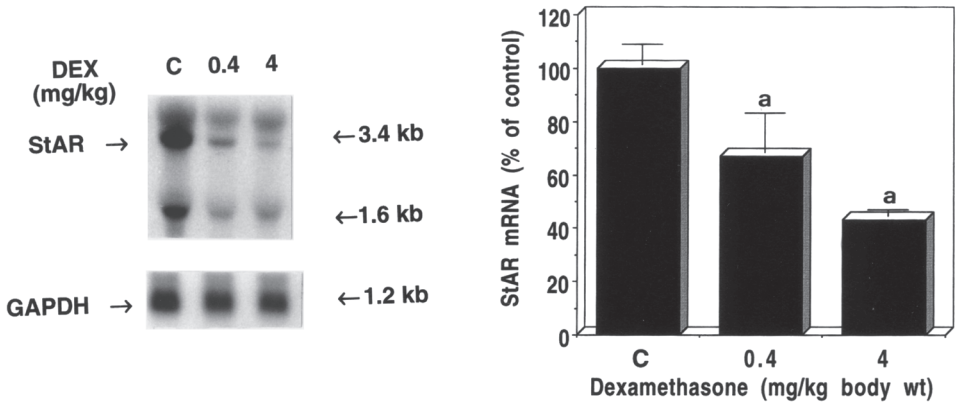

Fig. 6. Effect of in vivo treatment with dexamethasone (DEX) on testicular StAR mRNA levels. Rats were treated with PBS (C, control) or dexamethasone (0.4 and 4 mg/kg body wt) as described in Fig. 2. Total RNA was isolated from testicular cells. Samples of total RNA (25 μg) were analyzed for StAR mRNA by Northern blotting analysis. The positions of the 3.4- and 1.6-kilobase (kb) StAR mRNA and 1.2-kb GAPDH mRNA are indicated. Hybridization signals were quantitated using the Arcus II computer-assisted image system. Integrated optical density values for the 3.4-kb transcript of StAR were normalized to the GAPDH mRNA levels. The normalized values for the 3.4-kb StAR mRNA transcript were expressed as percentage StAR mRNA content of control cells. Values are the mean ± SEM; $n = 5$. a, $p < 0.05$ between control and concentration of 0.4 and 4 mg/kg body wt. A representative Northern blot is shown.

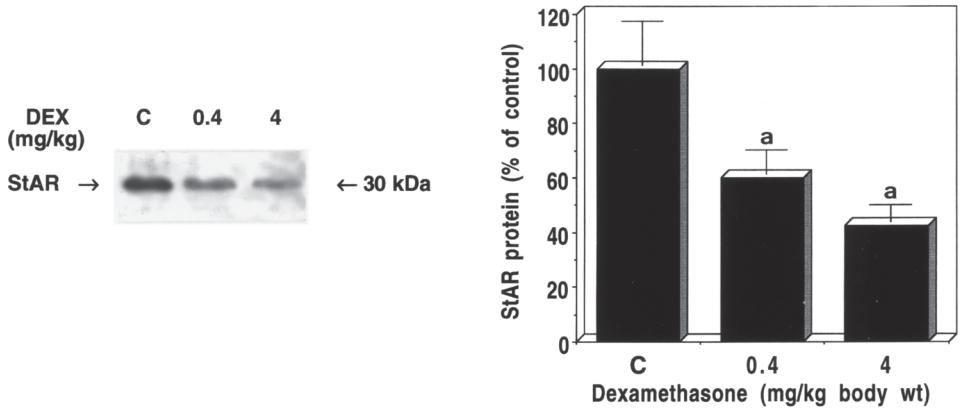

Fig. 7. Effect of in vivo treatment with dexamethasone (DEX) on testicular StAR protein levels. Rats were treated with PBS (C, control) or dexamethasone (0.4 and 4 mg/kg body wt) as described in Fig. 2. Whole cell extracts were prepared from testicular cells. For each sample, 50 μg of protein/lane were analyzed for StAR protein by Western blotting analysis. The position of the StAR protein is indicated. The StAR protein was detected by chemiluminescence and quantitated using the Arcus II computer-assisted image system at each treatment. Integrated optical density values were expressed as percentage of StAR protein content of control cells. Values are the mean ± SEM; $n = 5$. a, $p < 0.05$ between control and concentration of 0.4 and 4 mg/kg body wt. A representative Western blot is shown.

of the individual steroid precursors to testosterone was not affected by in vivo treatment with dexamethasone.

Effect of Treatment with Dexamethasone on Testicular StAR mRNA and Protein Levels

Because StAR is required for steroid hormone production and a reduction in StAR gene expression would likely result in a reduction in steroidogenesis, we then determined if expression of this protein is downregulated by dexamethasone in testicular cells. As shown in Figs. 6 and 7, the StAR mRNA and protein levels were significantly ($p < 0.05$) reduced upon treatment with dexamethasone.

Effect of Treatment with Dexamethasone on Testicular DAX-1 and SF-1 Protein Levels and Testicular Formation of DAX-1 and SF-1 Complex

Because DAX-1 decreases transcription of a StAR promoter-linked luciferase reporter gene *(28)*, we investigated whether dexamethasone alters DAX-1 protein levels leading to a decrease in StAR gene expression. As shown in Fig. 8, DAX-1 protein levels were significantly ($p < 0.05$) increased in testicular cells isolated from dexamethasone-treated rats.

Because SF-1 participates in the expression of StAR gene, the effect of dexamethasone on SF-1 protein expression was also investigated. As shown in Fig. 9, treatment with dexamethasone had no effect on SF-1 protein levels.

As stated in the Introduction, DAX-1 transcriptional repression can occur through its direct interaction with SF-1. We investigated whether dexamethasone affects the nature of complexformation between DAX-1 and SF-1. As shown in Fig. 10, a strong band was detected in anti-SF-1 precipitate of control cells, indicating the presence of a large amount of DAX-1–SF-1 complex in these cells. The amount of complexed DAX-1–SF-1 was increased in testicular cells obtained

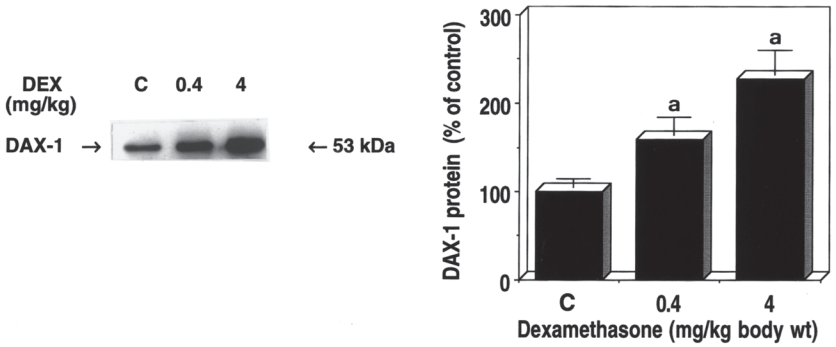

Fig. 8. Effect of in vivo treatment with dexamethasone (DEX) on testicular DAX-1 protein levels. Rats were treated with PBS (C, control) or dexamethasone (0.4 and 4 mg/kg body wt) as described in Fig. 2. Nuclear extracts were prepared from testicular cells. For each sample, 25 μg of protein/lane were analyzed for DAX-1 protein by Western blotting analysis. The position of the DAX-1 protein is indicated. After quantitation, integrated optical density values were expressed as percentage DAX-1 protein level of control cells. Values are the mean ± SEM; $n = 9$. a, $p < 0.05$ between control and concentration of 0.4 and 4 mg/kg body wt. A representative Western blot is shown.

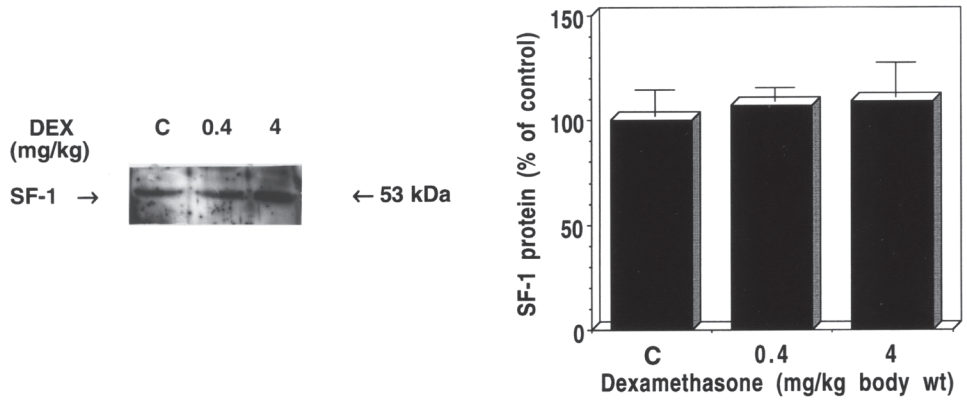

Fig. 9. Effect of in vivo treatment with dexamethasone (DEX) on testicular SF-1 protein levels. The conditions of this experiment are identical to those described for Fig. 8 with the exception that the immunoblots were analyzed for SF-1 protein. The position of the SF-1 protein is indicated. After quantitation, integrated optical density values were expressed as percentage SF-1 protein level of control cells. Values are the mean ± SEM; $n = 5$. A representative Western blot is shown.

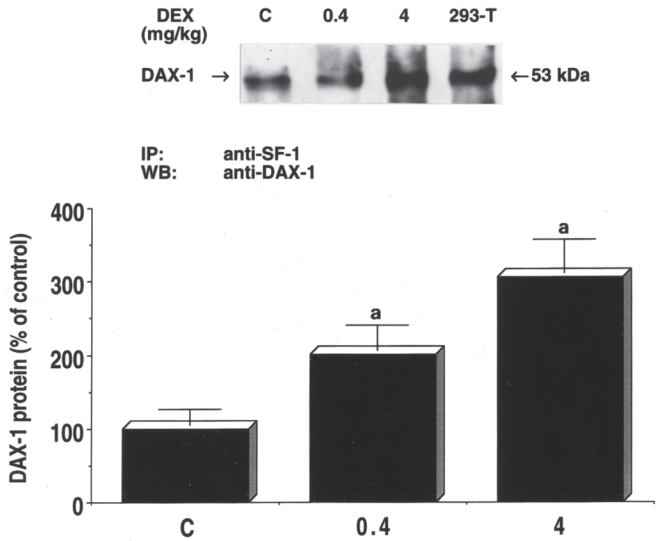

Fig. 10. Western blot analysis of the effect of dexamethasone (DEX) on the formation of DAX-1 and SF-1 complex. Rats were treated with PBS (C, control) or dexamethasone (0.4 and 4 mg/kg body wt) as described in Fig. 2. Nuclear extracts were prepared from testicular cells and immunoprecipitated with SF-1–specific antiserum. Immunoprecipitates were separated by SDS-PAGE and Western blotted with a DAX-1–specific antiserum. Extracts from 293T cells transfected with SF-1 served as a control for the Western blot. The position of the DAX-1 protein is indicated. After quantitation, integrated optical density values were expressed as percentage DAX-1 protein level of control cells. Values are the mean ± SEM; $n = 4$. a, $p < 0.05$ between control and concentration of 0.4 and 4 mg/kg body wt. A representative Western blot is shown.

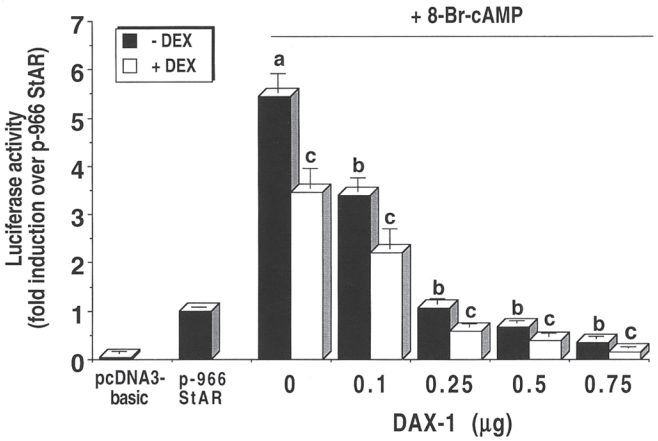

Fig. 11. Effect of increasing amounts of DAX-1 cDNA on 8-Br-cAMP–induced StAR promoter gene activity. Y-1 cells were transfected with p-966 StAR reporter construct (5 μg), and either an empty vector (pcDNA3) or increasing amounts of DAX-1 cDNA as indicated. The cells were treated with or without 8-Br-caMP (0.5 m*M*) in the presence or absence of dexamethasone (DEX; 1 μg/mL) for 24 h after which the cells were lysed and assayed for luciferase activity. Basal luciferase value for p-966 StAR was 106747 ± 19375 relative light units. Values are the mean ± SEM luciferase activity expressed as fold induction over p-966 StAR value (set at 1) for duplicate or triplicate observations averaged for three independent experiments. a, $p < 0.05$ when compared with p-966 StAR value; b, $p < 0.05$ when compared with DAX-1 = 0 in the control group (–DEX); c, $p < 0.05$ when compared with control (–DEX) value at each dose of DAX-1.

from dexamethasone-treated rats. The increased level was about 100–200% of that treated with PBS ($p < 0.05$).

Effect of Increasing Amounts of DAX-1 cDNA on 8-Br-cAMP-Induced StAR Promoter Reporter Gene Activity

Because our results have shown that dexamethasone caused a decrease in the levels of steady-state StAR mRNA and an increase in the levels of DAX-1 protein in testicular cells, we then determined whether DAX-1 could negatively regulate StAR gene expression in Y-1 adrenal tumor cells. The DAX-1 cDNA was co-transfected with the StAR promoter construct. The Y-1 adrenal tumor cell line was chosen because it expresses high levels of SF-1 but no DAX-1 *(28)* As shown in Fig. 11, stimulation of the Y-1 cells with 8-Br-cAMP (0.5 m*M*) resulted in a 5.4-fold increase in StAR promoter activity over the cells transfected with the StAR promoter construct alone. Dexamethasone reduced 8-Br-cAMP–induced StAR promoter activity by 37% ($p < 0.05$). Increasing amounts of DAX-1 cDNA caused progressive suppression of 8-Br-cAMP–induced StAR promoter activity. Luciferase activity in 8-Br-cAMP–treated cultures fell from 63% with 0.1 μg to 7% with 0.75 μg ($p < 0.05$) of the DAX-1 cDNA, relative to the non-DAX-1 expressing 8-Br-cAMP–treated cells. Dexamethasone suppressed 8-Br-cAMP–induced StAR promoter activity at all doses of DAX-1 cDNA used.

Discussion

In the present study we examined the effects of in vivo administration of the dexamethasone on testosterone production in vitro, on expression of StAR protein, and on DAX-1 in Leydig cells. We demonstrated that dexamethasone injection is able to significantly inhibit the ability of testicular cells to secrete testosterone in vitro in response to LH and 8-Br-cAMP. Dexamethasone injection also decreases StAR mRNA and protein levels in testicular cells. These results indicate that dexamethasone inhibits Leydig cell testosterone production by downregulating StAR gene expression. We also demonstrated that dexamethasone increases significantly testicular DAX-1 protein expression, providing a direct mechanism for the downregulation of StAR gene expression and inhibition of steroid production.

Our finding that treatment of rats with dexamethasone reduced the ability of testicular cells to secrete testosterone in vitro in response to LH and 8-Br-cAMP confirms reports that elevations in circulating glucocorticoids impair testicular function *(3,13,30)*. The complementary finding that exposure of dispersed testicular cells to dexamethasone in vitro reduces the release of testosterone provides direct evidence for an action of glucocorticoid within the testis; it thus agrees with earlier reports based on studies on purified mouse Leydig cells *(11)* and on Leydig cells from rat *(13)* and pig *(31)*.

Reports that the glucocorticoid receptor is present in Leydig cells *(13,32)* and that the glucocorticoid receptor antagonist RU486 reverses the inhibitory effect of glucocorticoids on LH-induced testosterone production *(13)* suggest that the effect of glucocorticoids is mediated through their receptors. Because little information is available on the synthesis of the glucocorticoid receptor and the process of downregulation of its hormone receptor in the testis in states of glucocorticoid excess, we do not know if the regulation of the glucocorticoid receptors by dexamethasone exists. Additional studies are necessary to define further the role that glucocorticoids play in the regulation of the glucocorticoid receptor gene expression in the testis. Several studies have shown that the events downstream of the glucocorticoid receptor involve suppression of the LH-induced transcription of steroidogenic enzymes, in particular P450 scc and 3β-HSD *(11,33)*, which are key regulators of testosterone biosynthesis, and also of 17α-hydroxylase/17-20-lyase *(10,17)*. It seems unlikely that the above-mentioned mechanism is effective in our present study. We found that the conversion of 22(R)-hydroxycholesterol to testosterone was not affected, suggesting that the decreased steroidogenic response of testicular cells to dexamethasone may be essentially localized to the transfer of cholesterol to the inner mitochondrial membrane. Whether dexamethasone treatment affects the levels of P450scc mRNA and protein in testicular cells under our experimental conditions remains to be investigated. Furthermore, the conversion of pregnenolone, 17-

hydroxypregnenolone, dehydroepiandrosterone, and androstenedione to testosterone were not affected. This suggests that dexamethasone may not affect the activity of 3β-HSD and 17α-hydroxylase/17-20-lyase. Because a time study on the conversion of pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone, and androstenedione to their direct metabolites was not examined in the present investigation, the conclusive demonstration that dexamethasone can affect the activity of 3β-HSD and 17α-hydroxylase/17-20-lyase needs direct measurement of the enzyme activity. The possibility that dexamethasone may act directly on the expression of 3β-HSD and 17α-hydroxylase/17-20-lyase also remains to be investigated. However, our finding that in vivo dexamethasone treatment impaired the steroidogenic responses to both LH and 8-Br-cAMP suggests that principal target for the steroid is downstream of the LH receptor. The demonstration that excessive exposure to glucocorticoids can induce apoptosis of rat Leydig cells *(34)* and thus potentially contribute to the suppression of testosterone levels when glucocorticoid concentrations are raised suggests an alternative mechanism of glucocorticoid action.

Results of the present study must be considered as pharmacologic as the effective inhibitory dose of dexamethasone, the most potent synthetic glucocorticoid, was 4.08×10^{-7} *M* (0.4 mg/kg body wt; 400 g of average body wt), which is greater than circulating levels of adrenal glucocorticoids under nonstressed conditions. Because when this dose was injected into animals for 7 d, we did not detect a significant inhibitory effect on serum testosterone. It is thus probable that the dose of 0.4 mg/kg body wt needs to be injected at a longer than 7-d interval in order to examine the dose effect of dexamethasone on serum testosterone. However, excessive production of glucocorticoids resulting from perturbations of the hypothalamic–pituitary–adrenal axis or the administration of large levels of glucocorticoid for therapeutic reasons has been shown to alter the normal activity of testicular function *(3,13,30)*. Interruption of testosterone production under these conditions may be explained, at least in part, by a direct glucocorticoid action on Leydig cells.

Whether the inhibitory mechanism of glucocorticoids is mediated prior or distal to cAMP formation was examined by investigation of the effect of dexamethasone on LH-stimulated cAMP production and also on 8-Br-cAMP–stimulated testosterone production in vitro. Dexamethasone treatment suppressed LH-stimulated cAMP and testosterone production. It appears that at least a part of the inhibitory mechanism of adrenal glucocorticoids is mediated prior to cAMP formation. Our data suggest that glucocorticoids may suppress LH-stimulation of adenyl cyclase activity. Direct measurement of the activities of adenyl cyclase is needed to further elucidate this point. In the present study, dexamethasone treatment also inhibited 8-Br-cAMP–stimulated testosterone production. This finding suggests an additional post-cAMP effect of gluccorticoids upon testosterone synthesis.

Our present study demonstrated that in rat testicular cells dexamethasone inhibits LH-induced testosterone production, whereas the activity of P450scc is not affected by dexamethasone treatment, indicating that the loss of steroidogenic capacity is not a result of inhibition of P450scc. Furthermore, dexamethasone decreases StAR mRNA and protein levels. This suggests that in rat testicular cells, the major inhibitory effect of dexamethasone on testosterone production is at the level of the regulation of the transcriptional expression of StAR. Diminished levels of StAR protein would likely result in a reduction in the capacity of testicular cells to produce testosterone, in response to gonadotropin, by limiting the availability of cholesterol to the P450scc complex. StAR protein is required for steroid hormone biosynthesis *(35)*, because it delivers cholesterol to the inner mitochondrial membrane *(19,20)*. The data presented here indicate that the dexamethasone-induced depression of testosterone production in testicular cells is due to the inhibition of StAR protein expression. This observation is highly consistent with previous studies in which inhibition of testosterone biosynthesis has been tightly correlated with StAR synthesis *(36–40)*. For example, agents and conditions that have been shown to result in a decrease in testosterone biosynthesis such as lipopolysaccharide *(36)*, interferon-γ *(37)*, leukemia inhibitory factor *(38)*, tumor necrosis factor-α *(39)*, and heat shock *(40)*, have all been demonstrated to decrease StAR protein content. Moreover, a StAR-dependent reduction in steroidogenesis could be manifested through a decrease in the expression and/or activity of the protein. In the present study, dexamethasone treatment reduced StAR mRNA levels. This finding suggests that the inhibitory effect of dexamethasone on StAR protein occurs as a result of a reduction in cAMP-mediated transcription of the StAR gene and/or StAR mRNA stability. The possibility that dexamethasone may exert a negative influence on StAR activity in testicular cells through post-translational modification (e.g., phosphorylation) of the protein *(41)* cannot be ruled out either.

With regard to the direct mechanism involved in the negative effect of glucocorticoids on StAR gene expression, we focused our attention on two transcription factors known to modulate StAR gene expression in opposite ways, DAX-1 and SF-1. DAX-1 represses StAR gene expression by binding to a hairpin structure located in the StAR promoter and blocks steroidogenesis *(27)*. In contrast, multiple binding elements for SF-1 have been reported in the 5'-flanking region of the StAR gene and are required for maximal promoter activity *(24)*. Results of the present study demonstrate a significant increase in DAX-1 protein expression in testicular cells, but no change in SF-1 expression following dexamethasone treatment in vivo. This finding provides a direct mechanism for the downregulation of StAR gene expression and inhibition of steroid production. SF-1 and DAX-1 have similar tissue distribution patterns and both are essential for the development of the adrenal cortex *(42)*. The over-

lapping tissue distributions and functional roles of SF-1 and DAX-1 in the adrenal gland and reproductive system raise the possibility that they might interact in one of several manners. In the present study, Western blot analysis of DAX-1 protein in anti-SF-1 precipitates demonstrates the presence of DAX-1 and SF-1 interaction in testicular cells. These results correlate with previous reports showing that SF-1 and DAX-1 were able to interact in yeast two–hybrid *(43)* and mammalian two–hybrid *(44)* analysis. Dexamethasone treatment increased the amount of complexed DAX-1–SF-1. Because dexamethasone increased DAX-1 level, but had no effect on SF-1 level, This finding indicates that dexamethasone has no effect on the interaction between DAX-1 and SF-1. Little is known about the mechanism by which DAX-1 represses its target genes. Two previous studies demonstrate a direct interaction between DAX-1 and SF-1, potentially recruiting co-repressors, such as NcoR (nuclear receptor co-repressor) to the transcription factor complex to stifle transcription of genes in response to external stimuli *(45,46)*. Our studies provide evidence that DAX-1 interacts with SF-1 in vivo. The elevation of DAX-1 may exert a dominant effect on StAR expression and attenuation of steroidogenesis in testicular cells.

As DAX-1 is reported here to repress 8-Br-cAMP–induced StAR promoter activity in Y-1 cells which do not express endogenous DAX-1 and this suppression was dose-dependent. These findings corfirmed a role for DAX-1 in the negative regulation of the StAR gene at the transcription level. Our findings were also consistent with the results in previous studies that overexpression of DAX-1 blocked the transcription of StAR *(28)*. Our studies also demonstrated that when an increased amount of the DAX-1 cDNA was cotransfected, dexamethasone further suppressed 8-Br-cAMP–induced StAR promoter activity. This would seem to indicate that upregulation of DAX-1 gene inhibits StAR gene expression by dexamethasone. Although the precise mechanism underlying the upregulation of DAX-1 protein expression by dexamethasone is not clear, our data provide the evidence that dexamethasone treatment increases endogenous DAX-1 expression and concordantly decreases StAR expression. Since glucocorticoids stimulate DAX-1 gene expression within the adrenal cortex and glucocorticoid receptor (GR) forms a transcription complex with SF-1, which potentiates DAX-1 expression *(47)*, dexamethasone-induced increases in the expression of DAX-1 in testicular cells may occur via the same mechanism. GR potentiation of DAX-1 gene may play a role in regulation of testicular steroidogenesis. The decrease in StAR expression strongly suggests the presence of an adrenal–testicular regulation loop.

In conclusion, the results of the present study indicated that in the rat, dexamethasone treatment increased endogenous DAX-1 expression and concordantly decreased StAR expression. Dexamethasone in vitro also suppressed StAR promoter activity when an increasing amount of the DAX-1 cDNA was transfected. These studies suggest that DAX-1 may interact with SF-1 and prevent SF-1-mediated transcription activation of the rat StAR gene.

Materials and Methods

Materials

Purified porcine pituitary LH (pLH; USDA-pLH-B-1; 1.7 U/mg) was obtained from the National Hormone and Pituitary Distribution Program (Bethesda, MD). Bovine serum albumin (BSA; fraction V, fatty-acid free), 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP), collagenase (310 U/mg), bovine pancreatic DNAase, 22(R)-hydroxycholesterol and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. Dexamethasone (dexamethasone phosphate) was obtained from Narn Guang Chemical Co. (Tainan, Taiwan). Ham's F12/Dulbecco's modified Eagle's medium (F12/DMEM; 1:1), fetal calf serum (FCS), and other culture supplies were purchased from Gibco-BRL (Grand Island, NY). [α-^{32}P]Deoxy-CTP (3000 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). The rabbit polyclonal antibody against DAX-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antiserum directed against a GST-fusion protein corresponding to the DNA binding domain of mouse SF-1 was purchased from Upstate Biotechnology (Lake Placid, NY) and used in the co-immunoprecipitation assay. Rabbit anti-SF-1 antiserum (provided by Dr. Bon-Chu Chung, Institute of Molecular Biology, Academia Sinica, Nankang, Taiwan) was generated by immunization with a full-length amino acid sequence for mouse SF-1. This antiserum was used in Western blotting. Mouse StAR cDNA was a generous gift from Dr. D.M. Stocco (Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX). Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was a generous gift from Dr. Hsiao-Sheng Liu (Department of Microbiology, National Cheng Kung University Medical College, Tainan, Taiwan). Murine DAX-1 cDNA was a generous gift from Dr. J.L. Jameson (Division of Endocrinology, Metabolism and Molecular Medicine, Northwestern University Medical School, Chicago, IL). The StAR promoter/luciferase plasmid expressing firefly luciferase driven by the –966-bp sequence of the StAR promoter (p-966 StAR) was a generous gift from Dr. D.M. Stocco. The plasmid CMV-β-galactosidase was a generous gift from Dr. J. Schwartz (Department of Physiology, University of Michigan Medical School, Ann Arbor, MI).

Animals and Treatments

Male Wistar rats [National Cheng Kung University Laboratory Animal Center (NCKULAC), Tainan, Taiwan], aged 10–12 wk, were maintained in 22–24°C rooms on a 12L:12D schedule (lights-on at 0600 h). Food and water were provided *ad libitum*. Animals were injected ip with dexamethasone (0.4 and 4 mg/kg body weight) or vehicle alone (phos-

phate-buffered saline; PBS) once daily for 7 d. Following 7 d of injection, and approx 18 h after the last injection, rats were decapitated after light ether anesthesia. Trunk blood was collected and serum was stored at –20°C until assayed for testosterone. The testes were excised and placed in ice-cold PBS for testicular cell isolation. At all times, the animals were treated as humanely as possible, following NCKULAC guidelines.

Isolation of Testicular Interstitial Cells

Decapsulated testes from each treatment group were incubated at 34°C for 25 min in F12/DMEM supplemented with 20 m*M* HEPES (pH 7.4), 2 m*M* glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B-250 in the presence of 0.1% BSA and heparin (10 IU/mL) containing 0.02% collagenase-DNAase. The medium was pregassed with 95%O_2–5% CO_2 for at least 3 h. The cell suspension was filtered through two layers of cheesecloth. After two subsequent washes in PBS, cells were then resuspended in culture medium (F12/DMEM–0.1% BSA) and cell viability quantitated by Trypan blue exclusion. Cell viability averaged 95–99%. Cells were utilized immediately for Western and Northern blottings. In some experiments, approx $0.9–1.5 \times 10^7$ cells were incubated in 0.5 mL culture medium in the presence or absence of LH or 8-Br-cAMP at 34°C with 95%O_2–5% CO_2 for 3 h in a shaking water bath. To evaluate the effects of dexamethasone on the conversion of a steroidogenic substrate to testosterone, testicular interstitial cells were incubated with or without 22(R)-hydroxycholesterol (1 µ*M*), pregnenolone (1 µ*M*), 17-hydroxypregnenolone (1 µ*M*), dehydroepiandrosterone (1 µ*M*), or androstenedione (1 µ*M* for 3 h. At the end of incubation, the spent media were centrifuged at 500*g* and stored at –20°C until assayed for testosterone. The testicular interstitial cells were comprised of approx 5–10% Leydig cells as determined by histochemical 3β-HSD staining *(48)*.

Y-1 Cell Culture

The mouse Y-1 adrenocortical tumor cells were a generous gift from Dr. Bernard P. Schimmer (Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontaria, Canada) and were grown in 75-cm^2 tissue culture flasks in F12/DMEM containing 10% FCS (F12/DMEM-10% FCS) at 37°C under 5%CO_2–air until needed for transfection studies. Cells were passed with 0.25% trypsin–1 m*M* EDTA and the resulting suspension of cells was centrifuged at 2500*g* for 5 min, followed by resuspending cells in fresh media and transferring them to six-well plates.

Nuclear and Whole-Cell Extract Preparation

Nuclear extract preparations from vehicle- or dexamethasone-injected testicular interstitial cells were performed essentially as described *(49)* using hypotonic buffer [20 m*M* HEPES, pH 7.9, 1 m*M* EDTA, 0.2% Nonidet P-40 (NP-40), 1 m*M* EGTA, 20 m*M* sodium fluoride (NaF), 1 m*M* sodium orthovanadate (Na_3VO_4), 1 m*M* sodium pyrophosphate decahydrate ($Na_4P_2O_7$), 1 m*M* dithiothreitol (DTT)] containing protease inhibitors [0.5 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL aprotinin, and 1 µg/mL leupeptin] to lyse the cells and high salt buffer (hypotonic buffer with the addition of 420 m*M* NaCl and 20% glycerol) for nuclear extraction. Cells were washed twice with ice-cold PBSV buffer (10 m*M* sodium phosphate, 150 m*M* NaCl, pH 7.4, 1 m*M* Na_3VO_4, 5 m*M* NaF) and then lysed in 0.5 mL hypotonic buffer in 1.5-mL microfuge tubes. The homogenates were pelleted by centrifugation at 13,800*g* at 4°C for 3 min. The pellets were resuspended in 150 µL high salt buffer and the samples were rotated for 30 min at 4°C. The nuclear lysates were then centrifuged at 13,800*g* for 20 min at 4°C, and the supernatant fluid was placed into a fresh microfuge tube. Whole-cell lysates were prepared by lysing testicular cells in buffer containing 50 m*M* HEPES (pH 7.0), 500 m*M* NaCl, 1% NP-40, and protease inhibitors (0.5 m*M* PMSF, 1 µg/mL aprotinin, and 1 µg/mL leupeptin). After centrifugation at 13,800*g* for 10 min at 4°C, supernatant was removed. Protein concentrations in the nuclear and whole-cell lysate extracts were determined by Lowry's method *(50)*. All samples were stored at –85°C until further analysis.

Western Blot Analysis

Fifty micrograms of whole-cell lysates and 25 µg nuclear extract were analyzed as described previously *(51)*. The protein samples were separated by 12.5% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and then the membranes were blocked in PBS containing 0.5% Tween 20 (PBST) and 4% nonfat dry milk for 1 h. The membrane was incubated with primary antibody (anti-DAX-1, 1:4000; anti-SF-1, 1:10000; or anti-StAR, 1:1000) in PBST with 0.1% BSA and immunoblot analysis performed using a horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (1:10000). Specific immunoactive bands were detected using the Western lightening Chemiluminescent Kit (NEN Life Science Products). Band intensities were determined using the Arcus II computer-assisted image system (PDI Inc., Huntington Station, NY). Values obtained were expressed as integrated optical density units.

Co-immunoprecipitation Assay

Co-immunoprecipitation was used to detect protein–protein interaction between DAX-1 and SF-1. Nuclear extracts (approx 500 µg) prepared from vehicle- or dexamethasone-injected testicular interstitial cells were immunoprecipitated using anti-DAX-1 (approx 1 µg) or anti-SF-1 (from Upstate) antibody in salt solution (150 m*M* NaCl, 20 m*M* HEPES, pH 7.9, 1 m*M* EDTA, 1 m*M* EGTA, 20% glycerol). After 2 h of mixing at 4°C, 30 µL of a 20% (w/v) slurry of protein A–agarose beads (Sigma) in salt solution was added and the samples were mixed for 1 h at 4°C. The antibody-containing pellet was recovered by centrifugation at 13,800*g* for 0.5 min and washed three times with 0.4 mL of

wash buffer (20 m*M* HEPES, pH 7.9, 1 m*M* EDTA, 0.2% NP-40, 1 m*M* EGTA, 20 m*M* NaF, 1 m*M* Na_3VO_4, 1 m*M* $Na_4P_2O_7$, 1 m*M* DTT) before 30 µL of SDS-PAGE sample buffer containing 1.7% SDS, 58.3 m*M* Tris-HCl (pH 6.8), 5% glycerol, 0.002% bromophenol blue, and 1% 2-mercaptoethanol was added. After heating at 100°C for 5 min, the supernatant was collected after centrifugation of the slurry for 1 min at 13,800*g*. Bound proteins were analyzed by immunoblotting with a SF-1-specific antiserum (from Dr. Chung) or with DAX-1-specific antiserum.

Northern Blot Analysis of StAR mRNA

Levels of StAR mRNA were evaluated by Northern blot analysis using a mouse StAR cDNA probe as previously described *(52)*. Total RNA (25 µg) from each sample was fractionated in a 1% agarose gel and transferred onto a nylon membrane and UV cross-linked. Northern blots were preincubated for 1 h at 60°C in QuikHyb solution (Stratagene, La Jolla, CA) and hybridized for 3 h at 60°C in the same solution containing heat-denatured ^{32}P-labeled StAR or GAPDH DNA and salmon sperm DNA. Mouse StAR and GAPDH probes were labeled by random priming with [α-^{32}P]deoxy-CTP using the Megaprime DNA Labeling System (Amersham). After hybridization, the blots were washed twice in 2X SSC/0.1% SDS at room temperature for 15 min each, and once in 0.1X SSC/0.1% SDS for 20 min at 42°C. After washing, the membrane was exposed to X-ray film at –85°C for 24 h for autoradiographic signals. The intensity of the bands on Northern blots was measured by the Arcus II computer-assisted image system. Values for StAR mRNA were normalized to values for the GAPDH.

Transfection

Y-1 cells (2×10^5 cells per well) were cultured in six-well plates overnight. The cells were transiently transfected by the calcium phosphate coprecipitation procedure *(53)* with 5 µg p-966 StAR plasmid, in the presence of pSK-DAX-1 or corresponding amounts of pcDNA3 vector plasmid per 35-mm well. After 24 h, cells were washed twice with PBS before being fed with fresh F12/DMEM-10% FCS. Cells were then incubated with or without dexamethasone (1 µg/mL) in the presence or absence of 8-Br-cAMP (0.5 m*M*) for 24 h before measurement of luciferase activity. A β-galactosidase expression vector, CMV-β-galactosidase, was used as an internal control of transfection efficiency.

Luciferase and β-galactosidase Assays

Following experiments, the cells were rinsed twice with PBS and incubated in 300 µL of reporter lysis buffer (100 m*M* potassium phosphate, 0.2% Triton X-100, 1 m*M* DTT) for 15 min at room temperature. The cells were then scraped into a 1.5-mL centrifuge tube and centrifuged at 13,800*g* for 2 min at room temperature. The supernatant fluid was placed in a 1.5-mL centrifuge tube and was either used immediately or stored at –85°C until luciferase activity was measured. For luciferase assay, 100 µL of the lysate was placed in a tube containing 360 µL luciferase buffer (25 m*M* glycylglycine, 15 m*M* $MgSO_4$, 4 m*M* EGTA, 0.27% Triton X-100, 15 m*M* potassium phosphate, 2 m*M* ATP, 1 m*M* DTT) in the Minilumat Luminometer (LB9056, Berthold, Germany). One hundred microliters of D-luciferin (0.2 m*M* in 25 m*M* glycylglycine, 15 m*M* $MgSO_4$, 4 m*M* EGTA, 2 m*M* DTT) was injected into the tube and the luminescence measured. Beta-galactosidase assay was performed exactly as described previously *(54)* using ONPG (*O*-nitrophenyl-β-D-galactopyranoside) (Acros Organics, Geel, Belgium) as the substrate with the exception that the reaction was stopped by placing the tubes into an ice-bath. The luciferase assay results were normalized to β-galactosidase activity to compensate for variations in transfection efficiency. Each treatment group contained at least triplicate cultures, and the experiment was repeated three times.

Radioimmunoassay (RIA) for Testosterone and cAMP

Quantitation of testosterone from serum and aliquots of the medium was performed by RIA as previously described *(55)*. The antiserum to testosterone-11-BSA was provided by Dr. G.D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO) and used at the dilution of 1:10,000 in 0.01 *M* Tris buffer (pH 7.4). The sensitivity of the assay was 6.25 pg per assay tube.

Testicular interstitial cells, obtained from vehicle- or dexamethasone-injected rats, were incubated at 34°C with 1-methyl-3-isobutyl xanthine (0.125 m*M*) in the presence or absence of LH (10 ng/mL) in 95%O_2–5% CO_2 for 3 h with shaking. cAMP was extracted from the cells with 65% ethanol and quantitated using Amersham's cAMP assay kit (Amersham), according to the manufacturer's instructions for the nonacetylation assay.

Statistical Analysis

Two means were compared using Student's *t*-test. Where there were more than two means, significant differences between means were determined by analysis of variance (ANOVA). The means were then analyzed by Fisher's PLSD multiple comparison.

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