Antiandrogenic Effect of Spirolactones: Mechanism of Action

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ABSTRACT. Spirolactones are aldosterone antagonists which inhibit the binding of aldosterone to the renal mineralocorticoid receptor. These molecules also possess an antiandrogenic effect which could be due, among other possibilities, to a peripheral antagonism of androgens. This hypothesis has been tested in the present study. From *in vivo* experiments, spironolactone and K^+ canrenoate appear to inhibit the binding of [^{3}H]5α-dihydrotestosterone [^{3}H]DHT to the cytosolic and nuclear receptor of the rat ventral prostate. The doses used are in the same range as those used for demonstrating the antimineralocorticoid effect of these molecules. *In vitro* incubations and *in vitro* displacement studies show that spironolactone and K^+ canrenoate are respectively about 20 and 100 times less effective than DHT in displacing 50% of 5×10^{-10} M [^{3}H]DHT from its receptor. Spirolactones are also able to compete with [^{3}H]DHT for the specific 8 S cytosolic receptor. Neither spironolactone nor K^+ canrenoate decreases prostatic 5α-reductase activity, even at a concentration as high as 10^{-5}M. It seems likely that spirolactones, besides their action on testosterone biosynthesis, exert their antiandrogenic activity via a peripheral androgen antagonism. (*Endocrinology* 97: 52, 1975)

SPIROLACTONES are aldosterone antagonists which are currently used in the treatment of primary hyperaldosteronism and essential hypertension. Kagawa first showed the antimineralocorticoid effect of spirolactones in adrenalectomized rats treated by deoxycorticosterone acetate (1). Spirolactones act *in vitro* by competitively inhibiting active Na^+ transport induced by aldosterone (2). At the molecular level, spirolactones have been shown to inhibit the first step of the mechanism of action of aldosterone, that is, its binding to kidney cytosol mineralocorticoid receptor (3–6).

One of the side effects most frequently encountered during chronic spirolactone therapy is the occurrence of sexual disorders induced by these drugs. Several mechanisms could account for these effects: Menard *et al.* (7) have recently reported that spironolactone decreased the activity of the 17α-hydroxylase and the content of cytochrome P-450 in the rat testis resulting in an impairment in testosterone biosynthesis.

Another possible mechanism could be a peripheral antiandrogenic effect of spirolactones at the level of the target organs. Such a mechanism is suggested by the experiments of Steelman *et al.* (8) and Rasmusson *et al.* (9) who have demonstrated that spironolactone antagonizes the androgen-stimulated weight gain of the seminal vesicle and ventral prostate. Such an androgen antagonism could be due either to the inhibition of prostatic 5α-reductase activity or to competition with 5α-dihydrotestosterone[1] (DHT) for the androgenic cytosolic receptor. The purpose of this study was to investigate these two possible effects of spirolactones on prostatic tissue.

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Materials and Methods

Animals

Adult male Wistar rats (200 g) were castrated by the scrotal route 24 h before sacrifice. On the day of experiment, the prostate was removed and rinsed with isotonic saline at 4 C. [1,2-^{3}H]5α-Dihydrotestosterone (^{3}H-DHT), 47 Ci/mmol, and [1,2-^{3}H]testosterone, 48 Ci/mmol, were purchased from Amersham and their purity checked

[1] The terminology used is: 5α-dihydrotestosterone (DHT), 5α-androstan-17β-ol-3-one; cyproterone acetate, 6α-chloro-17α-hydroxy-1α,2α-methylene-4,6-pregnadiene-3,20-dione-17-acetate.

before use as previously described (10). Spironolactone (SC 9420) and K^+ canrenoate (SC 14266) were kindly given by Searle, and 5α-dihydrotestosterone (Ikapharm) was recrystallized before use. Pure sucrose was obtained from Sigma and all other chemical products were analytical grade and purchased from Merck (Germany), unless otherwise indicated.

In vivo *experiments*

There were 3 rats in each experimental group. Animals in the control group (group I) were injected intraperitoneally with 0.5 ml of a mixture of ethanol, propanediol and physiologic saline. Rats in the other groups received either 200 μg of DHT (group II), 5 mg of spironolactone (group III) or 5 mg of K^+ canrenoate (group IV), dissolved in the same mixture. One hour later, all rats were injected intravenously with 20 μCi (4.3×10^{-10} M) of [^{3}H]DHT. Fifteen minutes after the last injection, the animals were killed and the prostate glands removed and homogenized in 4 ml of Tris HCl buffer 0.01M, EDTA 1 mM, pH 7.4, containing 10% glycerol and 0.05 mM of dithiothreitol. The homogenate was centrifuged at $700 \times g$ for 15 min and the cytosol ($105,000 \times g$) was prepared from the supernatant. The pellet obtained from the low speed centrifugation of the homogenate was resuspended in 2.2M sucrose and the nuclei purified by centrifugation for 1 h at $78,000 \times g$ on 2.2M sucrose. The bound and free radioactivity in the cytosol were separated using a dextran-charcoal method: 200 μl of cytosol were incubated for 90 min under agitation with 500 μl of dextran-coated charcoal (dextran 0.5%, charcoal 5%) and centrifuged at $2,500 \times g$ for 25 min; the supernatant was then counted. The nuclear pellet was resuspended in 2 ml of 0.4M KCl for 15 min, then centrifuged at $20,000 \times g$ for 20 min. An aliquot of the supernatant was taken for radioactivity measurement and another aliquot was taken for protein determination by the method of Lowry *et al.* (11).

In vitro *incubations*

Prostate slices (280 μm) were incubated with 5×10^{-9} M [^{3}H]DHT with or without competitors at 25 C for 25 min in incubation buffer A: $Na^+ = 133$, $K^+ = 6$, $Ca^{++} = 1$, $Mg^{++} = 0.5$, $Cl^- = 134$, $H_2PO_4^- = 6$, Tris HCl = 5, and glucose = 5 mM, pH 7.4. At the end of the incubation, the slices were homogenized at 4 C in Tris-HCl buffer, 0.01M, pH 7.4, containing 1 mM EDTA and 10% glycerol. The cytosol and the nuclei were then prepared as described above. Bound and free radioactivity in the cytosol were separated by dextran-charcoal and the nuclear radioactivity was extracted and counted as already described.

In vitro *displacement studies*

Twelve rat prostates were homogenized in 12 ml of Tris-HCl buffer, 0.05M, pH 7.4, containing 1 mM EDTA and 0.1 mM mercaptoethanol. The homogenate was then centrifuged at $1,000 \times g$ for 15 min and the resulting supernatant was incubated at 4 C for 180 min with 5×10^{-10} M [^{3}H]DHT in the presence or the absence of various competitors (DHT, spironolactone, K^+ canrenoate, cyproterone acetate[1]). Bound [^{3}H]DHT was separated from free steroid by the dextran-charcoal technique.

Linear sucrose gradients (5–20%) were prepared in the homogenization buffer containing 10% glycerol; 0.2 ml cytosol samples were then layered at the top of the gradients. Ultracentrifugation, fraction collection and the use of standard proteins were performed as already described (12).

Effects on 5α-reductase activity

Ventral prostates were removed from ether-anesthetized 200–250 g rats. Prostatic tissue was separated from connective tissue, then minced with scissors and pooled in cold buffer A (4 C). About 150 mg of tissue was incubated at 37 C for 2 h under oxygen in 2 ml of buffer A, pH 7.4, containing 2 U glucose-6-phosphate dehydrogenase, 10 pmol glucose-6-phosphate and 2.5 pmol TPN (Sigma) per incubation vial. Incubations were performed with 1.7×10^{-9} M [^{3}H]testosterone with or without 10^{-7} to 10^{-5} M spironolactone or K^+ canrenoate. After addition of 100 μg testosterone, 100 μg DHT and [^{14}C]DHT tracer for recovery, the tissue was homogenized and the steroids were extracted and purified by thin layer chromatography (TLC): benzene, then benzene: methanol (95:5). After acetylation, DHT acetate was chromatographed by TLC in benzene: ethyl acetate (90:10). The $^3H/^{14}C$ ratio was calculated.

Radioactivity measurements

Aqueous samples were counted after addition of 10 ml of Unisolve (Koch and Light) in a

TriCarb Scintillation spectrometer 3380 with an efficiency of 34%. ^{14}C- and ^{3}H-labeled compounds eluted from thin-layer plates were counted in a standard counting solution (toluene, 960 ml. Liquifluor (New England Nuclear), 40 ml).

Results

In vivo *experiments*

Fifteen minutes after *in vivo* injection of 4.3×10^{-10} M [^{3}H]DHT, radioactivity was found in the cytosol and nuclei of rat ventral prostate. The specifically bound radioactivity in cytosol was measured after removal of free radioactivity by dextran-charcoal. Displacement experiments showed that 200 μg DHT and 5 mg spironolactone could equally displace the bound radioactivity in both cytosol and nuclei (Fig. 1). K^+ canrenoate (5 mg) also inhibited the binding of the radioactivity to the cytosolic and nuclear receptor.

In vitro *displacement studies*

Very similar results were obtained by using *in vitro* incubation of rat ventral prostate slices (Table 1). A 5,000-fold excess of spironolactone displaced 75% of the bound radioactivity as did 500-fold excess of unlabelled DHT. In this experiment, as for the *in vivo* experiment, K^+ canrenoate appeared to be less potent than spironolactone since a 5,000-fold excess of this compound displaced only 50% the bound radioactivity.

Figure 2 shows complete *in vitro* studies: the 1,000 × *g* supernatant was incubated with 5×10^{-10} M [^{3}H]DHT and various competitors. Fifty per cent displacement of the bound radioactivity was obtained by 3×10^{-8} M spironolactone and

TABLE 1. Binding of [^{3}H]DHT in cytosol and nuclei during *in vitro* incubation of rat ventral prostate slices in the presence of competitors

Steroids	Cytosol dpm/mg protein	Nuclei dpm/mg protein
5×10^{-9} M [^{3}H]DHT	4,580	39,928
	4,183	39,133
+ 2.5×10^{-6} M DHT	1,195	11,390
	1,077	9,542
+ 2.5×10^{-5} M Spironolactone (SC 9420)	1,223	9,661
	1,038	10,041
+ 2.5×10^{-5} M K^+ Canrenoate (SC 14 266)	2,211	17,381
	2,570	20,948

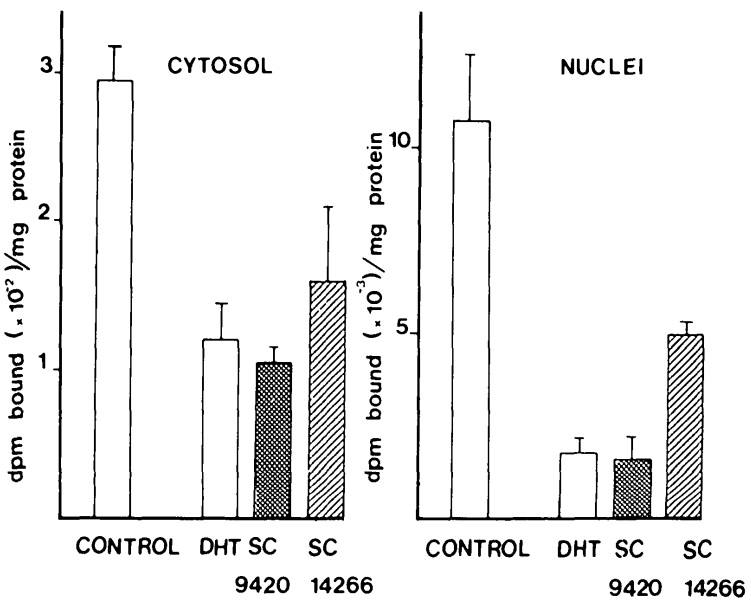

FIG. 1. Effect of DHT and of spirolactones on binding of radioactivity in prostatic cytosol and nuclei after injection of [^{3}H]DHT in orchidectomized rats. Rats were injected ip with solvent (control group), 200 μg DHT, 5 mg of spironolactone (SC 9420) or 5 mg K^+ canrenoate (SC 14266). One hour later the rats were injected iv with 10 μCi [^{3}H]DHT and killed 15 min later. The specifically bound radioactivity in the prostatic cytosol was counted after separation of bound from free steroid by the dextran-charcoal method. Nuclear bound radioactivity was counted after 0.4M KCl extraction. Each bar represents the mean of two experiments. There were 3 rats in each experimental group. Determination of cytosolic radioactivity was performed in quadruplicate (mean ± SD) and nuclear radioactivity was measured in duplicate.

4×10^{-6} M K^+ canrenoate. Cyproterone acetate produced about the same displacement of [^{3}H]DHT as spironolactone. When cytosol incubated with 5×10^{-9} M of [^{3}H]DHT was analyzed by sucrose gradient centrifugation, [^{3}H]DHT was specifically bound to an 8 S protein peak (Fig. 3). This peak was displaced by a 1,000-fold excess of unlabelled DHT or a 10,000-fold excess of spironolactone. K^+ canrenoate (10,000-fold excess) could also displace [^{3}H]DHT from its 8 S binding peak.

Effect of spirolactones on 5α-reductase activity

Finally, the possible effect of spironolactone and K^+ canrenoate on the 5α-reductase activity of rat ventral prostate was tested. As shown in Table 2, neither spironolactone nor K^+ canrenoate decreased 5α-reductase activity of rat ventral prostate, even at concentrations as high as 10^{-5} M.

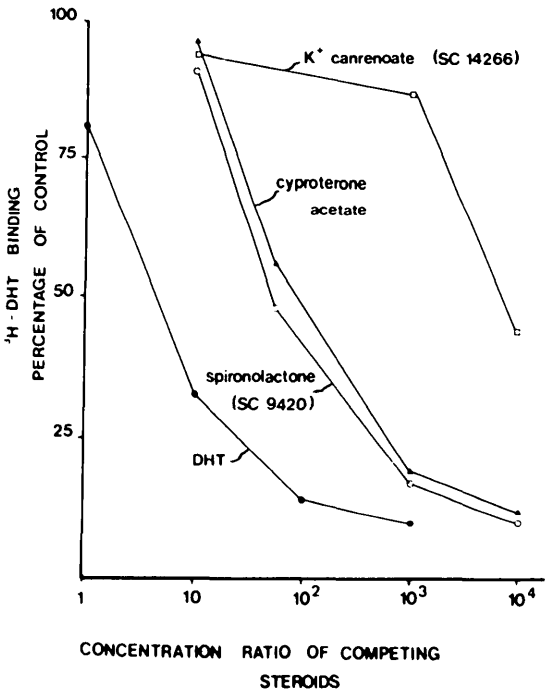

FIG. 2. *In vitro* incubation of prostatic supernatant with 5×10^{-10} M [^{3}H]DHT in the presence of various competitors. Bound DHT was separated from free DHT by the dextran-charcoal technique. The results are expressed as a percent of control. Each point is the mean of 3 experiments.

TABLE 2. Effect of spirolactone on prostatic 5α-reductase

Steroids (M)		mg Prostatic tissue/ incubation	% of Radioactivity recovered as [^{3}H]DHT/ incubation
Control (1.7×10^{-9} M [^{3}H]testosterone)		164 ± 24*	48 ± 6*
Spironolactone (SC 9420)	10^{-7}	151	49
		141	47
	10^{-6}	147	49
		170	45
	10^{-5}	148	46
		139	48
K^+ Canrenoate (SC 14 266)	10^{-7}	115	36
		158	45
	10^{-6}	89	47
		122	52
	10^{-5}	144	42
		156	45

* Mean of 4 values ± SD.

Discussion

Ever since the clinical introduction of spirolactones in diuretic therapy, there have been reports of associated sexual disorders. Gynecomastia is a common complication in spironolactone treated men, as well as decreased libido (13–16). Menstrual cycle abnormalities are very often observed in females (16). All of these effects disappear on withdrawal of the drug.

Spirolactones can interfere at different levels with the metabolism of androgens. Dymling *et al.* (17) have shown that 100 to 200 mg of K^+ canrenoate iv was able to lower the plasma level of testosterone significantly in normal men. Such an effect could be due to a change in testosterone metabolism or to reduced testicular secretion of testosterone. In favor of this latter hypothesis is the recent report of Menard

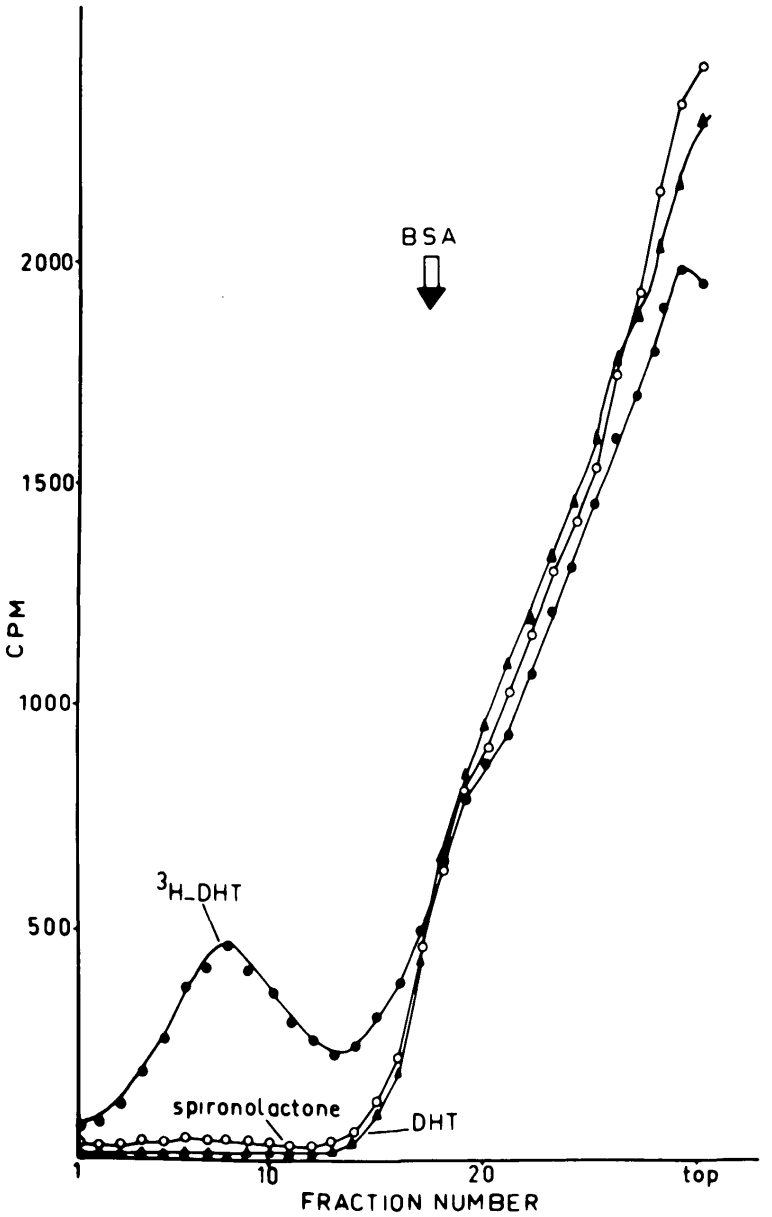

FIG. 3. Sedimentation patterns of prostatic cytosol after incubation with 5×10^{-9} M [^{3}H]DHT in the absence or in the presence of 5×10^{-6} M DHT or 5×10^{-5} M spironolactone. Five to 20% linear sucrose gradients were made in 0.05M Tris-HCl, 1.0 mM EDTA, 0.1 mM mercaptoethanol, pH 7.4, containing 10% glycerol. Samples were centrifuged at 55,000 rpm for 15 h at 2 C.

et al. (7) which demonstrates that daily treatment of male rats with spironolactone (200 mg/kg) for three days induces a 72% decrease in the *in vitro* formation of testosterone from progesterone.

However other mechanisms could account in part for the antiandrogenic effect of spirolactones. Steelman *et al.* (8) and Rasmusson *et al.* (9) have shown that spironolactone can antagonize the peripheral action of androgens in the castrate rat. One to 3 mg of spironolactone was able to significantly decrease the seminal vesicle and ventral prostate weights in the immature male castrate rat treated with a single dose of 0.5 mg of testosterone enanthate (9).

The aim of the present studies was to investigate further the peripheral antiandrogenic properties of spirolactones. Evi-

dence has been presented that spirolactones are able to compete *in vivo* and *in vitro* with DHT for the intracellular androgen receptor sites. *In vitro* experiments confirmed that both spironolactone and K^+ canrenoate were able to decrease the formation of cytosolic- and [^{3}H]DHT-chromatin-receptor complexes. That spironolactone was effectively able to compete with [^{3}H]DHT for the specific 8 S cytosolic receptor (18,19) was shown by the sucrose gradient experiment.

The doses used in the current study for demonstrating the inhibitory effect of spironolactone on DHT binding are similar to those used for proving its antiandrogenic and antimineralocorticoid activity. Inhibition of [^{3}H]DHT binding to cytosolic and nuclear androgen receptors *in vivo* was obtained with 5 mg of spironolactone, a dose which is close to that used by Rasmusson *et al.* (9) for testing the *in vivo* antiandrogenic effect (1 and 3 mg). Similarly, Kagawa showed that 1.6 mg of spironolactone was necessary to completely block the effect of 6 and 12 μg of deoxycorticosterone acetate in the adrenalectomized rat (1).

From complete *in vitro* displacement studies (Fig. 2), spironolactone appears to be about one-twentieth as effective as DHT in displacing 50% of 5×10^{-10} M [^{3}H]DHT bound to the receptor. K^+ canrenoate, on the other hand, appears to be an even less potent competitor than spironolactone for [^{3}H]DHT binding sites; *in vitro* competition experiments on a $1{,}000 \times g$ supernatant receptor (Fig. 2) show that K^+ canrenoate is about 100 times less effective than spironolactone in displacing 50% of [^{3}H]DHT binding.

Similarly, spirolactones inhibit aldosterone action by competing with aldosterone for the mineralocorticoid receptor. Funder *et al.* (20) found also a relatively low affinity of spironolactone for this receptor, since 4×10^{-8} M spironolactone inhibited the binding of 2×10^{-9} M aldosterone by only 54%. K^+ canrenoate appeared to be about 100 times less effective than spironolactone in displacing 50% of [^{3}H]aldosterone bound to the mineralocorticoid receptor (20). It is interesting to note that, as in the case of the androgenic receptor, the effect of opening the γ-lactone ring appears to decrease the affinity to the receptor.

The results of the present study should be compared with those obtained in similar experiments using cyproterone or cyproterone acetate, a well-known antiandrogen (21). This molecule has been previously reported to prevent [^{3}H]DHT binding to the cytosolic and nuclear androgen receptors, at about the same doses as those used in the present study (22,23). As in the case of spirolactones, it has no inhibitory effect on prostatic 5α-reductase (20). Spirolactones thus appear to produce their peripheral anti-androgenic effect via competition for the androgenic receptor.

The daily blood production rate of testosterone in men is about 5 mg and that of DHT is 0.3 mg (24). The doses of spirolactones commonly used in men (100–400 mg) represent, therefore, about 20–80 and 330–1330 times the production rate of testosterone and DHT, respectively. This study shows that the affinity of spironolactone for the androgenic receptor is about one twentieth of that of DHT. Therefore it seems likely that spirolactones, besides their action on testosterone synthesis, exert their antiandrogenic activity via a peripheral antagonism.

Acknowledgments

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