Effects of Antialdosteronics on Plasmatic Hormonal Levels and on Hepatic Sexual Steroid Receptors in Rat

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Spironolactone and potassium canrenoate are diuretics widely used for management of cirrhotic ascites. The administration of spironolactone frequently leads to feminization, which has been noted less frequently with the use of potassium canrenoate, a salt of the active metabolite of spironolactone. The use of these two drugs has been associated with decreases in serum testosterone levels and in case of spironolactone with a reduction in androgen receptor (AR) activity (8,10,19,20,23,25-28).

Considering the critical role of the liver in sex hormone metabolism the study about the effects of these drugs on liver sex hormone receptor state, could add new informations for understanding the mechanism of feminization by these two drugs. We report here the effect of spironolactone and potassium canrenoate treatment on the sex hormone receptor and androgen responsive protein status of the liver and the effects of these treatments on serum estrogen and testosteron levels.

MATERIALS AND METHODS

Animals

Three groups of adult male Sprague Dawley rats (240 gr b.w.) were studied. Animals in the control group (group 1) were

injected intraperitoneally with 0.5 ml of a solution of physiologic salin with TWEEN 80 (0.1% v/v). The rats in the other groups received 5 mg of spironolactone (group 2) or 5 mg of potassium canrenoate (group 3) dissolved in the same vehicle. All animals were injected daily for 21 days and killed by decapitation after that time.

Materials

Estradiol (E2) and Dihydrotestosterone were purchased from Steraloids, Wilton, NH. Spironolactone and Potassium Canrenoate were from SPA, Milan, Italy. Diethylstilbestrol (DEB), Leupeptin, Benzamidine, Bovine serum Albumin, Sodium Molybdate, Protamine Sulfate and other buffer component were purchased from Sigma Chemical Co., St. Louis, MO. Norit A and Dextran C were obtained from Fisher Scientific Co., Pittsburgh, PA. Radioactive (2,4,6,7,16,17)- 3H -E2 (131 Ci/mmol), 17 -methyl- 3H - methyltrienolone (3H -R1881) (79 Ci/mmol) and nonradioactive R1881 were obtained from New England Nuclear, Boston, MA. Instagel scintillation fluid was purchased from Packard Instrument Co., Downers Grove, IL. The radiolabeled steroids used in these studies were assayed periodically for purity by thin layer chromatography on silica gel G in ethyl acetate/hexane/ethanol (85:10:5), and were used only if purity was > 95%.

Assay of nuclear and cytosolic Sex-Steroid receptors

Nuclear and cytosolic Sex-Steroid receptors were evaluated by the method previously described by us (11,14,16-18).

Assay of androgen-responsive male estrogen binding protein

Assay for the determination of cytosolic content of a male specific estrogen binding protein (MEB) has been described previously (12,34). Briefly, cytosol is preincubated for 1 h at 0 °C with 500 nM DES to block the estrogen receptor. The cytosol is then incubated with 5 nM 3H-E2 in the absence and presence of 5 M unlabeled E2 for 2 h a 0 °C. Bound and free steroids are separated by centrifuge-assisted BioGel P-6 chromatography (34). This assay is quantitative for MEB and is linear over a broad range of protein concentration.

Other methods

Protein concentrations: method of Bradford (5).

DNA concentrations of homogenates and nuclear preparations: method of Burton (6).

Cytosolic receptor content for each animal was calculated on the basis of 3H -steroid bound/mg cytosol protein, and then corrected for yeld of cytosolic protein for gram of liver. Nuclear receptor content was calculated on the basis of 3H -steroid bound/mg DNA recovered in the nuclear preparation, and then corrected for the DNA content of the original homogenate. For each animal, the total liver content of receptor represents the sum of the cytosolic and nuclear content based on 1 gram of liver. Cytosolic contamination of nuclei was assayed by determining the activity of alcohol dehydrogenase (37) in the homogenate, cytosol and the washed nuclei preparation and was found to be less than 0.5% of that found in the nuclear preparation. The average recovery of DNA in the nuclear preparation was 72.8% of that in the homogenate.

Serum testosterone, estradiol and other hormones were determined by specific radioimmunoassays as described previously (38).

Equilibrium dissociation constants (Kd's) and the concentrations of binding sites were calculated by the method of Scatchard (35).

Statistical analyses were performed using the Student's t-test program.

Radioactivity content of samples was determined using a Packard TriCarb 5430 Liquid Scintillation System.

RESULTS

In spironolactone treated animals there is a significant decrease in testosterone levels; all other gonadal and pituitary hormone levels in this group are comparable to those of the control animals. In potassium canrenoate treated rats no change was observed in either testosterone or estradiol levels, although significant reductions in progesterone and DHEAS was found.

Cytosolic AR levels (cAR) are significantly higher in K canrenoate treated rats as compared with those observed in either control animals or spironolactone treated ones. There were no variations in the affinity of the cAR for its ligand in any groups studied. The Kd values for cAR were 0.26 ± 0.06 nM, 0.25 ± 0.08 nM and 0.31 ± 0.04 nM for group I, II and III respectively (Tab I).

The activity of nuclear androgen receptor (nAR), however, presents a markedly different picture, and is significantly decreased in the animals treated with either spironolactone or potassium canrenoate.

As a result of these variations, the total AR content of the liver changed significantly only in those animals treated with spironolactone (50% decrease from normal levels). Interestingly, the activity of total androgen receptors in the spironolactone treated animals shows a decrease proportional to that of plasma testosterone.

Tab I: Variation in specific 3H -R1881 in rat liver

	3H -R1881 specific binding (fmol/g liver)			
	TOTAL	NUCLEUS	CYTOSOL	PLASMA TESTOSTERONE (NG/ML)
CONTROL	364±62	202±46	162±9.9	1.77±0.23
SPIRO	170±38 (P<0.05)	26±7 (P<0.05)	144±24	0.78±0.20 (P<0.05)
K-CAN	347±61	21±8 (P<0.05)	326±42 (P<0.05)	1.53±0.32

As total hepatic androgen receptor activity and plasma testosterone values were reduced in the spironolactone-treated animals, the activity of a hepatic androgen responsive protein, a male specific estrogen binder (MEB), was also assessed (Tab II).

Tab II: Activity of an androgen responsive protein in rat liver

	MEB (pmol/mg protein)
CONTROL	2.41±1
SPIRO	0
K-CANREO	6.3±1.5 (p<0.05)

The activity of this protein is undetectable in the livers of the spironolactone treated animals; this loss of MEB activity is comparable to that seen in rats castrated at least 15 days prior

to sacrifice (14,36). However MEB level in k-carenoate treated rats is actually higher than that observed in the control rats.

To determine whether either of these drugs might interfer by competing for androgen binding to its receptor, we tested whether they were capable of competing for androgen receptor binding in an in vitro assay.

Spironolactone, at a 100 and 1000-fold excess, is partially effective as a competitor for 3H - R1881 binding in rat liver cytosol. However, the strong androgen R1881 and DHT are much more effective competitors than is spironolactone. In contrast, k-canrenoate is incapable of competing for 3H - R1881 binding at any concentration tested.

The activity of cytosolic estrogen receptors (cER) (Tab III) were virtually unchanged among the 3 groups, nor did the affinity of cER for E2 vary among the groups of animals (Kd values for cER were 0.26±0.07 nM, 0.45±0.12 nM and 0.36±0.08 nM for group I, II and III respectively). The activity of nuclear estrogen receptor (nER) was decreased significantly only in the spironolactone treated animals. However, the total hepatic estrogen receptor content does not vary in the 3 groups of animals studied. This latter finding is consistent with the similar plasma estradiol values in these three groups of animals.

Tab III: Variation in hepatic estrogen receptor activity in rat liver

	3H -E2 specific binding (pmol/g liver)			
	TOTAL	NUCLEUS	CYTOSOL	PLASMA ESTRADIOL (PG/ML)
CONTROL	623±115	178±33	445±61	9.52±1.73
SPIRO	549±89	16±12 ($P<0.05$)	533±150	7.82±3.04
K-CAN	650±222	209±44	441±94	9.91±1.13

DISCUSSION

Sexual dimorphism of liver function has been observed in both animals and humans (reviewed in 11).

In male rats, androgen is the major steroid hormone deter-

minant of the masculine pattern of liver function. Androgen stimulates the expression of both androgen receptor and certain enzymes and proteins such as the male specific estrogen binder MEB (14,36). This masculine pattern can be feminized by certain changes in serum hormone levels occuring after castration (14,36), chronic alcohol ingestion (13), estrogen treatment (21), portal vein ligation (15) and during liver regeneration (16,18).

Our results present that administration of spironolactone, resulted in a significant decrease in plasma of testosterone levels and hepatic androgen receptors (AR), and the complete loss of activity of the hepatic androgen responsive protein (MEB), that is a cytosolic protein which has a moderate affinity for estradiol, high binding capacity, and specificity for steroid estrogen (12,34,36). Its function in the cytosol of male rats is unknown, but may be that of an intracellular estrogen scavenger (12,34). The complete disappearance of MEB in rats treated with spironolactone is significant, first, in that its loss represents interruption of hepatic androgen response, and second, that its absence might lead to an increased intracellular estrogen concentration.

Plasma estradiol, estriol content, and the total ER activity in liver don't result to be influenced by spironolactone. It is possible that our treatment period was too short to produce a feminization of (i.e. an increase in) these activities. It is more likely, however, that in liver disease patients, feminization occurs because spironolactone treatment interferes in maintaining the androgenic influence necessary to sustain a masculine pattern of liver function. Such interference, coupled with an already compromised ability to excrete estrogens, may permit full feminization of liver and other peripheral tissues, such as the breast.

Patients with liver disease may also fail to clear efficiently other hormones such as prolactin which may enhance devolopment of gynecomastia. However, our experimental animals display no liver diseases; thus, treatment of normal male animals with spironolactone may not result in the pronounced feminization that has been noted in some liver disease patients.

Our result in the potassium canrenoate treated animals confirmed the clinical data indicating that this drug has less antiandrogenic effect than spironolactone. It has been demonstrated, in castrated animal, that potassium canrenoate does not inhibit cytochrome p-450 as spironolactone does, and produces significantly less androgenic antagonism. No decrease of plasma

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testosterone, total hepatic androgen receptor or MEB, and no inhibition of androgen binding to its receptor were found to occur in the liver of the potassium canrenoate treated animals.

In conclusion, the data reported in this paper demonstrate that spironolactone treatment of male rats decreased plasma testosterone, and exhibited a powerful anti-androgenic effect (low levels of hepatic androgen receptor and disappearance of MEB). We also demonstrated that potassium canrenoate did not show any of the anti-androgenic actions of spironolactone in the liver, suggesting that the clinical improvement reported in patients treated with potassium canrenoate as compared to spironolactone is the result of this biochemical difference. Also, our results indicate that in animals with no underlying liver disease, feminization, defined as an increase in plasma estrogen levels and hepatic ER content, does not occur.

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