Binding to R 1881 (Methyltrienolone) of Proteins from Human Benign Prostatic Hypertrophy

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Abstract

Cytosol of human benign prostatic hypertrophy bound to R 1881 in a high affinity manner. Most of the protein which bound to R 1881 was recovered in the precipitate of a 0-30% saturation of ammonium sulfate, and was eluted in the void volume on a Sephadex G-200 column. The binding of cytosol to R 1881 was more inhibited by progestins than by dihydrotestosterone and estradiol-17β. The binder therefore seemed to be different from dihydrotestosterone-binding protein. The R 1881-binding component extracted from the nuclei by 0.4M KCl bound also to dihydrotestosterone in a high affinity manner. Cytosol prelabeled with R 1881 was bound to the nuclei in a nonsaturable process, and the extraction pattern of R 1881 by 0.4M KCl from the nuclei was almost identical to that in the case of dihydrotestosterone as the ligand. These results suggested that a part of the cytosolic protein which bound to R 1881 entered the nuclei where it bound to nuclear such components as dihydrotestosterone-binding protein.

It has been recognized that human prostatic tissue contains androphilic protein(s) which possesses similar physicochemical properties to the androgen receptor of rodent prostate (Karr and Sandberg, 1979). Two kinds of androphilic proteins in tissues of human benign prostatic hypertrophy were separated by Sephadex chromatography (Kodama et al., 1977). These two proteins showed similar binding characteristics to dihydrotestosterone (5α-androstane-17β-ol-3-one) but rates of inhibition by other steroids were different (Kodama et al., 1978).

R 1881 (methyltrienolone, 17β-hydroxy-17α-methyl-εstra-4, 9, 11-triene-3-one) has been used for estimation of the binding to androgen (Bonne and Raynaud, 1975). It was claimed that R 1881 binds to the androphilic protein, but does not bind to testosterone-binding globulin in blood plasma (Menon et al., 1977, Krieg et al., 1978). To further clarify components binding to R 1881, the present study was performed, and the binding was compared with that to dihydrotestosterone.

Materials and Methods

Materials

Tissues of human benign prostatic hypertrophy were obtained surgically from patients admitted to the Chiba University Hospital. Immediately after removal, tissues were cut into small pieces, placed on ice, and carried to the laboratory. Some tissues were kept at -80°C until processing. Removed tissues were examined histologically to confirm their pathological state.

Tissue preparation

Tissues were homogenized in 5 volumes of 0.05M Tris-HCl buffer (pH 7.4) containing 1mM EDTA and 1mM β-mercaptoethanol (TEM buffer) using a
Preparation of acetone-dried cytosol

Cytosol was delipidized and precipitated by adding with 9 volumes of chilled acetone according to the method described by Ichii (1975). The acetone-dried cytosol was suspended in TEM buffer, homogenized briefly, then spun, and the resultant supernatant was used for binding studies.

Fractionation by ammonium sulfate

Cytosol was fractionated by the addition of ammonium sulfate at 4°C. The resultant precipitate was dissolved in TEM buffer, and dialysed for 1 hr during vigorous stirring.

Sephadex G-200 chromatography

Cytosol was chromatographed on a column filled with Sephadex G-200 (1 x 90 cm, Pharmacia, Sweden). The column was eluted with TEM buffer.

In some experiments, cytosol was incubated with 2.5 nM of [3H]-R 1881 (6, 7-[3H]-R 1881, specific activity 55.5 Ci/mm, Roussel-Uclaf, France, donated by Dr. J. P. Raynaud, Centre de Recherches, Roussel-Uclaf, 17a-methyl-[3H]-R 1881, specific activity 87.0 Ci/mm, New England Nuclear, U.S.A.) in the presence or absence of other additive steroids at 4°C for 3 hr. After incubation, dextran-coated charcoal suspension (0.5% Norit A, Wako Pure Chemicals, Japan, and 0.05% dextran T 70, Pharmacia, Sweden) was added, centrifuged, and the resultant supernatant was applied to a Sephadex G-200 column. In parallel experiments, 5α-dihydrotestosterone (1, 2, 4, 5, 7(n)-[3H]) 5α-dihydrotestosterone, specific activity 130 Ci/mm, The Radiochemical Centre, Amersham, England) replaced R 1881, and cytosol was incubated at 4°C for 2 hr.

Preparation of nuclear extract

Purified nuclei were suspended in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.4 M KCl and stirred at 4°C for 30 min. After centrifugation, the precipitate was reextracted in the same manner. The combined supernatant was referred to as the nuclear extract.

Sucrose density gradient centrifugation

The precipitate obtained by 0-50% saturation of ammonium sulfate of cytosol (0-50% fraction) was incubated either with 10 nM of [3H]-R 1881 for 3 hr or with 10 nM of [3H]-dihydrotestosterone for 2 hr at 4°C. The dextran-coated charcoal was added after incubation and 0.3 ml of the supernatant was applied on the top of a linear 5-20% sucrose gradient in TEM buffer (5 ml). Centrifugation was performed with a RPS66T rotor in an Hitachi Ultracentrifuge (80P, Hitachi Co., Japan) at 40,000 rpm for 16 hr. The gradient was fractionated in each 0.2 ml fraction using a density gradient fractionator (Isco, Model 640, U.S.A.). Radioactivity in the fractions was counted after the addition of toluene containing 0.4% PPO and 0.025% POPOP. Bovine serum albumin and human Immunoglobulin G (IgG) were used as the reference for calculation of the sedimentation coefficient.

Saturation analysis of the binding

Samples to be analyzed were incubated in 0.5 ml of TEM buffer containing various concentrations (0.25-5 nM) of [3H]-R 1881 at 4°C for 3 hr. Incubation of the nuclear extract was performed at 4°C for 18 hr. [3H]-Steroid was dissolved in 10 μl of ethanol and added to the incubation medium. When the effect of the addition of various steroids was examined, another 5 μl of the ethanol solution of the additives was added to the incubation tube, except that the radioinert R 1881 and R 5020 (17, 21-dimethyl-19-nor-4, 9-pregnadiene-3, 20-dione) were added in 10 μl of ethanol solution. Separation of free from bound steroid was performed with 0.05% dextran-coated 0.5% charcoal. An aliquot of the supernatant treated with dextran-coated charcoal was counted in 15 ml of toluene containing 0.4% PPO and 0.025% POPOP. The binding capacity and affinity were analyzed by the method of Scatchard (1949).

Binding of cytosol prelabeled with [3H]-R 1881 to nuclei

To prepare [3H]-R 1881-labeled cytosol, cytosol was incubated in the presence of 2.5 nM of [3H]-R 1881 at 4°C for 3 hr. After removal of the unbound steroid with dextran-coated charcoal, the labeled cytosol was incubated with purified nuclei obtained from human benign prostatic hypertrophy and suspended in TSMK buffer in a final volume of 2 ml at 25°C for 3 hr. To avoid the influence of a translocation inhibitor in cytosol (Simons et al., 1976) the concentration of cytosol in the incubation mixture was kept constant by adjusting the incubation medium with heat-inactivated (37°C for 30 min) steroid free cytosol. After incubation, the mixture was centrifuged at 1,000 × g for 10 min and the resultant precipitate was washed three times with TSMK buffer. The precipitate was solubilized in Soluene-350 (Packard, U.S.A.) and the radioactivity was counted after the addition of Instagel (Packard, U.S.A.).

In some experiments, nuclei incubated with [3H]-R...
Fig. 1. Binding to R 1881 of cytosol and of fractions separated by ammonium sulfate. Tissue preparations were incubated with various concentrations (0.25-5 nM) of [3H]-R 1881 at 4°C for 3 hr (6.0 mg as protein of cytosol/tube: open circle, 3.3 mg as protein of 0-30% fraction/tube: closed circle, 3.6 mg as protein of 30-50% fraction/tube: open triangle). Binding parameters to R 1881 were calculated from the Fig.; 2.3 × 10^{-9} M and 37 fmol/mg protein (cytosol), 2.5 × 10^{-9} M and 52 fmol/mg protein (0-30% fraction), 2.9 × 10^{-9} M and 12 fmol/mg protein (30-50% fraction) for Kd (dissociation constant) and maximum binding sites, respectively.

1881-labeled cytosol were extracted with 0.4 M KCl while stirring at 4°C for 30 min. After centrifugation, the precipitate was reextracted in the same manner. The radioactivity in each extract and ethanol extract of the final residual precipitate were measured after mixing with Instagel.

Analytical methods

Nucleic acids were extracted by the method of Hutchison et al. (1962) and determined by the colorimetric method described by Schneider (1960). Protein was measured either by the biuret method (Gornall et al., 1949) or by the ultraviolet absorption at 280 nm (Tombs et al., 1959) with bovine serum albumin as the calibration standard.

Results

Properties of protein which bound to [3H]-R 1881

Saturation analysis of the binding of cytosol from tissues of human benign prostatic hypertrophy to [3H]-R 1881 revealed a single high affinity binding (Fig. 1). When binding analysis was performed with acetone-dried cytosol, no high affinity binding was observed.

Cytosol was fractionated by the addition of ammonium sulfate into three fractions (0–30, 30–50, 50–70%). Whose binding properties were examined and high affinity binding was observed in the 0–30% and 30–50% fractions (Fig. 1). The binding capacity of the 0–30% fraction was higher than that of the 30–50% fraction when compared per unit of protein so that the 0–30% fraction seems to be rich in the [3H]-R 1881 binding component.

Cytosol was incubated with [3H]-R 1881 in the presence or absence of an excess amount of radioinert R 1881. After dextran-coated charcoal treatment to remove unbound steroid, an aliquot of the samples was applied to a Sephadex G-200 column (Fig. 2).
Fig. 3. Binding to R 1881 of void volume and IgG fractions separated by Sephadex G-200 chromatography. The 0–50% fraction of cytosol was chromatographed on a Sephadex G-200 column, and void volume and IgG fractions were obtained. Vo fraction (0.4 mg as protein/tube: open circle) or IgG fraction (0.3 mg as protein/tube: closed circle) were incubated with various concentrations (0.25–5 nM) of 3H-R 1881 at 4°C for 3 hr. Binding parameters to R 1881 were calculated from the Fig.; 1.4×10^{-9} M and 33 fmol/mg protein (Vo fraction), 8.0×10^{-9} M and 52 fmol/mg protein (IgG fraction) for Kd and maximum binding sites, respectively.

Sephadex chromatography of the cytosol prelabeled with 3H-dihydrotestosterone is also shown in the figure. Binding components to 3H-R 1881 were eluted mostly in the void volume fraction (Vo fraction), followed by fraction of or shortly after the elution site of hIgG (IgG fraction) and fraction at the site of bovine serum albumin. The addition of an excessive amount of radioinert R 1881 decreased the radioactivity in the Vo fraction. Therefore an R 1881 binding component showing low capacity binding was derived from the Vo fraction. The elution pattern for 3H-dihydrotestosterone binding components was different from that for 3H-R 1881 binding ones.

Fig. 4. Sucrose density gradient centrifugation of the 0–50% fraction after incubation with 3H-R 1881 or 3H-dihydrotestosterone. An aliquot of the 0–50% fraction (0.3 ml, 4.4 mg as protein) prelabeled with 3H-R 1881 (open circle) or 3H-dihydrotestosterone (closed circle) was applied on the top of 5–20% sucrose gradient. The gradient was centrifuged at 114,500×g for 16 hr, and each 0.2 ml fraction was collected and the radioactivity was counted. Position of markers (B.S.A.; bovine serum albumin [4.6S], IgG [7S]) is indicated.

2). Sephadex chromatography of the cytosol prelabeled with 3H-dihydrotestosterone is also shown in the figure. Binding components to 3H-R 1881 were eluted mostly in the void volume fraction (Vo fraction), followed by fraction of or shortly after the elution site of hIgG (IgG fraction) and fraction at the site of bovine serum albumin. The addition of an excessive amount of radioinert R 1881 decreased the radioactivity in the Vo fraction. Therefore an R 1881 binding component showing low capacity binding was derived from the Vo fraction. The elution pattern for 3H-dihydrotestosterone binding components was different from that for 3H-R 1881 binding ones.

Binding properties of the Vo and IgG fractions obtained from Sephadex G-200 chromatography of 0–50% fraction of cytosol were examined (Fig. 3). The Vo fraction showed a high affinity binding to 3H-R 1881, and binding affinity of the IgG fraction to this ligand seems to be lower than that of the Vo fraction.

The 0–50% fraction prelabeled with either 3H-R 1881 or 3H-dihydrotestosterone was fractionated in sucrose density gradient centrifugation (Fig. 4). In both cases, a small radioactive peak of 8.8S was observed.

Inhibition of 3H-R 1881 binding of cytosol by other steroids

Cytosol from human benign prostatic hypertrophy was incubated with various concentrations of 3H-R 1881 in the presence or absence of 10 nM of dihydrotestosterone
Fig. 5. Inhibition of the binding of cytosol to R 1881 by dihydrotestosterone or cyproterone acetate. Cytosol (6.0 mg as protein/tube) was incubated with various concentrations (0.25-5 nM) of $^3$H-R 1881 in the presence or absence of 10 nM of additives at 4°C for 3 hr. No addition (open circle), addition of dihydrotestosterone (closed circle) or cyproterone acetate (open triangle).

Fig. 6. Inhibition of the binding of cytosol to $^3$H-R 1881 by various steroids. Cytosol (4.7 mg as protein/tube) was incubated with 1 nM of $^3$H-R 1881 supplemented with various concentrations (10-1000 nM) of additives (Fig. 6). Progesterone, R 5020 and cyproterone acetate were potent inhibitors of the binding, but the inhibition by dihydrotestosterone and estradiol-17β was rather weak.

As was shown in Fig. 2, $^3$H-dihydrotestosterone-binding components were eluted both in Vo and IgG fractions by Sephadex G-200 chromatography. The effect of R 1881 on $^3$H-dihydrotestosterone binding of these two fractions was examined. While

or cyproterone acetate (Fig. 5). Analysis of the binding showed a competitive type inhibition by these two steroids.

To examine the effect of various steroids on the binding of cytosol to $^3$H-R 1881, cytosol was incubated with 1 nM of $^3$H-R 1881 supplemented with various concentrations (10-1000 nM) of additives (Fig. 6). Progesterone, R 5020 and cyproterone acetate were potent inhibitors of the binding, but the inhibition by dihydrotestosterone and estradiol-17β was rather weak.

As was shown in Fig. 2, $^3$H-dihydrotestosterone-binding components were eluted both in Vo and IgG fractions by Sephadex G-200 chromatography. The effect of R 1881 on $^3$H-dihydrotestosterone binding of these two fractions was examined. While
the addition of R 1881 did not evoke any effect on the binding of IgG fraction, the binding of the Vo fraction to $^3$H-dihydrotestosterone was inhibited by the R 1881 supplement (Fig. 7).

**Binding of nuclear extract to $^3$H-R 1881**

The nuclear extract from tissues of human benign prostatic hypertrophy showed high affinity binding to both $^3$H-R 1881 and $^3$H-dihydrotestosterone (Fig. 8). Binding parameters for these two ligands were almost identical.

The nuclear extract was incubated with 1 nM of $^3$H-R 1881 in the presence or absence of various concentrations (5–1000 nM) of a number of different steroids (Fig. 9). Dihydrotestosterone inhibited the binding to $^3$H-R 1881 significantly. Cyproterone acetate, R 5020 and estradiol-17β influenced the binding to a lesser extent. Therefore, inhibition of the binding of nuclear extract to $^3$H-R 1881 was quite different from that observed in the binding of cytosol.

**Binding of $^3$H-R 1881-labeled cytosol to nuclei and extraction of the bound cytosol from nuclei by salt extraction**

Various amounts of cytosol prelabeled with $^3$H-R 1881 were incubated with purified nuclei. In one series of experiments, the cytosol concentration in each incubation tube was varied (variable protein) and in the other, cytosol content was adjusted equally by adding heat-inactivated cytosol (constant protein). An almost linear relationship between the amount of the prelabeled cytosol incubated and the amount bound to nuclei was observed in both series of experiments (Fig. 10).

Purified nuclei were incubated with $^3$H-
Table 1. Extraction of nuclear bound radioactivity of \(^{3}\)H-R 1881-cytosol complex or \(^{3}\)H-dihydrotestosterone-cytosol complex with 0.4 M KCl solution.

<table>
<thead>
<tr>
<th></th>
<th>(^{3})H-R 1881</th>
<th>(^{3})H-dihydrotestosterone</th>
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<tbody>
<tr>
<td>Total nuclear bound radioactivity</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>1st extraction</td>
<td>37%</td>
<td>39%</td>
</tr>
<tr>
<td>2nd extraction</td>
<td>10%</td>
<td>14%</td>
</tr>
<tr>
<td>3rd extraction</td>
<td>7%</td>
<td>8%</td>
</tr>
<tr>
<td>Residual nuclear bound radioactivity</td>
<td>46%</td>
<td>39%</td>
</tr>
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</table>

Nuclear suspension (219 \(\mu\)g as DNA/tube) was incubated in the presence of cytosol prelabeled with \(^{3}\)H-R 1881 (5.0 mg as protein, specific activity 1923 dpm/mg protein) or cytosol prelabeled with \(^{3}\)H-dihydrotestosterone (5.0 mg as protein, specific activity 3701 dpm/mg protein) at 25\(^\circ\)C for 3 hr (\(^{3}\)H-R 1881) or for 2 hr (\(^{3}\)H-dihydrotestosterone). After incubation, nuclei were washed with TSMK buffer, then extracted with 0.05 M Tris-HCl containing 0.4 M KCl at 4\(^\circ\)C for 30 min during constant stirring. After centrifugation, the precipitate was extracted repeatedly. Finally, 1 ml of ethanol was poured into the precipitate, left at 35\(^\circ\)C for 16 hr, then the radioactivity in an aliquot of the supernatant was counted. This is referred to as the residual nuclear bound radioactivity.

a: amount of nuclear bound complexes were 21 and 40 fmol of \(^{3}\)H-R 1881 and \(^{3}\)H-dihydrotestosterone/mg DNA, respectively.

Fig. 10. Binding of cytosol prelabeled with \(^{3}\)H-R 1881 by nuclei. A series of increasing amounts of cytosol prelabeled with 2.5 \(\text{nM}\) of \(^{3}\)H-R 1881 (0.1–0.9 ml, 6.6 mg as protein/ml, specific activity 31 fmol of \(^{3}\)H-R 1881/mg protein) was added to constant amount of nuclei (147 \(\mu\)g as DNA/tube). The rest of the volume of incubation mixture was made up with TEM buffer (variable protein, open circle) or with heat-inactivated steroid-free cytosol (constant protein, closed circle). Incubations were performed at 25\(^\circ\)C for 3 hr.

R 1881-prelabeled cytosol or \(^{3}\)H-dihydrotestosterone-prelabeled cytosol and repeated extractions with salt solution were then performed (Table 1). The extraction patterns of the radioactivity from nuclei were almost identical in both of these experiments.

Discussion

It was observed that cytosol from human benign prostatic hypertrophy has components binding to R 1881 (Dubé et al., 1976b). Various incubation conditions for binding studies have been employed; Menon et al., (1978) studied binding at 0\(^\circ\)C for 20 hr and Shain et al. (1978) reported that binding at 4\(^\circ\)C was different from that at 15\(^\circ\)C, and that at 15\(^\circ\)C androgen was bound exclusively. In our preliminary experiments the binding of cytosol to R 1881 at 4\(^\circ\)C was similar to that at 15\(^\circ\)C, as shown by Menon et al. (1978). Moreover, more than 80\% of the amount of binding at 4\(^\circ\)C for 18 hr was achieved within 3 hr. Therefore, incubations of cytosol preparations in the present experiments were performed at 4\(^\circ\)C for 3 hr. Conditions for binding of nuclear extract seem to require longer incubation (Shain and Boesel, 1978), and in the present experiments the nuclear extracts were incubated at 4\(^\circ\)C for 18 hr.

It was reported that Kd and a number of maximum binding sites to R 1881 were ap-
proximately $10^{-9}$ M and 10–100 fmol/mg protein, respectively (Dubé et al., 1976b, Menon et al., 1978, Shain et al., 1978, Sirett and Grant, 1978 and Cowan et al., 1977). Similar binding parameters were also obtained in the present study.

Previous report showed that the binding ability of cytosol to dihydrotestosterone did not change after delipidization of cytosol by acetone (Kodama et al., 1977). However, the high affinity binding component to R 1881 was lost after treatment with acetone. Dihydrotestosterone-binding protein was concentrated in the precipitate of 30–50% saturation of ammonium sulfate, but the R 1881-binding component was observed in the 0–30% ammonium sulfate fraction. Although these two proteins had an identical sedimentation coefficient of 8.8S (Rosen et al., 1975, Attramadal et al., 1975), on Sephadex chromatography the R 1881-binding component was mainly located in the Vo fraction and the dihydrotestosterone-binding protein was detected in both Vo and IgG fractions. Inhibition by R 1881 of the binding of dihydrotestosterone to components of the Vo fraction was observed in the present study, but from differences in physicochemical properties and steroid specificity (Kodama et al., 1978) it seems that the R 1881-binding component is different from the dihydrotestosterone-binding protein.

Snochowski et al. (1977) reported that binding of cytosol from human benign prostatic hypertrophy to R 1881 was inhibited significantly by dihydrotestosterone, while Asselin et al., (1976), Dubé et al., (1976a, b) and Cowan et al., (1977) observed that the rate of inhibition of the binding by progestin was much higher than that of dihydrotestosterone. Menon et al., (1978) also confirmed the inhibition of the binding by progestins and assumed that the R 1881-binding component was a progestin receptor and not the androgen receptor. We also observed a similar inhibition pattern for the R 1881 binding of cytosol by progestin and dihydrotestosterone. The inhibition pattern of the nuclear binding component to R 1881 was different from that of the cytosol and the rate of inhibition by dihydrotestosterone was higher than that by progestin, as reported by several workers (Menon et al., 1978, Shain and Boesel, 1978, Sirett and Grant, 1978). From these observation, the nuclear R 1881-binding component seems to be the androgen receptor which is translocated from cytosol. Moreover, bound R 1881 was exchangeable with androgens supplemented under usual incubation conditions (Bonne and Raynaud, 1976, Ghanadian et al., 1978). Since the androgen-binding component(s) in R 1881 binding of human benign prostatic hypertrophy was separated from progestin-binding component(s) by triamcinolone acetonide (Asselin et al., 1979), it seems likely that cytosols from human benign prostatic hypertrophy contain both androgen and progestin binders.

Cytosol prelabeled with R 1881 bound to the nuclei in a non-saturable process. A similar observation has been made on the binding of dihydrotestosterone-androphilic protein complex to nuclear component(s) (Kodama and Shimazaki, 1979). Non-saturable binding of the hormone-receptor complex to nuclei was well discussed by Gorski and Gannon (1976) and Ichii et al. (1977).

From the results obtained in the present study, the translocation inhibitor described by Simon et al. (1976) does not seem to be present in human prostate, since under incubation conditions for the constant protein and the variable protein, binding of the cytosol of human benign prostatic hypertrophy prelabeled with R 1881 did not differ significantly.

Clark and Peck (1976) reported two kinds of estradiol-17β-binding sites discriminated by salt extraction in the nuclei of rodent uterus and they claimed that the salt-resistant binding was characteristic of the hormone action. In the present study, the nuclear bound cytosol prelabeled with
$^3$H-R 1881 and $^3$H-dihydrotestosterone was extracted in almost the same manner as 0.4 M KCl. However, the physiological significance of the salt-extractability of translocated cytosol component has been a matter of debate recently (Traish et al., 1977) and the problem is far from being solved.

Although it may be considered that androgen action is mediated by androgen-binding proteins in human prostate, further studies concerning the role of these proteins are awaited.

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