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PRONOUNCED EFFECTS OF CYCLOSPORIN A AND NVA^2-CYCLOSPORIN ON HEPATIC STEROID METABOLISM AND ENDOCRINE PARAMETERS IN MALE SPRAGUE–DAWLEY RATS

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Summary—Cyclosporin A (CsA) and the analogue NVA^2-cyclosporin (NVA^2-Cs) were administered by daily injections (i.p.) to male Sprague–Dawley rats for 7 days. At the 5 mg/kg dose level a significant increase in the ratio between 5α-reduction and 16α-hydroxylation of 4-androstene-3,17-dione was observed with both compounds. Serum levels of corticosterone were significantly increased in these rats. A daily dose of 50 mg/kg gave rise to more pronounced changes in the metabolism of 4-androstene-3,17-dione, with an almost 10-fold increase in the 5α/16α-ratio and a decrease in 6β-hydroxylation. At the high dose level serum levels of corticosterone increased >2-fold, whereas testosterone decreased. This decrease was more pronounced with CsA treatment (>40-fold) than with NVA^2-Cs (>3-fold). A significant decrease in the serum levels of luteinizing hormone (LH) was observed with high dose CsA treatment. High dose CsA and NVA^2-Cs reduced serum prolactin levels, 6- and 3.6-fold, respectively. A small but significant increase in the serum level of creatinine was observed only in rats receiving NVA^2-Cs, 50 mg/kg. The changes in hepatic steroid-metabolizing enzymes following CsA and NVA^2-Cs treatment indicate that both compounds influence male rat liver via neuroendocrine mechanisms previously shown to regulate a number of hepatic functions, including several steroid-metabolizing enzymes. These functions are regulated from the hypothalamus, through the secretory pattern of growth hormone. The observed effects of CsA and NVA^2-Cs might be due to an indirect action on growth hormone secretion, where an altered testosterone production exerts an influence at the hypothalamic level, and/or the result of a direct effect on the hypothalamus.

INTRODUCTION

Both cyclosporin A (CsA) and the analogue NVA^2-cyclosporin (NVA^2-Cs) exert potent effects as modulators of the immune response [1–3]. The exact mechanisms responsible for their influence on the immune system have not been elucidated. However, CsA has been shown to interact with the production of interleukins and the cellular response to stimulation by interleukins [4]. It has also been suggested that cyclosporins might modulate the immune system by influencing the polyamine biosynthetic pathway via specific antagonism of the prolactin receptor [5].

Following treatment with CsA many patients develop hypertrichosis [6, 7] and administration of CsA to nude mice has been shown to increase pelage after 6 days [8]. Gynecomastia has also been reported [9]. The endocrine background to these phenomena is unknown although CsA has been shown to affect the serum levels of several different hormones in experimental animals [10–12].

In a preliminary experiment we observed an altered metabolism of 4-androstene-3,17-dione (androstenedione) with liver microsomes from CsA treated male rats compared with controls. Androstenedione metabolism has been extensively used as a model substrate for studies on sex differentiated hepatic function in the rat and provides a useful tool in studies of endocrine interactions involving rat liver [13].

The present experiment was designed to investigate whether low (5 mg/kg) and high (50 mg/kg) daily doses of CsA and NVA^2-Cs for 1 week would affect hepatic steroid metabolism and, if possible, to identify the endocrine parameters responsible for the observed effects. Another purpose was to identify other endocrine

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Table 1. Effects of CsA and NVA²-Cs on the metabolism of androstenedione in microsomal preparations from male Sprague–Dawley rats

		Androstenedione metabolism (nmol/min/mg protein)				
		Hydroxylations				
Treatment	*n*	7α	6β	16α	5α-Reduction	5α/16α-Ratio
Control	6	0.34 ± 0.06	1.23 ± 0.14	1.69 ± 0.21	2.11 ± 0.37	1.26 ± 0.28
CsA						
5 mg/kg	5	0.31 ± 0.03	0.89 ± 0.11	1.46 ± 0.15	2.99 ± 0.99	2.12 ± 0.95[a]
50 mg/kg	5	0.44 ± 0.13	0.33 ± 0.12[a]	0.51 ± 0.18[a]	5.96 ± 1.86[a]	12.2 ± 4.3[a]
NVA²-Cs						
5 mg/kg	5	0.34 ± 0.14	1.06 ± 0.38	1.57 ± 0.36	3.11 ± 0.89	2.0 ± 0.48[a]
50 mg/kg	7	0.45 ± 0.14	0.32 ± 0.17[a]	0.47 ± 0.28[a]	5.28 ± 1.77[a]	11.2 ± 6.0[a]

Rats received daily injections of either 5 or 50 mg/kg body wt for 7 days. Controls were injected with vehicle only. Values are expressed as means ± SD and statistical analysis was performed using the Wilcoxon rank sum test ($P < 0.05$).
[a]Significantly different from controls.

changes of possible importance for the immune response and for adverse effects observed in humans.

MATERIALS AND METHODS

Fifty-six-day-old male Sprague–Dawley rats (Møllegaards Breeding Center, Skensved, Denmark) received daily i.p. injections (5 or 50 mg/kg) with either CsA (Sandimmun, 50 mg/ml) or NVA²-Cs dissolved in olive oil and cremophor B. Controls were injected with vehicle only. On day 8, rats were killed by decapitation and the livers were placed in ice-cold 0.25 M sucrose. Microsomes were prepared immediately according to Ernster *et al.* [14] and protein was determined according to Lowry *et al.* [15]. The microsomes were stored at −70°C. Androstenedione metabolism was determined as previously described [13]. Serum samples were collected at the time of killing and stored at −70°C until assayed.

Western blot analysis of the male-specific cytochrome $P\text{-}450_{16\alpha}$ (♂ > ♀), known to be the major enzyme catalyzing 16α-hydroxylation of androstenedione, and the female-specific cytochrome $P\text{-}450_{15\alpha}$ (♀ > ♂), active towards various steroid sulfates, was performed with microsomal preparations from all groups of rats. The SDS gel electrophoresis was performed essentially according to Laemmli [16], and the blotting procedure essentially according to Towbin *et al.* [17] using monoclonal antibodies towards cytochrome $P\text{-}450_{16\alpha}$ and $P\text{-}450_{15\beta}$ [18].

The serum levels of prolactin (PRL), luteinizing hormone (LH), corticosterone (CORT) and testosterone were determined by RIA as outlined previously [19]. Creatinine was determined enzymatically by reagents from Wako, Dusseldorf, Germany.

All results are expressed as means ± SD for each group of rats. Statistical analysis was performed using the Wilcoxon rank sum test and the level of significance was $P < 0.05$ [20].

RESULTS

Low doses (5 mg/kg) of CsA or NVA²-Cs did not significantly affect any of the cytochrome *P*-450-mediated enzyme activities towards androstenedione or the 5α-reduction pathway in rat liver microsomes (Table 1). However, when the 5α/16α-ratio was calculated both CsA and NVA²-Cs treated rats exhibited higher ratios than control rats. At the high dose level (50 mg/kg) both the 6β- and the 16α-hydroxylations were markedly decreased in rats receiving CsA/NVA²-Cs. 5α-Reduction as well as the 5α/16α-

Table 2. Effects of CsA and NVA²-Cs on the serum levels of PRL, LH, CORT, testosterone (T) and creatinine (CREAT)

Treatment	*n*	PRL (ng/ml)	LH (ng/ml)	CORT (nmol/l)	T (nmol/l)	CREAT (mg/dl)
Control	6	14.6 ± 5.9	0.59 ± 0.15	170 ± 107	7.7 ± 5.9	0.61 ± 0.04
CsA						
5 mg/kg	5	15.6 ± 8.3	0.43 ± 0.13	494 ± 226[a]	4.8 ± 4.2	0.63 ± 0.04
50 mg/kg	5	2.3 ± 1.3[a]	0.33 ± 0.08[a]	589 ± 219[a]	0.19 ± 0.19[a]	0.53 ± 0.05
NVA²-Cs						
5 mg/kg	5	12.3 ± 13.0	0.53 ± 0.18	448 ± 233[a]	6.0 ± 3.1	0.58 ± 0.04
50 mg/kg	7	4.0 ± 2.4[a]	0.58 ± 0.20	483 ± 324[a]	2.4 ± 2.3[a]	0.72 ± 0.12[a]

Values are expressed as means ± SD and statistical analysis was performed using the Wilcoxon rank sum test ($P < 0.05$).
[a]Significantly different from controls.

ratios were significantly increased compared with controls.

Western blot analysis revealed decreased levels of cytochrome $P\text{-}450_{16\alpha}$ and increased levels of $P\text{-}450_{15\beta}$ in liver microsomes from male rats receiving high dose treatment with CsA and NVA^2-Cs, respectively, compared with microsomes from intact control rats of both sexes (data not shown). Rats receiving low doses of either of the two substances were indistinguishable from the male controls.

CORT levels were significantly increased in all groups receiving CsA or NVA^2-Cs, the most pronounced effect being seen in rats receiving high dose CsA treatment (Table 2). At the high dose level both substances markedly influenced the endocrine status of the animals (Table 2). Whereas CsA reduced the S-PRL level to 16% of the level in controls the decrease in NVA^2-Cs treated rats was slightly less (27% of control).

Serum levels of testosterone were below the detection level (<0.1 nmol/l) in 4 out of 5 rats receiving high dose treatment with CsA and in 2 out of 7 rats on the same high dose of NVA^2-Cs.

A slight but significant decrease in the serum level of LH was observed in rats receiving high dose treatment with CsA. The creatinine level was significantly higher than in controls only in the group of rats receiving high dose treatment with NVA^2-Cs.

DISCUSSION

Administration of high doses of CsA and NVA^2-Cs (50 mg/kg) was shown to influence microsomal steroid-metabolizing enzymes in male rat liver. These enzymes are sex differentiated in adult rats, due to a neuroendocrine regulation via the hypothalamo–pituitary–liver axis, mediated by a sexual dimorphism in the secretory pattern of growth hormone (GH) [21, 22]. A male pattern of GH-secretion in the adult rat is dependent on circulating androgens [23]. Several endocrine manipulations, including castration, have been shown to feminize GH-secretion as well as sex differentiated liver functions in the male rat [24].

The ratio between 5α-reduction and 16α-hydroxylation of androstenedione has often been calculated to estimate the degree of feminization following endocrine manipulations of male rats [24]. Following low dose treatment (5 mg/kg) with both CsA and NVA^2-Cs in the present study a small but significant increase in 5α/16α-ratio was observed, whereas a marked increase was observed with high dose treatment. The observed effects represent only a partial feminization, comparable with that previously seen after castration of adult male rats [24]. Both the apparent increase in the female-specific cytochrome $P\text{-}450_{15\beta}$ and the fact that 7α-hydroxylation of androstenedione (♂ = ♀) was not affected after CsA or NVA^2-Cs treatment, supports the view that endocrine factors are major determinants for the observed effects of CsA on rat liver. Sikka *et al.* [10, 11, 25] demonstrated decreased serum levels of testosterone as well as of LH after treatment of adult male rats with CsA at a dose of 15 mg/kg or more for 30 days. They suggested that CsA exerts its effects on the hypothalamo–pituitary–gonadal axis primarily by an effect at the hypothalamic level [10] but other studies indicate that CsA has a direct influence on steroidogenesis in the rat testes [11, 26]. It is worth noting that administration of [^{3}H]CsA to mice, followed by whole body radiography, resulted in measurable amounts of radioactivity only in two areas in the brain, the area postrema region and the choroidal plexus [27]. Some radioactivity was also seen in the pituitary. In addition there was a low uptake in the adrenal cortex, indicating a hypothetical direct influence of CsA also on the production of corticosteroids.

The influence on LH secretion was limited to high dose CsA treated rats and no influence was seen in rats receiving NVA^2-Cs, supporting the view that both compounds act, at least partly, directly on the gonads. Independent of the mechanisms involved it is reasonable to assume that the decreased levels of testosterone following CsA/NVA^2-Cs treatment lead to a partial feminization of the androgen-dependent male pattern of GH-secretion, with concomitant alterations of rat liver function [23].

The nephrotoxicity of CsA seems to be dependent on the hepatic capacity to metabolize the compound [28] and, consequently, it is tempting to speculate that CsA might alter its own metabolism through the endocrine interaction(s) observed in the present study. Such an interaction with enzymes central for the pharmacokinetics of the compound might be an essential part of the mechanisms responsible for toxicity.

The increased levels of CORT even at low levels of CsA/NVA^2-Cs provides additional evidence for the multiendocrine effects induced. Studies in humans have so far not yielded any conclusive data with respect to possible effects on endogenous production and turnover of cortico-

steroids. However, simultaneous treatment with CsA and low dose prednisolone has been reported to decrease prednisolone clearance [29]. An effect on hepatic forms of cytochrome *P*-450 was suggested as a possible explanation for this interaction. Although the mechanisms are still unclear it is tempting to speculate that the elevated levels of CORT observed here might be part of the explanation to the occurrence of hypertrichosis in humans.

In contrast with previous studies our data show that high dose treatment with CsA as well as with NVA^2-Cs results in a pronounced decrease of serum PRL levels. The acute effects of low dose CsA treatment on PRL secretion previously reported involve a rapidly increased serum level [12]. Both hyper- and hypoprolactinemia has been reported to result in a compromised immune state [30, 31] and accumulating evidence indicates that PRL plays a central role in maintaining immune function [5]. Cyclosporin has been suggested to exert its immunosuppressive action by interfering with PRL binding sites at the surface of T lymphocytes possibly through an inhibition of the polyamine biosynthetic pathway [32].

On the basis of present knowledge it is difficult to give a clearcut explanation to the observed decrease in serum PRL levels. As, however, circulating PRL exerts a negative feedback control on its release from lactotrophs in the anterior pituitary [12] it could be hypothesized that CsA/NVA^2-Cs might interact with PRL receptors at the pituitary level. Secondary effects must also be considered as thyroid hormone, glucocorticoids and sex steroids have been shown to influence both PRL action and its secretion [33–36].

NVA^2-Cs has recently been introduced as an alternative immunosuppressive drug, due to less nephrotoxicity. However, the effects of CsA and NVA^2-Cs on the parameters studied here exhibit a similar pattern. Although the NVA^2-Cs induced changes in some cases are less pronounced it could be noted that the only significant increase in serum creatinine levels was seen in rats receiving high dose treatment with NVA^2-Cs.

Our data demonstrate that treatment with CsA as well as NVA^2-Cs exerts a multiendocrine influence not only on the serum levels of several hormones but also on several enzymes involved in GH-dependent hepatic metabolism of steroids. Further studies are needed to evaluate the role of GH-secretion as a regulator of the hepatic metabolism of CsA/NVA^2-Cs and other drugs used for immunosuppression and possibly also as a potential modulator of immune response *per se*.

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