Journal of Steroid Biochemistry, Vol. 9, pp. 325–330

0022-4731/78/0501-0325$02.00/0

THE USE OF METHYLTRIENOLONE IN THE MEASUREMENT OF THE FREE AND BOUND CYTOPLASMIC RECEPTORS FOR DIHYDROTESTOSTERONE IN BENIGN HYPERTROPHIED HUMAN PROSTATE

R. GHANADIAN, G. AUF, P. J. CHALONER and G. D. CHISHOLM
Prostate Research Laboratory, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London, W12 0HS, England

(*Received* 8 *July* 1977)

SUMMARY

The bound and free cytoplasmic receptors for dihydrotestosterone (DHT) were measured in 15 benign hypertrophied human prostates using a specific radioligand [^{3}H]-methyltrienolone ([^{3}H]-R1881) in an exchange assay. The specificity of R1881 to androgen receptors was studied using several steroid competitors. The results showed that the mean ±S.E.M. for the total binding sites was 80.3 ± 10.1 fmol/mg protein. Unoccupied binding sites (free) were measured either by preloading with DHT or by direct measurement. The values (mean ±S.E.M.) were 5.2 ± 0.75 fmol/mg protein and 8.5 ± 0.95 fmol/mg protein respectively. The bound receptor which is the difference between total and unoccupied binding sites gave a value of 73.6 ± 9.5 fmol/mg protein. The results suggest that most of the receptor population is bound to endogenous DHT.

INTRODUCTION

The presence of a specific androgen receptor which binds dihydrotestosterone (DHT) has been found by several investigators [1–3]. In addition to this specific receptor, the existence of sex hormone binding globulin (SHBG) which binds DHT has also been reported in the human prostate [4–7]. In the normal prostate and particularly the hypertrophied prostate, the concentration of DHT is high [8]. It is quite possible that most of the androgen receptors in the tissue are bound to endogenous DHT and therefore only a small proportion of the receptors are available for measurement. This together with the contamination of tissue with SHBG could explain the failure to detect androgen receptors [5, 9].

Recently, a new synthetic androgen methyltrienolone (R1881) was reported [10] to have a high affinity for DHT receptors in the prostate without binding to SHBG. This compound was employed in an exchange assay [11] which was originally reported by Katzenellenbogen *et al.*[12]. The two major criteria for using exchange assay with R1881 (a) binding to the receptor and not SHBG and (b) binding to the total receptor population and not just to the available sites, prompted us to use this technique for the assay of androgen receptors in human prostate. The disadvantage of this method is reported to be the binding of R1881 to progesterone binding receptors [13] although the existence of such a receptor in the prostate has not been established. In the present investigation we report on further specificity studies on R1881 together with a modification of the procedure by Bonne and Raynaud[11] in the study of prostatic tissue from 15 patients with benign prostatic hypertrophy.

MATERIALS AND METHODS

Steroids. The following steroids were used: 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) (DHT). Progesterone (4-pregnene 3,20 dione) and cortisol (11β,17,21 trihydroxy-4-pregnene, 3,20 dione) were all purchased from Sigma Chemicals; a solution of 10 nmol/ml of each was prepared in absolute ethanol. [1,2,6,7-^{3}H]-Progesterone (S.A. 87 Ci/mmol) was purchased from Radiochemical Centre, Amersham) stock solution of 50 pmol/ml of Methanol was used. Methyltrienolone (17β-hydroxy-17α methyl 4,9,11-estratrien-3-one) (R1881) (Roussel Uclaf). Stock solution of 50 pmol/ml of [^{3}H]-R1881 (S.A. 58.2 Ci/mmol) was prepared in a solution of toluene–methanol (9:1, v/v). Radioinert R1881 (10 nmol/ml) was prepared in ethanol. All solutions were stored at 4°C.

Buffers. Buffer A was Tris (20 nM) containing EDTA (1.5 nM) and mercaptoethanol (2 mM). Buffer B was tris (10 mM) containing EDTA (1.5 mM) and sucrose (250 mM). Both buffers were adjusted to pH 7.4 using HCl and stored at 4°C. Two solutions (1 and 2) of dextran coated charcoal were prepared in buffer B. Solution 1 consisted of 5% water washed Norit GSX charcoal (Hopkins and Williams) and 0.5% dextran T-70 (Pharmacia). Solution 2 consisted of 1.25% water washed Norit GSX charcoal and 0.625% dextran T-70. Both solutions were stored at 4°C.

Solvents. Ethanol, Methanol, Toluene and Triton X-100 were all of Analar grade (B.D.H.).

Column chromatography. Sepharose 6B (Pharmacia Fine Chemicals).

Scintillation fluid. Toluene–Triton X-100 (2:1) containing 0.4% 2,5-diphenyloxazole (Koch–Light Laboratories).

Tissue. Prostatic tissues were obtained from patients with benign prostatic hypertrophy (BPH) undergoing retropubic prostatectomy. Tissue was immediately transferred to a container and placed in crushed ice. All experiments were performed within 1 h of operation.

Preparation of cytosol. The following steps were carried out at 0–4 C. A piece of prostatic tissue was taken from the centre of an adenoma, sliced, minced, blotted with filter paper and weighed. The minced tissue was placed in a pre-cooled B24 tube and homogenised in buffer B (1:3, w/v). Homogenisation was carried out using an Ultra Turrax type TP18-10 (Junks and Kunkle AG) using 5 strokes each 10 s with 30 s cooling intervals. The homogenate was then centrifuged at 105,000 *g* for 1 h at 4°C. The resulting supernatant (cytosol fraction) was cleanly pipetted. Special care was taken to avoid contamination from the lipid layer on the top.

Removal of free endogenous hormone. Free endogenous hormone was removed from the cytosol fraction by incubating the cytosol with charcoal buffer (solution 1) in a ratio 10:1, v/v at 0–4°C for 10 min. The steroid bound to charcoal was precipitated by centrifugation at 3700 *g* at 4°C for 20 mins. Aliquots of 100 μl of the cytosol were then used for protein estimations [14]. The remaining cytosol was used to assay both unoccupied and total binding sites.

Specificity of R1881 binding with cytoplasmic androgen receptors. Aliquots of 105,000 *g* preparation of the cytosol were incubated with 5 nM of either [^{3}H]-R1881 or [^{3}H]-progesterone in the presence and absence of various concentrations of progesterone, DHT and R1881 at 0°C for 16 h. Unbound steroids were removed by dextran coated charcoal treatment and the binding in 100 μl aliquots was measured (see procedure for exchange assay). In further experiments cortisol (100 nM) was added in order to block the binding of progesterone to corticosteroid binding globulins (CBG).

Sepharose 6B gel filtration. Aliquots of the 105,000 *g* cytosol (0.3 ml) were incubated with 50 nM of either [^{3}H]-R1881 or [^{3}H]-progesterone at 15°C for 16 h. Unbound steroids were removed by incubating the cytosol with 150 μl of dextran charcoal solution 2 at 0–4 C for 10 min. Following centrifugation at 1000 *g* for 15 min, the supernatant was made up to 1.8 ml with buffer B. This was then eluted on precalibrated column (55 cm × 2.5 cm) of Sepharose 6B (15) at a flow rate of 40 ml/h with buffer A in the presence or absence of 10% v/v glycerol. Fractions (4 ml) were collected in an LKB 7000 fraction collector. Aliquots 2 ml of each fraction were transferred to scintillation vials and scintillation fluid (15 ml) was added. The radioactivity was counted in a scintillation spectrometer and the results were expressed in counts/min (c.p.m.) per 4 ml of fraction.

Measurement of unoccupied binding sites. Unoccupied binding sites in the cytosol were measured either directly or after preloading the cytosol with 10 nM DHT by incubating at 0–4°C for 1 h. In either case the method was as follows:

For triplicate assay 6 test tubes containing 1 nM [^{3}H]-R1881 with and without 5000 nM radioinert R1881 were evaporated to dryness and subsequently transferred to crushed ice. Aliquots of 0.1 ml cytosol were added to each tube and incubated at 0–4°C for 1 h. The bound [^{3}H]-R1881 was separated from free R1881 by adding 50 μl dextran charcoal (solution 2) which was then vortexed and incubated at 0–4 C for 10 min. After centrifugation at 1000 *g* for 15 min, aliquots of 100 μl of the resulting supernatant were transferred to 10 ml of scintillation fluid. The radioactivity was counted in a scintillation spectrometer (SL40 Intertechnique).

Preparation for the measurement of total binding sites. In order to saturate unoccupied binding sites, cytosol stripped of free endogenous hormone was incubated with 10 nM DHT at 0–4 C for 1 h. The excess of DHT was then removed by adding charcoal buffer (solution 1) in a ratio of 1:10, v/v. Following centrifugation at 1000 *g* for 20 min, aliquots of 100 μl of the supernatant were removed for protein estimation [14].

Before embarking upon the measurement of the total binding sites by exchange assay, some parameters influencing this estimation were evaluated. It was found that maximum exchange occurred within 12–16 h of incubation at 15°C. It was also found that the saturation of total binding sites was achieved at a concentration range of 30–50 nM [^{3}H]-R1881. Both of these results are in accordance with the findings reported previously [11].

Measurement of the total binding sites by exchange assay. Portions (100 μl) of the saturated cytosol were incubated with [^{3}H]-R1881 (20–50 nM) in the presence or absence of 5000 nM radioinert R1881 at 15°C for 16 h. Separation of free from bound [^{3}H]-R1881 was achieved by adding 50 μl of dextran charcoal buffer (solution 2) at 0–4°C for 10 min. After centrifugation at 1000 *g* for 15 min aliquots of 100 μl of the supernatant were transferred to 10 ml scintillation fluid and the radioactivity counted in a scintillation spectrometer.

Calculation of the results. The results were calculated as suggested by Katzenellenbogen[12]. The difference in c.p.m. between cytosol labelled with [^{3}H]-R1881 and [^{3}H]-R1881 + R1881 was taken as a measure of the quantity of DHT receptor in the cytosol. It was therefore possible to express the final results in terms of fmol of specific receptor per mg of cytoplasmic protein. The difference between the total and unoccupied binding sites was considered as

bound receptor. For further detail see legend to Fig. 3.

RESULTS

1. *Specificity study*

When [^{3}H]-R1881 was incubated in the presence of steroid competitors, it was found that radioinert R1881 caused the greatest inhibition (75%) followed by progesterone (63%) and DHT (55%) (Fig. 1a). When similar concentrations of these steroids were in competition with [^{3}H]-progesterone in the absence of cortisol, the main competitor was progesterone (50%) followed by DHT (20%) whilst R1881 produced no inhibition (Fig. 1c). It was interesting to note that the binding of [^{3}H]-progesterone to the cytosol was approximately 50% higher than that of [^{3}H]-R1881.

In order to eliminate the interference of CBG binding to progesterone, the experiment was repeated in the presence of a high concentration of cortisol. No change was observed in the binding of [^{3}H]-R1881 (Fig. 1b) and the results were similar to those in Fig. 1a. However, in the case of [^{3}H]-progesterone the binding to the cytoplasmic fraction was considerably reduced to only 62% to that of [^{3}H]-R1881. In this case none of the radioinert steroids competed for the binding of [^{3}H]-progesterone (Fig. 1d).

2. *Chromatographic behavior of the labelled cytosol*

In order to study the binding characteristics of [^{3}H]-R1881 and [^{3}H]-progesterone to the cytosol of BPH, aliquots of the labelled cytosol were prepared under exchange conditions and subjected to Sepharose 6B column chromatography in the presence and absence of 10% (v/v) of glycerol. The results are shown in Fig. 2.

Both R1881 and progesterone were bound to a protein fraction at a molecular weight of 2.8×10^5 daltons which corresponds to the 8 *S* androgen receptor (unpublished data). No evidence was observed for the specific binding of R1881 to other protein components. Progesterone was bound less avidly than R1881, the percentage binding being 60% of that of R1881. The addition of glycerol produced identical results. In the presence of excess of unlabelled R1881 or progesterone the peak corresponding to 2.8×10^5 daltons was abolished.

3. *Total binding sites*

The concentration of the total binding sites in 15 patients with benign prostatic hypertrophy was 80.3 ± 10.1 fmol/mg protein (mean ± S.E.M.). The values ranged from 28.5 to 162.2 fmol/mg protein. A typical example of the calculation of results is shown in Fig. 3.

4. *Unoccupied binding sites*

In 7 prostatic samples, unoccupied binding sites were measured directly without preloading the cytosol with DHT. The mean ± S.E.M. was 8.5 ± 0.95 fmol/mg protein ranging from 4.5–11.7 fmol/mg protein.

In order to evaluate the effect of preloading the cytosol with DHT on unoccupied binding sites, cytosol fractions from 8 samples of prostatic tissue were incubated with 10 nM DHT at 0–4°C for 1 h. This was then followed by dextran coated charcoal treatment and binding measurement as described in Methods. The mean ± S.E.M. was 5.2 ± 0.75 fmol/mg protein ranging from 3.3–9.9 fmol/mg protein.

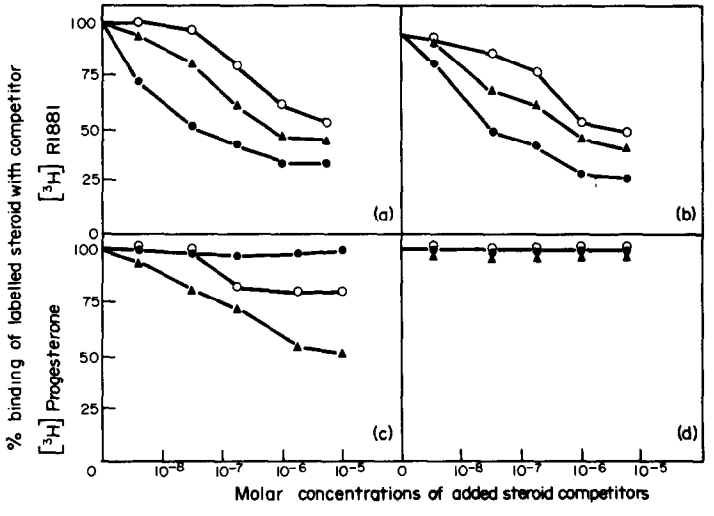

Fig. 1. Specificty of R1881 binding to androgen receptors in human BPH. Cytosol was incubated with either [^{3}H]-R1881 or [^{3}H]-progesterone in the presence of R1881 (●——●), progesterone (▲——▲) or DHT (○——○) at 0°C for 16 h. Binding was measured following dextran charcoal treatment. a. Cytosol was incubated with 5 nM [^{3}H]-R1881 in the presence of either R1881, progesterone or DHT. b. The same as a. but cortisol (10^{-7} M) was included. c. Cytosol was incubated with 5 nM [^{3}H]-progesterone in the presence of either R1881, progesterone or DHT. d. The same as c. but cortisol (10^{-7} M) was included.

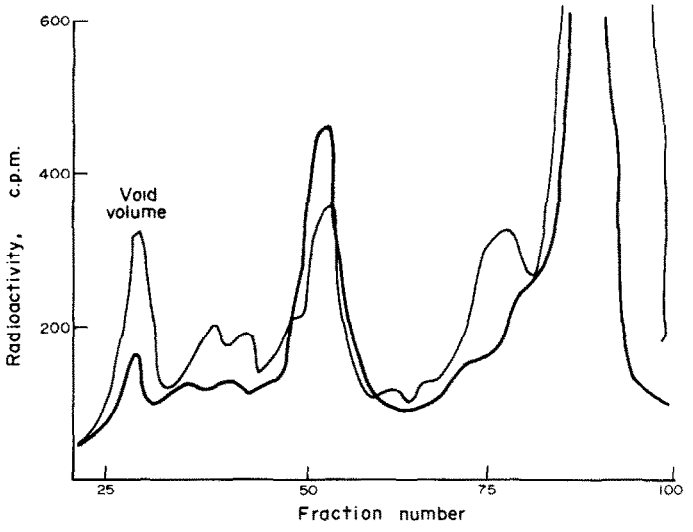

Fig. 2. Elution profile of BPH cytosol labelled either with [^{3}H]-R1881 (——) or [^{3}H]-progesterone (——) on Sepharose 6B column. Peak between fraction No 47–59 correspond to a protein with the molecular weight of 2.8×10^5 daltons. Peak between fractions 77–92 correspond to free and loosely bound steroid.

5. *Ratio of bound to free receptor*

The mean ± S.E.M. for bound receptor was 73.6 ± 9.5 fmol/mg (ranging from 20.5 to 152). The results are shown in Table 1. There was a significant difference in the value for bound and free binding sites whether the free sites were loaded with DHT or directly measured. The bound receptor sites were found to be from 2.6 to 17.8 times greater than the free sites.

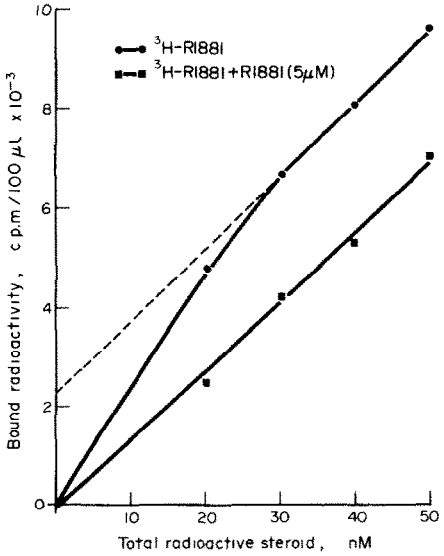

Fig. 3. Cytosol of BPH was saturated with DHT and then incubated at 15°C with different concentrations of [^{3}H]-R1881 in the presence or absence of 5000 nM radioinert R1881 for 16 h. The difference between total bound R1881 (●) (P) and unspecifically bound R1881 (■) (Po) is expressed as total specific bound receptor = (P − Po × dilution factor)/(Total added radioactivity × protein concentration).

DISCUSSION

The use of a radioligand which specifically binds to the cytoplasmic DHT receptor of the prostate and not to SHBG provided the basis for developing a direct measurement of the androgen receptors. In the present investigation, such a ligand was used in an exchange assay to measure the whole population of specific DHT receptors.

The suitability of methyltrienolone (R1881), a potent synthetic androgen for this purpose was originally reported by Bonne and Raynaud[10, 11]. Although these workers did not report data on the binding specificity of R1881 in BPH cytosol, they concluded [11] that R1881 may bind to progesterone receptors. The present studies on the specificity of R1881 for androgen receptors suggest that progesterone could compete with the binding of R1881 in BPH cytosol. However, when the cytosol was labelled with [^{3}H]-progesterone there was no effect of R1881 on this binding. The fact that R1881 did not compete with the binding of [^{3}H]-progesterone in the prostate suggests that either this binding is not to a specific progesterone receptor or, if there is a progesterone receptor, then R1881 did not bind to it. In view of the fact that progesterone binds to CBG, this would not allow "progesterone receptor" to exhibit its presence and therefore a high level of cortisol was introduced into the incubation medium. The results demonstrated that in the presence of cortisol the test steroids (R1881, DHT or progesterone) did not compete in the binding of [^{3}H]-progesterone within the cytosol. This suggests that the binding was mainly to some high capacity binding component. It is of note that Karsnia *et al.*[16] reported the binding of [^{3}H]-progesterone to a protein component in the cytosol of rat prostate. The molecular weight of this

Table 1. Concentration of free and bound receptor proteins for dihydrotestosterone (DHT) in the cytoplasmic fraction of benign hypertrophied human prostate. Unoccupied (free) receptors were measured with and without preloading with DHT

	Concentration of receptors (fmol/mg protein)			
Patient No.	Unoccupied (free) (With preloading)	Total	Bound	Bound:Free ratio
1	9.9	162.2	152.3	15.4
2	6.3	73.4	67.1	10.7
3	4.1	70.8	66.7	16.3
4	5.3	60.0	55.7	10.5
5	4.3	30.7	26.4	6.1
6	3.3	40.0	36.7	11.1
7	4.6	70.0	65.4	14.4
8	3.6	51.2	47.6	13.2
Mean ± S.E.M.	5.18 ± 0.75 (Without preloading)			
9	6.6	74.2	67.6	10.2
10	8.0	28.5	20.5	2.6
11	4.5	84.5	80.0	17.8
12	7.8	83.2	75.4	9.7
13	10.0	112.9	102.0	10.3
14	10.6	131.2	120.6	11.4
15	11.7	131.0	119.3	11.7
Mean ± S.E.M.	8.46 ± 0.94	80.3 ± 10.06	73.6 ± 9.5	11.4 ± 0.99

binding component was 50,000 daltons and it was stable at 60°C. These characteristic features do not comply with those of progesterone receptors identified in other tissues [17].

Further studies on Sepharose 6B column chromatography indicated that both progesterone and R1881 bind to a single specific binding component with a molecular weight of 2.8×10^5 daltons which is similar to that of androgen receptor in the prostate of the rat [18], human BPH (unpublished observation) and monkey prostate [19]. Our results demonstrate that while progesterone could compete with the binding of R1881 to the androgen receptor, under these experimental conditions no evidence was shown in favour of a progesterone receptor in BPH.

In a similar type of study Asselin *et al.*[13] reported that progesterone and R5020 were strong competitors for the binding of R1881 in the human prostate. They also showed that R1881 strongly binds to cytosol of the oestrogen treated human uterus. Based on their binding studies of R5020 to the cytosol of the human BPH, they concluded "the presence of progestin binding components or of an atypical androgen receptor in human BPH cytosol". Although we agree that progesterone competes with R1881 neither our results nor those of Asselin *et al.* provide evidence for the presence of a progesterone receptor.

In this study DHT was used in preference to R1881 for saturating the unoccupied binding sites prior to the exchange assay. This approach ensures an homogenous exchange with [^{3}H]-R1881 since all the bound receptors are already occupied by the endogenous DHT. Furthermore, it has been shown that the rate of dissociation of DHT from the receptor is faster than that of R1881 [11].

Free receptor sites were measured before and after preloading the cytosol with DHT. This step was initially performed to determine whether R1881 will exchange with the endogenous hormone for the occupied receptor at 0°C. Although the differences in the results for the two situations could be explained in terms of partial exchange at 0°C, it is equally feasible that the added DHT may have been bound to SHBG and/or undergone metabolism to diols. Therefore the possibility exists that not all the unoccupied binding sites were saturated. As this constitutes only a small difference, we suggest that this step could be omitted.

The results suggest that the free receptors consitute only a very small proportion of the total receptor sites. A similar conclusion was also reached by Rosen *et al.*[20]. Using a radioimmunoassay, they estimated the concentration of DHT in the 8 *S* region of the glycerol gradient of the cytoplasmic fraction of benign hypertrophied prostate and found that the ratio of free to bound receptor was approximately 1:9. However, they did not measure the free receptor directly. The concentration of total receptor in their experiment was estimated to be 2.6 pmol/g of wet tissue. If the results of our experiments are expressed in terms of wet weight of the tissue rather than protein content, the mean value for the total site is 2.4 pmol/g wet tissue.

The results clearly demonstrate that short term incubation of cytosol at 0–4°C would only label a very small proportion of the receptor population. This observation explains the failure of several workers to detect cytoplasmic androgen receptors [5, 9, 21].

Acknowledgements—The authors wish to thank the Cancer Research Campaign for financial support. Mr. P. J. Cha-

loner was supported by a Grant from St. Peter's Hospitals. We are indebted to Dr. J. P. Raynaud of Roussel Uclaf, France for the generous supplies of R1881. We also wish to thank Mr. C. B. Smith for performing Sepharose chromatography and Mrs. P. Dodi for her excellent secretarial assistance.

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