Modulation of the Testicular Steroidogenic Pathway by Cyclosporin A in Adult Rats Pretreated with Diethylstilbestrol[1] (44115)

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Abstract. **Treatment of male Fischer-344 rats with diethylstilbestrol (DES) induces hyperprolactinemia and alterations in testicular steroidogenesis. Cyclosporin A (CsA), an immunosuppressive drug, is believed to act by opposing the effects of prolactin (PRL) and was reported to influence testicular function. We have examined the effect of CsA on gonadal function in rats pre-treated with DES. Male rats were implanted with DES-containing silastic capsules. After 19 weeks, the capsules were removed, and, starting 2.5 weeks later, rats were treated for 1 week with CsA. At sacrifice, plasma and testes were collected. Testis fragments were incubated with or without 12.5 mIU of human chorionic gonadotropin (hCG)/ml at 32°C for 4 hr. As expected, plasma PRL levels were increased in DES exposed rats, while testicular, seminal vesicle, and prostate weights were reduced. Cyclosporin A treatment did not further modify these parameters. Treatment with CsA and/or DES decreased circulating levels of testosterone, while testosterone content in the testes was not modified. Although CsA did not affect *in vitro* release of testosterone, it reduced the stimulatory influence of DES pretreatment on testicular responses to hCG *in vitro*. Plasma and testicular content of progesterone (P) was increased by DES administration but was not affected by CsA. Both CsA and DES administration decreased plasma 17-OH-Progesterone (17-OH-P) levels, but only CsA decreased testicular 17-OH-P contents. Combined exposure to CsA and DES enhanced the stimulatory effect of hCG on the accumulation of 17-OH-P in the media. Media estradiol levels were not affected by treatment with either CsA or DES. The present results indicate that CsA may interfere with the enhancement of the conversion of P to testosterone in DES-treated rats. As CsA did not change plasma PRL or gonadotropin levels, a direct effect of the drug on the testicular function is suspected.** [P.S.E.B.M. 1997, Vol 215]

Cyclosporin A (CsA), a potent immunosuppressive drug (1), is widely used for the prevention of graft rejection in organ and bone marrow transplantation (2, 3). The use of CsA was associated with dose-related hepatotoxicity and nephrotoxicity (4, 5). Along with these nonendocrine actions, there is evidence that CsA also affects pancreatic islets (6, 7) as well as the hypothalamic-pituitary-adrenal (8, 9) and the hypothalamic-pituitary-gonadal axes (10, 11). However, it is not clear whether these effects are exerted at the hypothalamic, pituitary, or directly at the adrenal or gonadal levels. In this context, there is evidence that CsA induces hypogonadotropic hypogonadism in adult male rats mediated through the hypothalamic-pituitary axis (12). However, in prepubertal male rats this immunosuppressor can reduce plasma testosterone (T) levels despite an elevation of LH, indicating that the drug directly inhibits testosterone synthesis (11). Moreover, the mechanism(s) of CsA actions responsible for these

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effects remain to be elucidated. One of the proposed mechanisms of CsA action on the immune system involves antagonism of prolactin (PRL) effects on the lymphocytes (13, 14), and PRL can influence gonadotropin release and testicular function (15–18). In previous studies we have examined possible interactions between the effects of CsA and PRL on the hypothalamic-pituitary axis and demonstrated that CsA may alter hypothalamic and pituitary function, in some measure counteracting the regulatory influence of PRL at the hypothalamic level, but not interfering with the actions of PRL on anterior pituitary function (19, 20).

Therefore, the present study was designed to examine the effects of treatment with CsA *in vivo* on testicular function in normal and in hyperprolactinemic rats. Hyperprolactinemia was induced by treatment with subcutaneous implants of a synthetic estrogen, diethylstilbestrol (DES). Animals were exposed to CsA treatment after the removal of the capsules—that is, during the period when hyperprolactinemia persists while the synthetic estrogen is no longer present in the circulation, and testicular function recovers (17).

Materials and Methods

Animals. Eight-week-old male Fischer 344 rats were purchased from Harlan-Sprague-Dawley (Indianapolis, IN) and kept under controlled conditions (22°–24°C, lights on 0700–1900 h) with free access to food and tap water. Silastic capsules containing approximately 8 mg DES (length of drug-filled space, 5 mm; i.d. = 1.56 mm; o.d. = 2.39 mm) or identical empty capsules were implanted under the skin on the back. After 19 weeks, the capsules were removed (DES and control groups), and, starting 17 days later, the animals were treated daily for 1 week with CsA (5 mg/kg body wt/day, s.c.) or with vehicle.

CsA Solution. Cyclosporin A was generously provided by Sandoz S.A. (Basel, Switzerland). Fifty milligrams were dissolved in 500 μl of absolute ethanol and further diluted with sesame oil to a final concentration of 5 mg/ml. Vehicle was prepared in the same way.

Testes Incubation. One day after the last injection of CsA, the animals were decapitated between 9 and 10 AM, trunk blood was collected, and the testes were removed, weighed, decapsulated, and cut into fragments of approximately equal size. These fragments were weighed and incubated in 2 ml of Krebs Ringer bicarbonate buffer containing glucose (1 mg/ml) and human chorionic gonadotropin (hCG) at 12.5 mIU/ml or no gonadotropins. The incubations were carried out in 30-ml glass beakers in a Dubnoff metabolic incubator at 31° ± 1°C with constant flow of O_2:CO_2 mixture (95:5) without preincubation. After 4 hr of incubation, the media were collected, centrifuged, and stored frozen for subsequent determinations of estradiol (E_2), progesterone (P), 17-OH-progesterone (17-OH-P), and testosterone (T) levels (21).

RIA Procedures. Plasma PRL, T, 17-OH-P, and P were measured by radioimmunoassay (RIA) (22, 23). The testicular and the incubation media T levels were determined using RIA procedures described previously (22). The measurements of testicular and of incubation media P and 17-OH-P levels, as well as those of incubation media E_2 levels, were done using solid-phase RIA procedures (24).

Statistics. The results were analyzed by analysis of variance followed by Student-Neuwman-Keul's multiple-range test.

Results

As expected, DES exposure markedly increased plasma PRL levels as compared to the empty capsule (control) group. Cyclosporin A treatment did not change plasma PRL levels either in control (treated with empty capsules) or in DES-treated animals (Table I).

On the day CsA treatment was started, rats that had been exposed to DES weighed much less than the control animals, which were implanted with empty capsules (Fig. 1; initial body weight). At the end of CsA treatment, body weight gain expressed as growth index (final body weight/initial body weight × 100) was higher in both groups of rats exposed to DES compared with their respective control animals (Fig. 1, right panel). Treatment with CsA did not affect growth index in either empty-capsule–treated animals or DES-treated animals (Fig. 1).

Diethylstilbestrol-exposed rats showed a significant reduction in absolute testes, seminal vesicle, and prostate weights (Fig. 2, top panel) that was evident also when the weights were expressed in relation to body weight (Fig. 2, bottom panel). Treatment with CsA did not modify either absolute or relative (mg tissue/g body wt) weights of any of the three organs studied (Fig. 2).

Progesterone levels in plasma and in the testes were higher in rats exposed to DES than in the control (empty capsule) group. However, the plasma/testicular P ratio was not modified by DES exposure. Cyclosporin A did not change plasma or testicular P levels or plasma/testicular P ratio in either control or DES groups (Fig. 3, top panel). Treatment with DES decreased plasma 17-OH-P without affecting its levels in the testes, and thus the plasma/testicular 17-OH-P ratio was diminished in rats treated with DES compared with empty capsule groups (Fig. 3, middle panel). Cyclosporin A administration reduced plasma and testicular 17-OH-P levels in control rats, but not in DES animals, without altering the plasma/testicular 17-OH-P ratio in either group (Fig. 3, middle panel). Plasma T, but not testicular T, was decreased by DES therapy, resulting in reduced plasma/testicular T ratio in DES treated animals (Fig.

Table I. Effects of Cyclosporin A (CsA) and Diethylstilbestrol (DES) on Plasma PRL (PRL) Levels in Male Rats

	Control		DES	
	Veh	CsA	Veh	CsA
PRL (ng RP-3/ml)	7.5 ± 1.2 (10)	11.5 ± 1.7 (10)	542 ± 68[a] (9)	496 ± 70[a] (10)

Note. Animals were treated with DES capsules (DES) or with empty capsules (control) for 19 weeks, had their capsules removed, and, starting 17 days later, were treated for 1 week with daily s.c. treatment of 5 mg CsA/kg body wt or vehicle (Veh). Blood was collected by decapitation 1 day after the last injection of CsA. Numbers in parentheses indicate the number of animals in each group. [a]$P < 0.05$ versus the respective control group.

3, bottom panel). Cyclosporin A administration reduced plasma T levels only in the control (empty capsule) group with no modification of plasma/testicular T ratio in either control or DES animals (Fig. 3, bottom panel).

Figure 4 shows testicular ratios of T/P (left panel), 17-OH-P/P (middle panel), and T/17-OH-P (right panel). In animals exposed to DES, these ratios of testicular steroid levels were not altered from those calculated for the control group. Treatment with CsA decreased 17-OH-P/P ratio and increased T/17-OH-P in control rats but not in DES-exposed animals.

During *in vitro* incubations, testicular tissue from rats that had been exposed to DES *in vivo* released significantly more T than the amount released by testicular tissue from control animals (Fig. 5, bottom left). This effect was significant under both basal and human chorionic gonadotropin (hCG)–stimulated conditions, but was particularly striking in the presence of hCG. Although CsA treatment did not affect accumulation of T in the media under basal conditions (i.e., in the absence of hCG) in either group (control or DES), it reduced the stimulatory effect of hCG on *in vitro* T release in incubations of testes from DES-exposed animals (Fig. 5, bottom left). Basal and hCG-stimulated accumulation of E_2 in the media were not affected by prior exposure of the animals to either CsA or DES (Fig. 5, bottom right). Basal P and 17-OH-P release did not change after DES or CsA treatment (Fig. 5, top panels). However, DES pretreatment led to significantly greater hCG-induced release of 17-OH-P in incubations of the testes from both vehicle and CsA-treated animals compared with control (empty capsule) groups (Fig. 5, top right).

Data on the accumulation of different steroids in the media of testicular incubations were used to calculate *in vitro* ratios (Fig. 6). Basal *in vitro* E_2/T ratio was not modified by previous *in vivo* exposure to DES or CsA. However, the increase in media E_2/T ratio in the presence of hCG was attenuated by DES pretreatment compared with the control group (Fig. 6, bottom left). Media

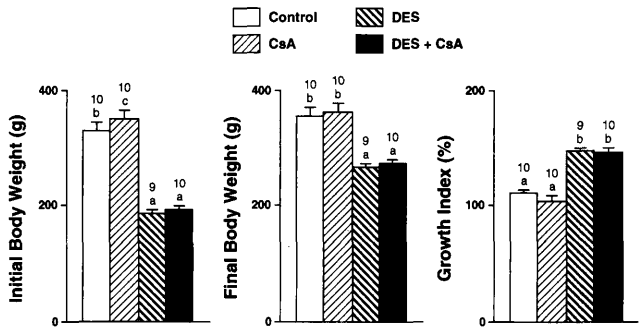

Figure 1. Body weight and growth index (%) in animals that were treated with empty (control) or DES-filled silastic capsules for 19 weeks, when they had their capsules removed and, starting 17 days later, were treated for 1 week with daily s.c. injections of 5 mg CsA/kg body wt or vehicle. Numbers at the top of columns indicate the number of animals in each group. Values are expressed as mean ± SEM. Values without the same letter are significantly different ($P < 0.05$).

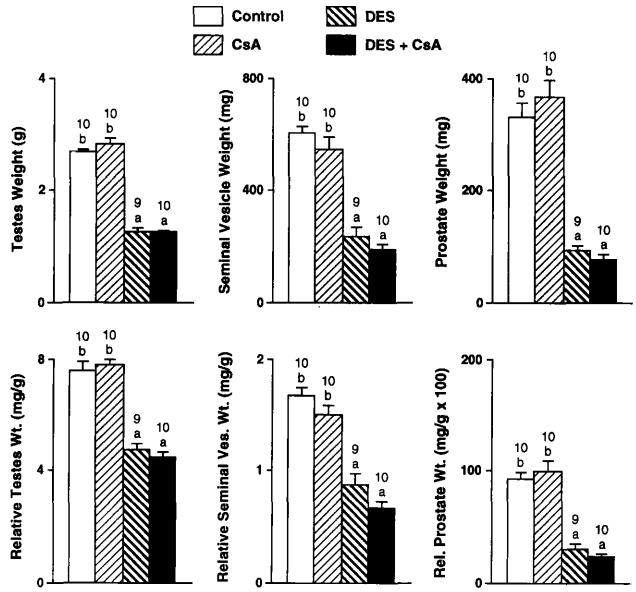

Figure 2. Absolute (top) and relative (bottom) testes, seminal vesicle, and prostate weights in animals that were treated with empty (control) or DES-filled silastic capsules for 19 weeks, when they had their capsules removed and, starting 17 days later, were treated for 1 week with daily s.c. injections of 5 mg CsA/kg body wt or vehicle. Numbers at the top of columns indicate the number of animals in each group. Values are expressed as mean ± SEM. Values without the same letter are significantly different ($P < 0.05$).

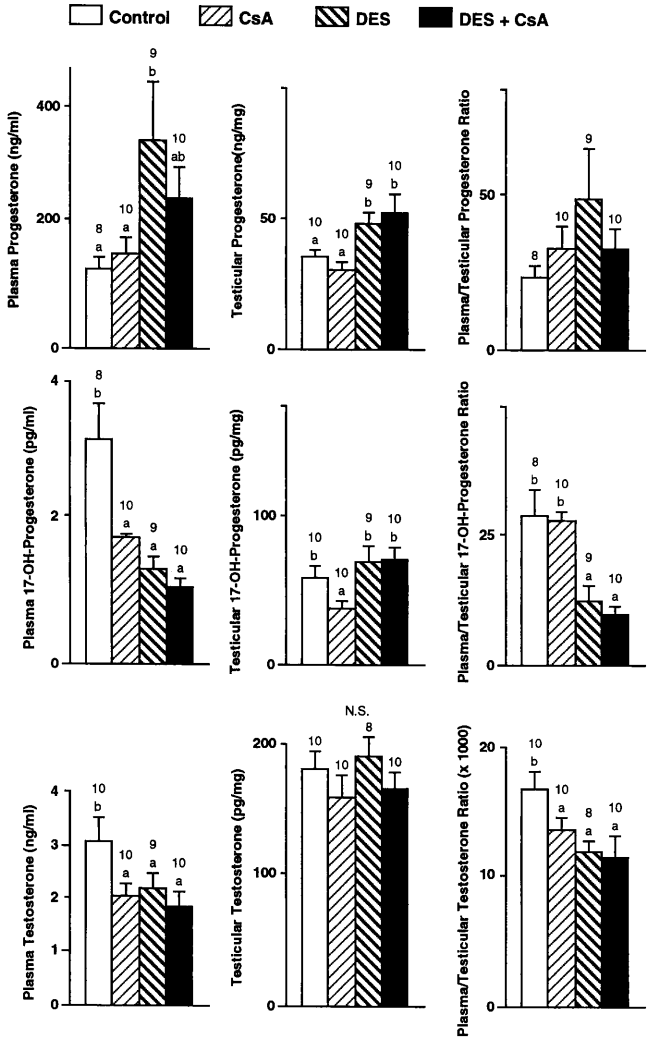

Figure 3. Plasma, testicular, and plasma/testicular ratio of progesterone (top panel), 17-OH-progesterone (middle panel), and testosterone (bottom panel) in animals that were treated with empty (control) or DES-filled silastic capsules for 19 weeks, when they had their capsules removed and, starting 17 days later, were treated for 1 week with daily s.c. injections of 5 mg CsA/kg body wt or vehicle. Numbers at the top of columns indicate the number of animals in each group. Values are expressed as mean ± SEM. Values without the same letter are significantly different ($P < 0.05$).

T/17-OH-P basal ratio in the control group was not modified by exposure to CsA *in vivo* or by hCG *in vitro*. Treatment with DES increased media T/17-OH-P ratio under basal conditions. Treatment with CsA diminished this ratio in DES animals and attenuated the decrease induced by hCG in the DES-treated group (Fig. 6, bottom right). Media T/P in the control group was not affected by either hCG or CsA. However, DES pretreatment markedly increased media T/P ratio compared with the empty capsule group. This ratio was diminished after CsA therapy of DES animals. Media T/P ratio was not modified by hCG in DES-treated groups (both vehicle and CsA), and thus remained higher than in the corresponding normal control (empty capsule) groups (Fig. 6, top left). Media 17-OH-P/P ratio was increased by hCG in each of the groups examined. Cyclosporin A administration did not modify media 17-OH-P/P ratio under basal conditions or in the presence of hCG in either of the studied groups (empty capsule and DES-treated animals) (Fig. 6, top right).

Discussion

The present results indicate that CsA at the employed dose level leads to a reduction in plasma T concentration without affecting testicular testosterone concentration or the *in vitro* response to hCG stimulation in normal adult rats. However, in rats pretreated with DES, CsA partially attenuated the capacity of the testes to convert P into T and interfered with the stimulatory action of hCG on *in vitro* T release.

Significant reduction in body weight and in absolute weights of the testes, seminal vesicles, and prostate in hyperprolactinemic (DES-treated) rats was demonstrated previously (17, 25). However, in the present study, the effects of DES exposure on the male reproductive system were evident also when organ weights were expressed per unit of body weight, in contrast to our previous findings (17). This difference was undoubtedly due to a much longer interval between the removal of the DES capsule and the autopsy in the previous study compared with the present study. Cyclosporin A treatment did not produce changes in body (25) and testicular weights or in the weights of the other reproductive organs in either control or DES-pretreated animals, in agreement with some authors (11, 25, 26) but differing from others (27, 28). These differences can be related to different doses of CsA and different length of treatment with the drug in the various studies.

Cyclosporin A decreased plasma T in control animals, but the testicular concentration of T was unchanged. Reduction in T levels was previously described in both sexually mature and immature CsA-treated rats (11, 27, 29). However, these authors also found decreased intratesticular levels of T using different doses of CsA and different duration of treatment than those employed in the present study (1–2 mg/kg body wt in prepubertal animals and 10–40 mg/kg body wt in sexually mature rats versus 5 mg/kg body wt in the present study; and 14–45 days in previously published reports versus 7 days in the present work). Using a lower dose of CsA in adult male rats (7.5 mg/kg body wt administered orally for 28 days), Rajfer *et al.* (26) did not find the previously described inhibitory effect on testicular T, although plasma levels of T tended to be suppressed. Vexian *et al.* (30) found an increase in the activity of 5 α-reductase in peripheral tissues, after CsA treatment (7.5 or 10 mg/kg/day) in humans. Moreover, Krieger *et al.* (31) found a decrease in plasma T during CsA therapy (25 or 40 mg/kg body wt s.c., for 6 days) with a reduction in testicular T with the higher dose. Collec-

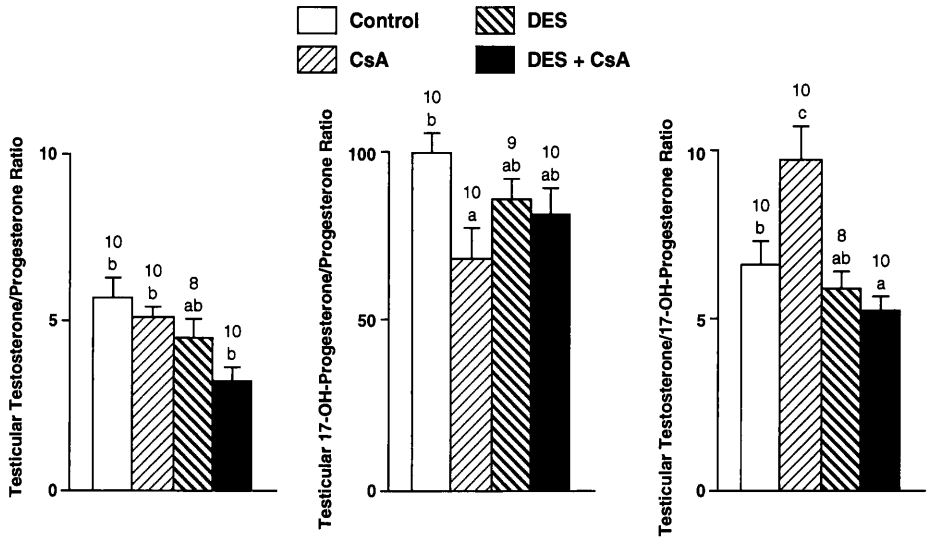

Figure 4. Testicular testosterone/P, 17-OH-P/P, and testosterone/17-OH-P ratios in animals which were treated with empty (control) or DES-filled silastic capsules for 19 weeks, when they had their capsules removed and, starting 17 days later, were treated for 1 week with daily s.c. injections of 5 mg CsA/kg body wt or vehicle. Numbers at the top of columns indicate the number of animals in each group. Values are expressed as mean ± SEM. Values without the same letter are significantly different ($P < 0.05$).

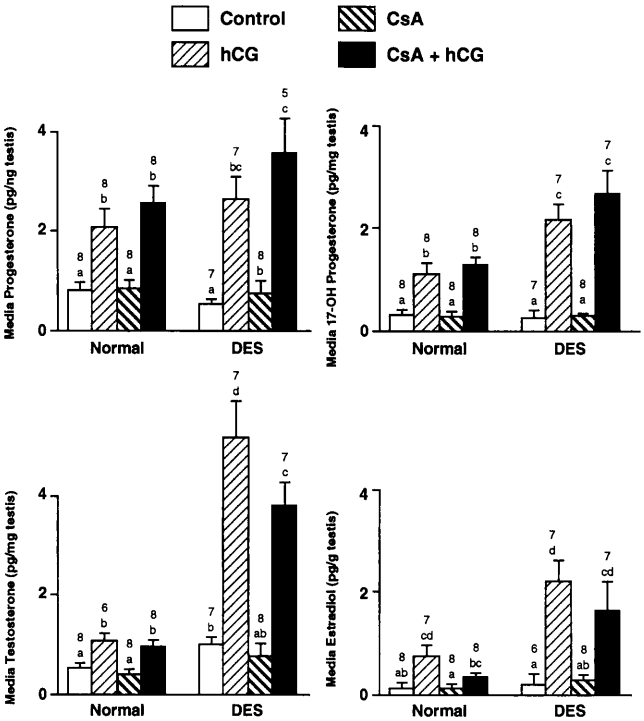

Figure 5. Levels of testosterone (bottom left panel), estradiol (bottom right panel), P (top left panel), and 17-OH-P (top right panel) in the media from testicular incubations in the presence or absence of human chorionic gonadotropin (hCG). The testes were obtained from animals that were treated with empty (control) or DES-filled silastic capsules for 19 weeks, when they had their capsules removed and, starting 17 days later, were treated for 1 week with daily s.c. injections of 5 mg CsA/kg body wt or vehicle. Numbers at the top of columns indicate the number of animals in each group. Values are expressed as mean ± SEM. Values without the same letter are significantly different ($P < 0.05$).

tively, these data suggest differential effects of the drug on the endocrine system that are dependent on dose and length of treatment. In normoprolactinemic control (empty capsules) rats treated with CsA, 17-OH-P levels were significantly decreased in plasma and in the testes, whereas P levels were not modified. Seethalakshmi *et*

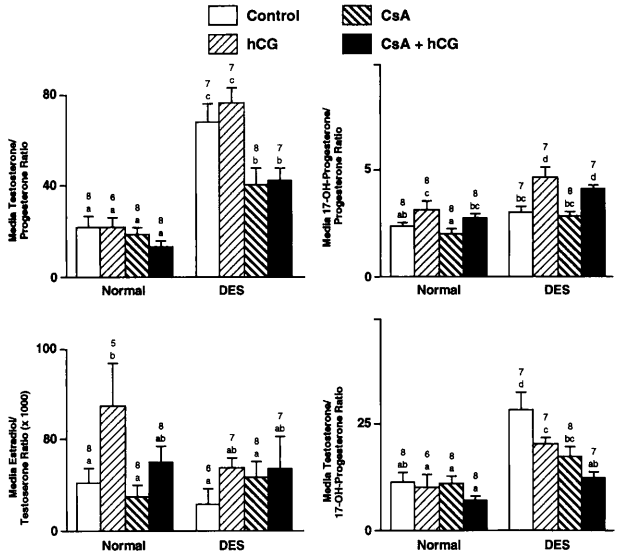

Figure 6. Ratios of estradiol/testosterone (bottom left panel), testosterone/17-OH-P (bottom right panel), testosterone/P (top left panel), and 17-OH-P/P (top right panel) in the media from testicular incubations in the presence or absence of hCG. The testes were obtained from animals that were treated with empty (control) or DES-filled silastic capsules for 19 weeks, when they had their capsules removed and, starting 17 days later, were treated for 1 week with daily s.c. injections of 5 mg CsA/kg body wt or vehicle. Numbers at the top of columns indicate the number of animals in each group. Values are expressed as mean ± SEM. Values without the same letter are significantly different ($P < 0.05$).

al. (11), using very low doses of CsA in prepubertal rats, found a significant increase in the intratesticular concentrations of pregnenolone and 17-OH-P and a concomitant decrease in P and T with no changes in the activity of 17-α-hydroxylase and 17,20 lyase. This would imply that the effects of CsA on testicular steroidogenesis are age dependent, as was suggested for the ovaries in an earlier report from our laboratory (32). In the present study in adult rats, we found a reduction in plasma and testicular levels of 17-OH-P and in the testicular 17-OH-P/P ratio, thus suggesting that 17-α-hydroxylase activity might have been reduced, in agreement with results obtained by others using higher doses of CsA (27, 31).

Testicular T levels were not modified by CsA treatment, although the testicular T/17-OH-P was increased, thus suggesting an increase in the 17,20-lyase activity. However, Sikka *et al.* (33), using different doses of CsA (50, 500, and 5000 ng/ml) in *in vitro* studies, found a decrease in the activity of this enzyme with the highest dose. With the two lower doses (therapeutic levels) the activity of this enzyme did not change. Seethalakshmi *et al.* (11) reported a decrease in the activity of 17,20-lyase when CsA was administered in low doses to prepubertal animals. Under these experimental conditions, plasma levels of CsA ranged between 235 and 333 ng/ml, while in the present study the concentration of the drug in plasma was 182 ng/ml. The differences between the effects of these treatments on steroids levels within the testes could be due to the differences in plasma CsA levels, although it has been reported that the effects of CsA on testicular function and fertility in adult rats were not dependent on the values of the drug in plasma (28). The effects of CsA on the steroidogenic pathway in the testes might depend mainly on the age at which the animals are treated with the drug.

When we analyzed the accumulation of T, P, 17-OH-P, and E_2 in the media after incubating the testes of normoprolactinemic (control) animals that had been treated with CsA or vehicle, we did not find any effects of CsA on either basal levels of these steroids or on their responses to hCG. It has been reported that exposure to CsA *in vitro* did not inhibit basal secretion of T but diminished T release after hCG stimulation (34), although another author did not find the latter effect (33). Suppression of peripheral T levels was apparently not due to CsA action on the pituitary because in animals treated with CsA in the present study, we did not detect any changes in plasma PRL, LH, or FSH levels as was previously described using the same experimental design (19).

Cyclosporin A can modify the function of the hypothalamic-pituitary-adrenal axis (8) and act directly on the adrenal gland (9, 35) to reduce adrenal corticosterone production. This action perhaps could have contributed to the decrease in plasma T levels found in the present study. Other mechanisms of CsA actions on the testis could be postulated on the basis of the immunosuppressive actions of this drug and the recent appreciation of complex physiological and pathological interactions between the immune and the endocrine system. It should also be mentioned that the response of hyperprolactinemic animals to CsA in the present study may be somehow related to the method used to induce hyperprolactinemia, namely DES treatment. Although DES treatment was terminated 2.5 weeks before commencing CsA administration, and testicular activity was obviously recovering, the possibility of residual (direct or indirect) effects of DES on Leydig cell function cannot be eliminated.

Pretreatment with DES decreased plasma T levels without affecting the concentration of this hormone in the testes. These results differ from previous findings in our laboratory (17), which included normalization in plasma T after removal of DES capsules despite persistent suppression of plasma LH and FSH levels. Moreover, in rats rendered hyperprolactinemic by pituitary grafting, plasma T levels are normal, but LH levels are diminished (36, 37). In DES-exposed animals in the present study, plasma gonadotropin levels were lower than in the control (empty capsule) group as we have shown previously (19), confirming earlier data in hyperprolactinemic male rats (17, 36, 38). The presence of normal testicular T concentrations and the consequent reduction in the plasma/testis T ratio in DES-treated animals in the present study probably represents a particular stage of recovery of testicular function after removal of DES capsules. At the time of autopsy, testicular weight in DES-treated rats was suppressed to less than 50% of the control values and thus normal concentration of T in the testes of these animals implies a marked reduction in the total testicular content of T, in keeping with reduced plasma T levels. Suppression of testicular weight obviously involves major reduction of the volume of seminiferous tubules (which constitute approximately 90% of the testis). This would lead to relative enrichment of interstitial cells. Therefore, suppression of T levels in the Leydig cells would be compatible with "normal" concentration of T in the whole testis. Cyclosporin A administration did not modify plasma or testicular T in DES-treated rats, in keeping with the presence of normal levels of PRL, LH, and FSH in these animals after treatment with this immunosuppressor (19). Plasma and testicular P levels were increased after DES treatment, possibly suggesting an increase in the activity of 3-β-hydroxysteroid dehydrogenase which is responsible for the conversion of pregnenolone to P. In contrast, plasma 17-OH-P was decreased while its testicular content was unchanged in DES-treated rats. These results, together with the decrease in plasma T but not in the concentration of T in testes and lack of modifications in the testicular ratios of T/P, 17-OH-

P/P, or T/17-OH-P, suggest that the activities of 17-α-hydroxylase-C17,20-lyase were not modified in these animals despite persistent hyperprolactinemia and reduction in gonadotropin release (39). Cyclosporin A administration in DES-treated rats did not affect plasma or testicular levels of T, P, and 17-OH-P, or the calculated ratios between the levels of these steroids compared with animals pretreated with DES and vehicle. This was not unexpected in view of the absence of significant changes in plasma PRL, LH, and FSH levels after CsA treatment of DES-exposed rats (19).

Prior exposure to DES *in vivo* increased both basal T release and T response to hCG stimulation in testicular incubations. Basal release of P, 17-OH-P, and E_2 in the testes were unchanged after DES treatment, but the release of 17-OH-P after hCG stimulation was increased. Cyclosporin A treatment did not affect basal release of T, P, 17-OH-P, or E_2 *in vitro,* but decreased the response of T to hCG in rats treated with DES. These results suggest that treatment with CsA can attenuate some of the effects of hyperprolactinemia on the testes.

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