Low Molecular Weight Component of Androgen Receptor in Cytosols from Benign Hypertrophic Human Prostate treated with High KCl Solution

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Abstract

Cytosol of the benign hypertrophic human prostate was prepared in a low salt medium and then the concentration of salt was increased to 0.4 M with KCl (0.4 M KCl-cytosol). This preparation showed a high affinity binding to R 1881 and the binding was specific for androgens. These results suggest that the binding of the preparation to R 1881 was due mainly to the cytosolic androgen receptor. The R 1881 binding component in the 0.4 M KCl-cytosol was sedimented at 3S by sucrose density gradient centrifugation. The small sedimentation coefficient of the binder seems to be due to the high concentration of salt and not to degradation by proteolytic enzymes in the preparation. The molecular weight, Stokes radius and frictional ratio of this binding component were 32,000, 25.9 Å and 1.24, respectively.

It is well known that the human prostate contains several species of protein which show a high affinity binding to androgens (Menon *et al.*, 1977, Shain and Boesel, 1978). Among these androphilic proteins, androgen receptor, testosterone-binding globulin from blood plasma and progestin-binding protein have been identified in the tissues of the human prostate. As androphilic proteins in the prostate are heterogeneous, it seems to be difficult technically to measure the amounts of androgen receptor separately from other binders. However, using triamcinolone acetonide (9 α -fluoro-11 β , 16 α , 17, 21-tetraol-pregna-1,4-dien-3,20-dione cyclic 16, 17 acetal with acetone) and R 1881 (methyltrienolone, 17 β -hydroxy-17 α -methyl-estra-4,9,11-trien-3-one), several workers claimed that binding of the androgen receptor could be detected in the presence of other androphilic proteins in cytosol of the human prostate (Zava *et al.*, 1979, Asselin *et al.*,

1979).

It was also observed that, in a high ionic solution, androgen receptor of rat prostate dissociated into small components (Liao *et al.*, 1975). However, there has been little information about the components of androgen receptor in the human prostate. The present study was undertaken to clarify the properties of the component of androgen receptor which was formed in cytosol of the benign hypertrophic human prostate due to high ionic strength.

Materials and Methods

Steroids

Labeled and unlabeled R 1881 ((17 α -methyl-³H) 17 β -hydroxy-17 α -methyl-estra-4,9,11-trien-3-one, specific activity 87 Ci/mmol) and R 5020 ((17 α -methyl-³H) 17,21-dimethyl-19-nor-pregna-4,9-dien-3,20-dione, specific activity 87 Ci/mmol) were purchased from New England Nuclear (Boston, Mass. U.S.A.). Triamcinolone acetonide was obtained from Sigma Chemical Co., (St. Louis, Mo. U.S.A.). Dihydrotestosterone (17 β -hydroxy-5 α -androstan-3-one) was donated from the

Teikoku Hormone Co. (Kawasaki, Japan).

Materials

Tissues of the benign hypertrophic human prostate used in this study were obtained by retropubic procedure from patients admitted to 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 3 volumes of 0.01 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM β -mercaptoethanol and 10% (w/v) glycerol (TEMG buffer) using a glass homogenizer. The homogenate was filtered through nylon cloth, and centrifuged at $105,000\times g$ for 60 min to obtain cytosol (low salt cytosol). Concentrated KCl solution in TEMG buffer was then added to the low salt cytosol to make the final concentration of 0.4 M with respect to KCl (0.4 M KCl-cytosol).

Saturation analysis of the binding

The 0.4 M KCl-cytosol was incubated in 0.5 ml of TEMG buffer containing 0.4 M KCl, a series concentration of ^3H -R 1881 and 1000-fold molar excess of triamcinolone acetonide at 4°C for 20 h. ^3H -R 1881 and triamcinolone acetonide were dissolved in 10 μl of ethanol and added to the incubation mixture. All binding experiments were performed in the incubation medium containing 10 mM Na_2MoO_4 and 0.5 mM leupeptin (Sigma Chemical Co., St. Louis, Mo. U.S.A.) except where otherwise indicated. When binding of the low salt cytosol prepared in TEMG buffer was observed, 0.4 M KCl was omitted from the incubation mixtures. R 5020 binding of the low salt cytosol and 0.4 M KCl-cytosol was estimated by incubating with various concentrations (0.25–5 nM) of ^3H -R 5020 at 15°C for 3 h (Kodama *et al.*, 1981).

After incubation, 0.5 ml of TEMG buffer containing 0.05% dextran (T 70, Pharmacia, Uppsala, Sweden)-coated 0.5% charcoal (Norit A, Wako Pure Chemicals, Osaka, Japan) was added to the incubation mixture, centrifuged, and the radioactivity in an aliquot of the supernatant was counted to obtain the total binding. In parallel incubations, 1000-fold molar excess of unlabeled R 1881 or R 5020 was added to the incubation tube for estimation of the nonspecific binding. The specific binding was calculated from the total binding by subtracting the nonspecific binding. The dissociation constant (K_d) and binding capacity (maximum binding sites) were determined by the method of Scatchard (1949).

Sucrose density gradient centrifugation

The 0.4 M KCl-cytosol was incubated in TEMG buffer containing 5 nM ^3H -R 1881, 0.4 M KCl, 10 mM

Na_2MoO_4 and 0.5 mM leupeptin in the presence or absence of 5 μM triamcinolone acetonide at 4°C for 20 h. After incubation, unbound steroid was removed with TEMG buffer containing 0.05% dextran-coated 0.5% charcoal, centrifuged, and an aliquot of the mixture was applied on the top of a 5–20% sucrose gradient in TEMG buffer which contained 0.4 M KCl, 10 mM Na_2MoO_4 and 0.5 mM leupeptin (5 ml). Except where otherwise indicated, centrifugation was performed in a Hitachi Ultracentrifuge (80 P-7, Hitachi Co., Tokyo, Japan) using a RPS 50-2 rotor at $215,000\times g$ for 16 h. After centrifugation, the gradient was fractionated into 0.2 ml fractions by a density gradient fractionator (Isco, Model 640, Lincoln, Nebraska, U.S.A.). Human immunoglobulin G (IgG, 7.0S), equine myoglobin (Myo, 2.1S) and bovine serum albumin (BSA, 4.6S) were used as references for sedimentation. In parallel incubations, 5 μM radioinert R 1881 was added to the incubation medium to observe the location of non-specific binding.

When the effect of high concentration of salt on the R 1881 binding complex in the low salt cytosol was observed, the low salt cytosol was incubated with ^3H -R 1881 in the absence of 0.4 M KCl. After incubation, the incubation mixture was treated with dextran-coated charcoal, and then the salt concentration was adjusted to 0.4 M with respect to KCl. The incubated cytosol treated with high salt was left at 4°C for 60 min, and an aliquot was subjected to sucrose density gradient centrifugation.

Sephadex G-200 Chromatography

The 0.4 M KCl-cytosol was incubated in TEMG buffer containing 5 nM ^3H -R 1881, 0.4 M KCl, 10 mM Na_2MoO_4 , 0.5 mM leupeptin and 5 μM triamcinolone acetonide in the presence or absence of 5 μM unlabeled R 1881 at 4°C for 20 h. After incubation, dextran-coated charcoal was added, centrifuged, and an aliquot of the resultant supernatant was applied to a column filled with Sephadex G-200 (1×87 cm, Pharmacia, Uppsala, Sweden). The column was eluted with TEMG buffer containing 0.4 M KCl, 10 mM Na_2MoO_4 and 0.5 mM leupeptin. Each 2 g fraction was collected. Estimation of molecular weight, Stokes radius and frictional ratio was performed according to the method described by Siegel and Monty (1966).

Analytical Procedures

Protein was determined by the biuret method (Gornall *et al.*, 1949). Radioactivity was determined in toluene containing 0.4% PPO and 0.01% POPOP using a liquid scintillation spectrometer (LKB 1215 Rackbeta, Wallac OY, Finland). Radioactivity in eluates of Sephadex G-200 were estimated in the presence of Insta-Gel (Packard, U.S.A.).

Results

Binding of the 0.4 M KCl-cytosol to ^3H -R 1881 in the presence or absence of triamcinolone acetonide

Time course of the binding of the 0.4 M KCl-cytosol to ^3H -R 1881 in the presence or absence of 1000-fold molar excess of triamcinolone acetonide was examined (Fig. 1). The binding increased gradually with time, and the specific binding reached almost the maximum level after incubation of 16 h. The presence of triamcinolone acetonide did not influence the time course of the binding. Therefore, subsequent incubations of the 0.4 M KCl-cytosol for determination of ^3H -R 1881 binding were performed for 20 h.

The 0.4 M KCl-cytosol was incubated with

various concentrations of ^3H -R 1881 in the presence or absence of triamcinolone acetonide to estimate the binding parameters (Fig. 2). The 0.4 M KCl-cytosol showed a binding of single high affinity nature to ^3H -R 1881 and influence of triamcinolone acetonide on the binding seems to be, if any, relatively small. This suggests that the 0.4 M KCl-cytosol contains a ^3H -R 1881 binding component, the binding of which is relatively resistant to addition of triamcinolone acetonide.

To examine whether the 0.4 M KCl-cytosol contains component(s) bound to progestin or not, the 0.4 M KCl-cytosol and the low salt cytosol were independently incubated with ^3H -R 5020 (Fig. 3). The low salt cytosol showed a high affinity binding to ^3H -R 5020, but in the 0.4 M KCl-cytosol no specific binding to ^3H -R 5020 was detected.

Fig. 1. Time course of the binding of 0.4 M KCl-cytosol to ^3H -R 1881.

Cytosol of the benign hypertrophic human prostate was prepared in 10 mM Tris-HCl-1 mM EDTA-1 mM β -mercaptoethanol-10% glycerol buffer and concentrated KCl solution was added as to make the concentration of KCl 0.4 M (0.4 M KCl-cytosol). The 0.4 M KCl-cytosol (2.5 mg as protein/tube) was incubated with 2.5 nM ^3H -R 1881 in the presence (closed symbol) or absence (open symbol) of 2.5 μM triamcinolone acetonide at 4°C for the various time intervals indicated. All binding experiments in this and the following Fig. were performed in the medium containing 0.4 M KCl, 10 mM Na_2MoO_4 and 0.5 mM leupeptin except where otherwise indicated. Total binding ($\blacksquare$), specific binding ($\bullet$), nonspecific binding ($\blacktriangle$).

Fig. 2. Binding of 0.4 M KCl-cytosol to ^3H -R 1881.

The 0.4 M KCl-cytosol (2.8 mg as protein/tube) was incubated with various concentrations (0.25–5 nM) of ^3H -R 1881 in the presence (closed circle) or absence (open circle) of 1000-fold molar excess of triamcinolone acetonide at 4°C for 20 h. K_d and maximum binding sites were calculated from the Fig. as 0.7×10^{-9} M and 19 fmol/mg protein in the presence of triamcinolone acetonide and 0.9×10^{-9} M and 25 fmol/mg protein in the absence of triamcinolone acetonide, respectively.

Fig. 3. Binding of 0.4 M KCl-cytosol and of low salt cytosol to ^3H -R 5020.

The 0.4 M KCl-cytosol (2.9 mg as protein/tube, closed circle) or the low salt cytosol (2.9 mg as protein/tube, open circle) were incubated with various concentrations (0.25–5 nM) of ^3H -R 5020 at 15°C for 3 h. 0.4 M KCl was omitted from the incubation mixture for the determination of the binding with the low salt cytosol. K_d and maximum binding sites of the binding of the low salt cytosol were calculated from the Fig. as 0.7×10^{-9} M and 22 fmol/mg protein, respectively.

Inhibition of ^3H -R 1881 binding in the 0.4 M KCl-cytosol by various steroids

The 0.4 M KCl-cytosol was incubated with 1 nM ^3H -R 1881 in the presence or absence of triamcinolone acetonide supplemented with various concentrations (10–1000 nM) of unlabeled additives (Fig. 4). Binding to ^3H -R 1881 of the 0.4 M KCl-cytosol was significantly inhibited by R 1881 and dihydrotestosterone, but R 5020 was a weak inhibitor irrespective of the presence or absence of triamcinolone acetonide. Triamcinolone acetonide inhibited the binding slightly. The addition of triamcinolone acetonide to the incubation did not modify the pattern of inhibition by various steroids.

Sedimentation and chromatographic analysis of the binding

The 0.4 M KCl-cytosol prelabeled with 5 nM ^3H -R 1881 in the presence or absence of triamcinolone acetonide was fractionated in the sucrose density gradient centrifugation (Fig. 5A). A peak of 3S was evident irrespective of the presence of triamcinolone acetonide. In incubation with an excessive amount of

Fig. 4. Inhibition of ^3H -R 1881 binding in 0.4 M KCl-cytosol by various steroids.

The 0.4 M KCl-cytosol (2.1 mg as protein/tube) was incubated with 1 nM ^3H -R 1881 in the presence of various concentrations (10–1000 nM) of radioinert competitors supplemented with (closed symbol) or without (open symbol and cross) $1 \mu\text{M}$ triamcinolone acetonide at 4°C for 20 h. The binding was expressed as a percentage of that incubated in the absence of competitors (19 fmol/mg protein with triamcinolone acetonide and 21 fmol/mg protein without triamcinolone acetonide). circle (dihydrotestosterone), triangle (R 1881), square (R 5020), cross (triamcinolone acetonide).

Fig. 5. Sucrose density gradient centrifugation in high salt solution of 0.4 M KCl-cytosol (A) or of cytosol (B) bound with ^3H -R 1881.

- A: The 0.4 M KCl-cytosol was incubated with 5 nM ^3H -R 1881 and 5 μM triamcinolone acetonide at 4°C for 20 h. After separating unbound steroid an aliquot (0.3 ml, 1.0 mg as protein) was applied on the top of a 5-20% sucrose gradient containing 0.4 M KCl, 10 mM Na_2MoO_4 , 0.5 mM leupeptin and 10% glycerol, and centrifuged at $215,000 \times g$ for 16 h. Each 0.2 ml fraction was collected and the radioactivity in each fraction was counted (closed circle). In parallel incubations, triamcinolone acetonide was omitted (open circle) or 5 μM unlabeled R 1881 was added (open triangle). The site of the binding component is indicated by the white arrow. Black arrows indicate the location of markers (IgG; human immunoglobulin G (7.0S), Myo; equine myoglobin (2.1S), BSA; bovine serum albumin (4.6S)).
- B: The low salt cytosol was incubated with 5 nM ^3H -R 1881 in the medium containing 10 mM Na_2MoO_4 and 0.5 mM leupeptin supplemented with (closed circle) or without (open circle) 5 μM triamcinolone acetonide. After removing unbound steroid, concentration of KCl in the supernatant was adjusted to 0.4 M and left at 4°C for 60 min. An aliquot (0.3 ml, 0.8 mg as protein) was fractionated by sucrose density gradient centrifugation. open triangle; nonspecific binding.

unlabeled R 1881, this peak was not observed.

The low salt cytosol was incubated with ^3H -R 1881 in the presence or absence of triamcinolone acetonide. After incubation, dextran-coated charcoal was mixed and centrifuged and then the KCl concentration in the resultant supernatant was adjusted to 0.4 M. An aliquot of this mixture was analyzed in the sucrose density gradient centrifugation (Fig. 5B). A radioactive peak of 3S was also observed regardless of the presence of triamcinolone acetonide.

To estimate the sedimentation coefficient of

the binding components accurately, the 0.4 M KCl-cytosol prelabeled with ^3H -R 1881 was fractionated with more centrifugal force (Fig. 6). A peak of 3S was again confirmed, separating from the radioactivity of the first few fractions due to dissociated free ^3H -steroid.

To examine whether proteolytic enzymes in the sample were involved in the formation of 3S peak or not, the 0.4 M KCl-cytosol was incubated with 5 nM ^3H -R 1881 in the incubation medium without leupeptin, and further analyzed in the sucrose density gradient centrifugation (Fig. 7). A radioactive peak was

Fig. 6. Sedimentation profile of ^3H -R 1881-bound 0.4 M KCl-cytosol in high salt solution. The 0.4 M KCl-cytosol was incubated with ^3H -R 1881 described in Fig. 5A and an aliquot (0.3 ml, 0.9 mg as protein) was analyzed by sucrose density gradient centrifugation performed at $215,000\times g$ for 25 h. Total binding (closed circle), nonspecific binding (open triangle).

Fig. 7. Sedimentation profile of ^3H -R 1881-bound 0.4 M KCl-cytosol prepared in the medium without leupeptin.

The 0.4 M KCl-cytosol was incubated with ^3H -R 1881 and an aliquot (0.3 ml, 0.9 mg as protein) was analyzed by sucrose density gradient centrifugation. All procedures were performed in the medium omitted leupeptin. Total binding (closed circle), nonspecific binding (open triangle).

Fig. 8. Sephadex G-200 chromatography of 0.4 M KCl-cytosol prelabeled with ^3H -R 1881.

The 0.4 M KCl-cytosol was incubated with 5 nM ^3H -R 1881 and $5\text{ }\mu\text{M}$ triamcinolone acetonide at 4°C for 20 h. After dextran-coated charcoal treatment, an aliquot (3.1 mg as protein) was applied to the Sephadex G-200 column ($1\times 87\text{ cm}$). The column was eluted with TEMG buffer containing 0.4 M KCl, 10 mM Na_2MoO_4 and 0.5 mM leupeptin at a flow rate of 5.1 ml/h. Each 2 g of the eluate was collected and the radioactivity in an aliquot of the eluate was counted (closed circle). Nonspecific binding (closed triangle). Vo; void volume, IgG; human immunoglobulin G, Myo; equine myoglobin, BSA; bovine serum albumin.

observed in the same fraction as that incubated with leupeptin. It was also confirmed that a 3S peak was obtained when leupeptin was added to the homogenization medium. Therefore, the possibility of digestion by proteolytic enzyme at the time of homogenization and tissue fractionation could be neglected.

The 0.4 M KCl-cytosol labeled with ^3H -R 1881 in the manner described above was applied to a Sephadex G-200 column (Fig. 8). Some radioactive materials appeared just after the void volume and at the site of BSA, but a considerable amount of binding component was eluted between the elution site of BSA and that of Myo. The first peak seems to be due to aggregation of the third binding component, since the latter shifted to a void volume fraction after dialysis and rechromatography.

The small binding component eluted at the elution site of BSA might be other binding protein, but the nature of this binding was not further examined.

Together with the results of sedimentation experiments, the main binding component in the 0.4 M KCl-cytosol was assumed to be a low molecular weight component that sedimented at 3S on sucrose density gradient centrifugation and was eluted between the elution site of BSA and that of Myo in Sephadex G-200 column chromatography. The molecular weight of this binding component was calculated as approximately 32,000, and Stokes radius and frictional ratio were estimated as 25.9 Å and 1.24, respectively.

Discussion

A higher rate of inhibition by progestins than dihydrotestosterone was observed in the binding to R 1881 of cytosol from the human prostate, but when the incubation mixture for the binding included triamcinolone acetonide, dihydrotestosterone exhibited a potent inhibition of the R 1881 binding (Hicks and Walsh, 1979). Therefore, the R 1881 binding of the human prostatic cytosol in the presence of triamcinolone acetonide seems to be attributable to the binding of androgen receptor. The addition of molybdate stabilizes the binding to R 1881 in the cytosol from the human prostate as has been observed in the rodent prostate (Gaubert *et al.*, 1980, Noma *et al.*, 1980, Trachtenberg *et al.*, 1981). Based on these observations the determination of the specific binding by the androgen receptor in the human prostate has been improved, and the current method used for the examination of receptor in the human prostatic tissues includes triamcinolone acetonide and molybdate in the incubation medium (Shimazaki *et al.*, 1981).

In the present study, it is revealed that the human prostatic cytosol treated with 0.4 M KCl shows a high affinity binding to R 1881, and this binding seems to be attributable to

that of the androgen receptor. The rate of binding to androgens in the rat prostate was not influenced by the ionic strength of the incubation medium (Fang *et al.*, 1969). Incubations of the 0.4 M KCl-cytosol from the human prostate in the present experiments were performed in the presence of triamcinolone acetonide but the omission of triamcinolone acetonide from the incubation media did not cause significant changes in the R 1881 binding. In addition, since no 0.4 M KCl-cytosol binding of progestin was observed, therefore, the R 1881 binding of the 0.4 M KCl-cytosol seems to be due to the binding by the androgen receptor. The reason why the binding to progestin is inhibited in high ionic medium is not clear but the binding component with progestin in the human prostatic cytosol does not seem to be steroid receptor but another binding protein (Kodama *et al.*, 1981). It is possible that the binding of the latter to ligand is easily influenced by ionic strength.

Many reports indicated that the androgen receptor of the rat prostate showed a sedimentation coefficient of 8S in low salt solutions (Liao, 1975, Gorski and Gannon, 1976, Bradlow and Gasparini, 1979). However, 3S component has been generally observed in androgen receptors of the various androgen-target tissues of the rodent (Lea *et al.*, 1979, Wilson and French, 1979). It is assumed that androgen receptor sedimented in 8S is the complex of 3S component and other macromolecules (Wilson and French, 1976, Liao, 1977), although it is still discussed that the 8S androgen receptor is a dimer consisting of small subunits, as was suggested concerning estrogen receptor (Gorski and Gannon, 1976). In cytosols of the human prostate, the R 1881 binding component which has a sedimentation coefficient of approximately 8S in low ionic medium was observed (Kodama *et al.*, 1980). Under the high ionic conditions, it was reported that the molecular size of the dihydrotestosterone-binding component was decreased (Mainwaring and Milroy, 1973). In the present study, it can be shown that the sedimentation coeffi-

cient of the human androgen receptor is 3S in the high salt solution and this 3S component still possesses the binding ability to R 1881 under the high ionic conditions. Furthermore, the 8S binding protein which binds to R 1881 dissociates to 3S in the presence of 0.4 M KCl. Among the steroid hormone binding protein so far examined, 3S component which exhibits the intact binding ability to androgens seems to be the smallest one. However, the 3S binding component in the human prostate observed in the present study is not likely to be an artificial product of proteolytic digestion, since omitting leupeptin, the potent inhibitor of protease (Umezawa, 1976, Aoyagi *et al.*, 1969, Sherman *et al.*, 1980, Hazato and Murayama, 1981), from the incubation medium as well as from the homogenizing medium, did not modify the sedimentation profile of the R 1881 binding component in the 0.4 M KCl-cytosol. Recently a 3S subunit of estrogen receptor called mero-receptor was derived from the 8S receptor by treatment with trypsin or chaotropic salt, NaSCN (Miller, 1980, Sica *et al.*, 1976, Bailly *et al.*, 1980, Sherman *et al.*, 1978, Erdos *et al.*, 1977, Vallet-Strouve *et al.*, 1976). The androgen receptor of the human prostate observed in the present study dissociates more easily to the smallest component in high salt solution. Moreover, the 3S androgen receptor still contains R 1881 binding site, and this seems to be similar to that reported in the mero-receptor. Further studies to clarify the physiological significance of 3S binding component will be awaited.

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