EFFECT OF SPIRONOLACTONE ON ANDROGEN-DEPENDENT PROTEINS IN THE VENTRAL PROSTATE OF THE RAT

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SUMMARY

The effect of spironolactone on five androgen-dependent proteins in the ventral prostate of the rat was investigated by two-dimensional gel electrophoresis. Spironolactone was given to intact male, castrated and androgen-stimulated castrated rats. It has been shown that spironolactone had no influence on the synthesis or accumulation of the androgen-dependent proteins in intact animals. However, spironolactone suppressed the restoration of the major androgen-dependent protein of low molecular weight in castrated rats given testosterone. The mechanism by which spironolactone exerts its anti-androgenic activity was shown to be unrelated to its capacity to inhibit the synthesis or accumulation of the five androgen-dependent proteins studied in this investigation.

INTRODUCTION

Spironolactone, the aldosterone antagonist, has been shown to cause regression of prostatic and seminal vesicle tissue in the rat (Basinger & Gittes, 1974). Several investigators have reported the anti-androgenic activity of spironolactone in man and rat (Steelman, Brooks, Morgan & Patanelli, 1969; Corvol, Michaud, Menard, Freifeld & Mahoudeau, 1975). The observation that spironolactone is able to block the effects of exogenous androgen in the rat suggests that interaction of the compound with androgen receptors at the target tissue may occur (Bonne & Raynaud, 1974). Spironolactone has been shown to inhibit competitively the binding of dihydrotestosterone to the cytosolic androgen receptor of the rat ventral prostate (Corvol et al. 1975; Pita, Lippman, Thompson, & Loriaux, 1975) and more recently to human androgen-binding protein in prostate and prepuce from newborn infants (Rifka, Pita, Vigersky, Wilson & Loriaux, 1978).

Several groups of workers have investigated the androgen-dependent proteins synthesized in the rat ventral prostate (Heyns, Peeters & Mous, 1977; Parker, Scrace & Mainwaring, 1978; Lea, Petrusz & French, 1979). The synthesis of androgen-dependent proteins is believed to be mediated by the androgen receptor in the prostate. Thus any displacement of androgen from the androgen receptor by a competitor such as spironolactone may be expected to influence the synthesis or accumulation of androgen-dependent proteins. This study has investigated the effect of spironolactone on the synthesis and maintenance of a major group of androgen-dependent proteins in the rat ventral prostate.

MATERIALS AND METHODS

Male Sprague–Dawley rats (Charles River, Wilmington, Massachusetts, U.S.A.) aged 3 months and of 250 g body wt were used. Of two groups of intact rats, one remained...
untreated and the other animals received 25 mg crystalline spironolactone (G. D. Searle Co., Arlington Heights, Illinois, U.S.A.) each day for 6 days.

Four groups of castrated rats were used. Six days after castration one group was killed, a second group was given 25 mg spironolactone each day for 6 days beginning immediately after castration, a third was given 25 mg spironolactone and 1.25 mg testosterone propionate (G. D. Searle) each day for 6 days and the fourth group was given 1.25 mg testosterone propionate only each day for 6 days. The doses of testosterone propionate given were the same as those used by Parker et al. (1978) and by Higgins, Burchell & Mainwaring (1976) in rats of similar weight. The dose was not intended to be physiological but was used to prime the production of androgen-dependent proteins. The vehicle in all cases was propylene glycol. The protein profiles from either intact or castrated rats were not influenced by propylene glycol. The prostates of two rats were pooled from each group for each experimental point.

Fractionation of total prostatic protein

The ventral prostate was dissected and immediately frozen in liquid N₂. Subsequently 25 mg frozen tissue were placed in a ground-glass homogenizer with 200 µl 1% sodium dodecyl sulphate (Bio-Rad Laboratories, Richmond, California, U.S.A.), 8 M-urea and 2.5% dithiothreitol (Bio-Rad Laboratories) (Wilson, Hall, Stone & Rubin, 1977). After homogenization at 25 °C, 60 mg dry ultra-pure urea (Schwarz-Mann, Orangeburg, New York U.S.A.) and 75 µl 10% Nonidet P-40 (Particle Data Laboratories Ltd, Elmhurst, Illinois, U.S.A.), 1-6% 5-7 ampholine and 0-4% 3-10 ampholine (LKB Instruments, Rockville, Maryland, U.S.A.) were added and homogenization continued until the tissue was completely dissolved.

The homogenate was centrifuged at 160 000 g for 1 h at 25 °C in a Beckman Airfuge. The supernatant fraction (50 µl) was applied to isoelectric gels as described by O’Farrell (1975) for the first dimension. The second dimension electrophoresis was carried out on 14% polyacrylamide slabs, 0.75 mm in thickness, and stained for 1 h in 2-5% Coomassie Blue (R-250, Bio-Rad Laboratories) in 10% acetic acid : 40% methanol (v/v) and destained in 10% acetic acid : 40% methanol, (v/v), two changes being made in 15 h.

Protein determination

Sections of the two dimensional gels containing stained protein were excised and placed in 0.6 ml dimethylsulphoxide. The gel slice was shaken for 15 h which quantitatively removed the dye from the gel slice (determined in control samples by absorption densitometry on the gel slice at 600 nm) and protein was quantified by absorption spectroscopy at 600 nm.

Incorporation of L-[³⁵S]methionine

Ventral prostate tissue was excised from rats taken from each group and minced in Dulbecco’s modified Eagle’s medium without methionine under sterile conditions. The minced tissue was then incubated for 4 h with 0.5 mCi L-[³⁵S]methionine (Amersham, Radiochemical Centre, Arlington Heights, Illinois, U.S.A.; 750 Ci/mmol) for 4 h at 37 °C in 95% air and 5% CO₂.

Stained protein spots were excised from the two-dimensional gels, the acrylamide was solubilized in a mixture of one part 4 M-NH₂OH : five parts NCS tissue solubilizer (New England Nuclear, Albany, New York, U.S.A.) : 50 parts Aquasol (by vol.; New England Nuclear) and counted after shaking at 25 °C for 15 h.

RESULTS

The ventral prostates of intact animals treated with spironolactone were substantially diminished in size as compared with those of untreated intact animals. The weight decrease...
in the ventral prostate from spironolactone-treated animals was approximately 60%. This
was similar to the percentage decrease in weight reported by Basinger & Gittes (1974).
The proteins of the rat ventral prostate which had isoelectric points (pI) in the pH range
from 4 to 7 are shown in Pl. 1, fig. 1. The protein labelled β corresponded closely to the pI and
molecular weight of the androgen-regulated low molecular weight protein described by
Parker et al. (1978) and by Heyns & DeMoor (1977) as a subunit of prostatic binding protein.
After castration, the quantity of β protein, as expected, dropped markedly over an interval of
6 days. Stained gels of prostatic proteins from a castrated rat and a castrated rat given
spironolactone are shown in Pl. 2, figs 3 and 4. The protein spot denoted by β appeared to be
smaller by visual inspection. Spectrophotometric determination of the quantity of
Coomassie Blue dye bound by the β protein in control and test animals is shown in Table 1.

Table 1. Quantification of stained protein spots excised from two-dimensional gels
(The β spot focused at isoelectric point (pI) 5.4 and mol. wt 13,000 and the A spot at pI 5.5
and mol. wt 45,000. Proteins were extracted from total tissue of rat ventral prostate.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>β</th>
<th>A</th>
<th>β : A</th>
<th>Average β : A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>1.44</td>
<td>0.33</td>
<td>4.36</td>
<td>4.39</td>
</tr>
<tr>
<td>Untreated</td>
<td>1.38</td>
<td>0.31</td>
<td>4.42</td>
<td></td>
</tr>
<tr>
<td>Spironolactone (25 mg)</td>
<td>1.5</td>
<td>0.38</td>
<td>3.93</td>
<td></td>
</tr>
<tr>
<td>Spironolactone (25 mg)</td>
<td>1.3</td>
<td>0.28</td>
<td>4.46</td>
<td>4.29</td>
</tr>
<tr>
<td>Castrated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spironolactone (25 mg) + testosterone propionate (1.25 mg)</td>
<td>0.49</td>
<td>0.35</td>
<td>1.38</td>
<td>1.34</td>
</tr>
<tr>
<td>Spironolactone (25 mg) + testosterone propionate (1.25 mg)</td>
<td>0.51</td>
<td>0.36</td>
<td>1.30</td>
<td></td>
</tr>
<tr>
<td>Spironolactone (25 mg)</td>
<td>0.30</td>
<td>0.28</td>
<td>1.07</td>
<td>1.11</td>
</tr>
<tr>
<td>Spironolactone (25 mg)</td>
<td>0.41</td>
<td>0.35</td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td>Testosterone propionate (1.25 mg)</td>
<td>1.7</td>
<td>0.41</td>
<td>4.15</td>
<td>4.15</td>
</tr>
</tbody>
</table>

The data for each sample were obtained from an independent experiment and the average ratio of β : A for each
group of animals is listed. Measurements were made on a Gilford Model 250 Spectrophotometer. Only one gel was
run of the stimulated castrated rats for quantitation. Numerous other gels were run but they all appeared the same.

In all cases the quantity of dye bound to the β protein was also compared with the dye bound in a protein which was not markedly altered by androgens. The protein chosen was the spot
labelled A which migrated with the characteristic pI and molecular weight of actin (Garrels & Gibson, 1976). When the dye bound in A was averaged over four gels obtained from a
sample of normal prostate, the average absorbance at 600 nm was found to be 0.3. The
average absorbance of dye bound in A from samples from castrated animals was 0.31, the
same as the value of average absorbance for A from castrated rats treated only with
spironolactone (Table 1). The average absorbance of A from intact rats treated with
spironolactone and castrated rats treated with spironolactone and testosterone propionate
was 0.33 and 0.35 respectively (Table 1). The difference from the average of 0.32 for the
normal samples was less than the 15% experimental error determined from the four normal
samples. The loading of the sample for the castrated animal treated with testosterone was
about 25% larger than for the other samples given in Table 1 and consequently the
absorbance of dye eluted from the A and B spots was about 25% greater than the values for
the other samples listed in Table 1. Therefore, the quantity of dye bound in the A spot
appeared to be independent of the treatment given. Thus the comparisons of the quantity of
dye bound by the β protein with a protein such as A, serving as an internal control, is a valid
method of comparing the dependence of the β protein on treatment. Alternatively, the
absolute amounts of dye bound in the β protein spot could be compared provided that the
same amount of protein was applied to the gels. Both methods of analysis gave the same conclusion.

Treatment with spironolactone of normal control intact male rats did not change the amount of β protein accumulated at the end of a treatment period of 6 days. As seen in Pl. 1, figs 1 and 2 (control and control plus spironolactone), the β protein appeared comparable on both photographs. The data is quantified in Table 1. After castration for 6 days (Pl. 2, fig. 3) and after castration with spironolactone treatment (Pl. 2, fig. 4) the β protein had regressed relative to A. The absolute amount had decreased by almost 25%. On the other hand, giving spironolactone and testosterone propionate simultaneously to a rat castrated 6 days previously blocked the restoration of the β : A ratio. The absolute quantity of β on the gel was slightly above the value as seen in the castrated rats (Pl. 3, fig. 5). That the β protein was androgen-dependent is demonstrated in Pl. 3, fig. 6 in which testosterone treatment begun 6 days after castration had restored the β : A ratio and the absolute quantity of β protein.

The group of spots 1–4 (Pl. 1, figs 1, 2 and Pl. 3, figs 5, 6) were acidic, high molecular weight (pI < 5.5 < 50 000) proteins whose synthesis was decreased after castration. In Pl. 3, fig. 6 the above numbered spots were also restored in castrated rats given stimulation with testosterone propionate for 6 days. We refer to this group of proteins as the high molecular weight, androgen-dependent (HMAD) proteins. These proteins also reappeared on the stained gel from castrated rats given spironolactone and testosterone propionate; the β protein levels, however, remained close to the level found in castrated animals (Table 1). The HMAD proteins did not appear by visual inspection of Pl. 1, fig. 2 to be drastically influenced by spironolactone treatment. These conclusions were in agreement with the data obtained by labelling the tissue with L-[35S]methionine.

Table 2 shows the quantification of incorporation of label after incubation for 4 h with radioactive methionine in the spots designated 1, 2, 3, 4, β and A. The rates of synthesis of the β and A proteins of the HMAD group were similar for the control animals, control rats treated with spironolactone and castrated rats treated with testosterone. The 10% increase in incorporation of label into the β protein in spironolactone-treated intact compared with intact animals was not considered to be significant since the amount of label uptake varied over a 15% range from gel to gel with the same sample. The prostates from castrated and spironolactone-treated castrated animals showed large declines in the rates of synthesis of the HMAD and β proteins but normal levels of incorporation in the A protein. On the other hand, the prostate from castrated rats treated with both spironolactone and testosterone incorporated near normal levels of label into the HMAD group but not into the β protein.

Table 2. Quantification of uptake of L-[35S]methionine (counts/min) into protein spots excised from two-dimensional gels

<table>
<thead>
<tr>
<th>Protein spot</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>β</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intact</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>550</td>
<td>490</td>
<td>360</td>
<td>650</td>
<td>5210</td>
<td>1075</td>
</tr>
<tr>
<td>Spironolactone (25 mg)</td>
<td>485</td>
<td>380</td>
<td>375</td>
<td>710</td>
<td>5820</td>
<td>1125</td>
</tr>
<tr>
<td><strong>Castrated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>47</td>
<td>31</td>
<td>28</td>
<td>42</td>
<td>270</td>
<td>985</td>
</tr>
<tr>
<td>Spironolactone (25 mg)</td>
<td>36</td>
<td>27</td>
<td>25</td>
<td>27</td>
<td>215</td>
<td>990</td>
</tr>
<tr>
<td>Spironolactone (25 mg) + testosterone propionate (1-25 mg)</td>
<td>210</td>
<td>195</td>
<td>175</td>
<td>290</td>
<td>312</td>
<td>1215</td>
</tr>
<tr>
<td>Testosterone propionate (1-25 mg)</td>
<td>610</td>
<td>425</td>
<td>410</td>
<td>775</td>
<td>5750</td>
<td>1170</td>
</tr>
</tbody>
</table>
Effect of spironolactone on prostatic proteins

The stained gel in Pl. 3, fig. 5 also supports this conclusion. That is, the HMAD proteins had reappeared and accumulated, but the quantity of β was still comparable with the level seen in castrated animals (Table 1).

The protein spots labelled C and Z (the protein spot C focuses at pl 5.6 and molecular weight of 40 000 and Z focuses at pl 6.1 and molecular weight of 80 000) appeared to be enhanced in prostates from castrated, spironolactone-treated castrated and spironolactone- and testosterone-treated castrated rats (Pl. 2, figs 3, 4 and Pl. 3, fig. 5) relative to the analogous protein spots in Pl. 1, figs 1, 2 and Pl. 3, fig. 5. It was apparent from Pl. 3, fig. 5 that spironolactone treatment blocked the ability of testosterone to reduce the synthesis of Z and C to normal levels.

DISCUSSION

The anti-androgenic activity of spironolactone has been studied extensively (Loriaux, Menard, Taylor, Pita & Santen, 1976) and it has been shown that spironolactone interferes with the binding of androgen to the androgen receptor of the rat ventral prostate (Corvol et al. 1975; Pita et al. 1975). Although the level of spironolactone given to intact animals was sufficient to reduce ventral prostate mass by at least 60% neither a reduction in the rate of synthesis nor the accumulation of the HMAD and β proteins were detectable (Pl. 1, figs 1 and 2, Tables 1 and 2). Evidence that spironolactone was capable of exerting an anti-androgenic effect was deduced from the experiment in which spironolactone and testosterone were given simultaneously to rats castrated 6 days previously. It was observed that the full restoration of β protein was blocked but β protein was fully restored to the intact level of synthesis and accumulation when no treatment with spironolactone was given.

There are a number of possible explanations for the inability of spironolactone to affect the androgen-dependent proteins as markers of androgen action. The level of exogenous spironolactone may not have been sufficient to inhibit the biological effectiveness of endogenous testosterone even though the dose was sufficient to reduce the mass of prostate and seminal vesicle by 60% over a regimen lasting for 6 days. The dose of spironolactone used in this study was five to ten times in excess of doses used to prove anti-androgenic or anti-mineralocorticoid activity in rats. At higher doses of spironolactone the reduction of testosterone formation in the testis would complicate interpretation of any changes in the protein patterns (Stripp, Menard & Zampaglione, 1973).

An alternative explanation for the lack of effect of spironolactone on the androgen-dependent proteins studied in this investigation is based on a model proposed by Roy (1976). As applied to rat ventral prostate the hypothesis that both a primary (constitutive) and a secondary (inducible) receptor exist could explain the inability of spironolactone to block synthesis of androgen-sensitive proteins in the intact animal. The growth and differentiated characteristics of the ventral prostate could depend on the primary receptor which is regulated by a developmental programme while secretory activity may be fine-tuned by the secondary receptor. If the primary receptor is sensitive to spironolactone and the secondary receptor insensitive to the drug, the secondary receptor would be capable of maintaining androgenic induction in the treated intact animal. In the castrated animal the spironolactone sensitivity would be conferred by the sole presence of active primary receptor. Parker et al. (1978) have observed that the anti-androgen, cyproterone acetate, almost completely inhibits the stimulatory effect of testosterone on the low molecular weight androgen-dependent proteins in the ventral prostate of castrated rats. The partial induction of the HMAD proteins in the castrated animal treated with testosterone and spironolactone would seem to imply that their synthesis is more sensitive than that of β to the amount of androgen that is not displaced from the primary receptor by spironolactone.

An alternative explanation for the differential induction is that the receptor/acceptor system for the β gene differs from the receptor/acceptor apparatus for the HMAD genes and
that the β gene is more sensitive to spironolactone than the HMAD-gene receptor. The results provided here are insufficient to decide among the various models of spironolactone interaction with androgen receptors that have been discussed. To explicate the mechanism of spironolactone anti-androgenicity, more experimentation utilizing specific androgen-dependent markers is required.

A summary of conclusions based on the data reported here is as follows: (1) spironolactone treatment in a dose sufficient to cause substantial weight loss of the ventral prostate does not impair the ability of the organ to synthesize and accumulate the androgen-dependent proteins, HMAD and β; (2) spironolactone given to a castrated animal which is simultaneously receiving a testosterone dose sufficient to restore the HMAD and β proteins is capable of blocking the restoration of β protein to normal levels but does not appear to block the enhancement of accumulation of the HMAD proteins; (3) spironolactone appears to block the decrease of C and Z proteins normally seen after 6 days of testosterone stimulation given to castrated animals.

The authors would like to thank Jane Tucker and David Wagoner for their expert technical assistance.

REFERENCES


DESCRIPTION OF PLATES

(Gels were stained in 2.5% Coomassie Blue in 10% acetic : 40% methanol (v/v) for 1 h and destained in 10% acetic acid : 40% methanol (v/v), two changes being made in 15 h. The plates are reduced by 25% of the size of the stained gels.) The plates show stained two-dimensional gels. In every case 250 µg protein was layered on the first dimension isoelectric gel with the exception of the gel in Fig. 6 where 310 µg was used. Spot number 1 focused at isoelectric point (pI) 5.0 and molecular weight 70 000 (pI 5.0/70 000), spot 2 at pI 5.2/70 000, spot 3 at pI 5.3/80 000, spot 4 at pI 5.3,68 000, spot A at pI 5.5/45 000, spot B at pI 5.4/13 000, spot C at pI 5.8/40 000, spot Z at pI 6.1/80 000 and the right hand margin of the gels was at pI 7.0. In each case the proteins were extracted from total tissue of rat ventral prostate after various treatments as described in Materials and Methods.

PLATE 1

Fig. 1. Untreated intact rat.
Fig. 2. Intact rat treated with 25 mg spironolactone each day for 6 days.

PLATE 2

Fig. 3. Rat castrated for 6 days before autopsy.
Fig. 4. Rat castrated for 6 days, then given 25 mg spironolactone each day for 6 more days before autopsy.

PLATE 3

Fig. 5. Rat castrated for 6 days, then given 1.25 mg testosterone propionate and 25 mg spironolactone each day for 6 more days before autopsy.
Fig. 6. Rat castrated for 6 days, then given 1.25 mg testosterone propionate each day for 6 more days before autopsy.

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