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IDENTIFICATION AND PARTIAL CHARACTERIZATION OF THE CYTOPLASMIC ANDROGEN RECEPTOR IN BOVINE OVARIAN CAPSULE

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Summary—[^{3}H]Dihydrotestosterone (DHT) binding to a specific protein in the cytosol of bovine ovarian capsule was studied *in vitro*. The specific androgen-binding protein in the cytosol was analyzed by chromatographic and ultracentrifugal techniques. From Scatchard analysis, the dissociation constant was 7.4 nM and the number of binding sites was 58.8 fmol/mg protein. Testosterone and 17α-methyltrienolone (R1881) compete for [^{3}H]DHT binding. In the presence of sodium molybdate and at low salt concentrations, the steroid–protein complex sediments as a 9S form, while in the presence of high salt, it sediments at 3.5S. In the absence of molybdate or in the presence of high salt, the 9S form dissociates in a temperature-dependent manner into smaller units. These properties are consistent with the presence of a typical androgen receptor in the bovine ovarian capsule.

INTRODUCTION

It has been reported that collagenase and plasminogen activator, which are produced by the Graafian follicle, play a significant role during ovulation [1–4]. We have confirmed that human and bovine ovarian capsules mainly consist of connective tissue components such as collagen and acidic glycosaminoglycans [5]. Enlargement of the ovary and thickening of the ovarian capsule are known to be histological changes observed in polycystic ovarian disease (PCO). In this disease, high levels of circulating plasma androgen [6–9] and of ovarian venous androstendione and testosterone [10] are characteristically observed. It is interesting that successive administration of testosterone to rhesus monkeys has been reported to induce PCO-like changes in ovaries [11]. These facts have suggested that andogens may be related to the metabolism of connective tissue in the ovarian capsule. Currently, it is not possible to collect sufficient capsular tissue with which to examine the above diseases. Therefore, we have examined the possibility that bovine ovarian capsule may be used as a model system for study. In this report, we demonstrate that androgen receptors are present in the bovine ovarian capsule.

EXPERIMENTAL

Tissue source

Bovine ovarian capsules were obtained at a local slaughterhouse. The ovaries were immediately excised after killing and washed sufficiently with cold saline. The capsule (tunica albuginea) was completely separated from medulla, and follicles in the capsule were removed as far as possible, again washed with cold saline, and then stored at −80°C until use.

Reagents used

The following reagents were commercially obtained; 5α-dihydro[1,2,4,6,7-^{3}H]testosterone (sp. act. 110 Ci/mmol, [^{3}H]DHT) and 17α-methy-[^{3}H]trienolone ([^{3}H]R1881) and unlabeled R1881 from New England Nuclear, Boston, MA, U.S.A.: 1-*O*-methyl-α-D-glucopyranoside (α-methyl-D-glucoside), γ-globulin (Cohn fraction II), androstendiol, androstendione, cortisol (C), DHT, dehydroepiandrosterone (DHA), oestradiol-17β (E_2), estriol (E_3), progesterone (P), testosterone (T) from Sigma, St Louis, MO, U.S.A.: bovine serum albumin (5×, crystallized) from ICN Pharmaceuticals, Cleveland, OH, U.S.A.; pronase E (one million tyrosine units/g) from Kakenseiyaku C., Bunkyo, Tokyo, Japan; Sephadex G-50, Concanavalin A (Con-A) Sepharose 4B and dextran T-70 from Pharmacia, Uppsala, Sweden. Antipain from Protein Research Foundation, Minoh, Osaka, Japan. Other reagents used were of analytical reagent grade.

Preparation of cytosol fraction

The thawed and minced tissue was homogenized in 10 vol of 20 mM Tris–HCl/3 mM EDTA/10 mM Na_2MoO_4/1.5 mM 2-mercaptoethanol/0.15 mM antipain (pH 7.4) (buffer A) using a glass homogenizer. The homogenization was done twice for 30 s each, with cooling in ice for 30 s after the first homogenization, at 900 rpm. The homogenate was centrifuged at 105,000 *g* for 60 min at 4°C, in a Hitachi 65P ultracentrifuge using a 65T rotor.

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Assay for androgen-receptor

Aliquots (0.5 ml) of the cytosol were incubated with 5.0 mM of [^{3}H]DHT in the presence or absence of a 200-fold excess of unlabeled DHT at 4°C for 2 h. At the end of the incubation period, the incubation mixture was filtered on Sephadex G-50 to remove unbound [^{3}H]DHT by the method of Godefroi and Brooks[12]. The column (1 × 25 cm) of Sephadex G-50 was previously equilibrated and eluted with buffer A. The radioactivity in each fraction (1 ml) was counted in Bray's solution using Aloka model 902 liquid scintillation counter. The radioactivity in the void volume fractions was used for analysis of the inhibitory effect of other steroids toward the [^{3}H]-DHT binding, and also calculation of the dissociation constant and the number of binding sites [13].

Sucrose density gradient analysis

To remove the unbound [^{3}H]DHT, the cytosol labeled with [^{3}H]DHT was mixed with an equal volume of dextran coated-charcoal (0.1% charcoal and 0.01% dextran T-70), incubated at 0°C for 5 min and then centrifuged at 15,000 *g* for 10 min. The supernatant (0.2 ml) was laid on a linear 5–20% of sucrose density gradient in 4.0 ml of buffer A/10% (w/v) glycerol, and then centrifuged at 150,000 *g* for 18 h using a Hitachi RPS 65T swing rotor at 4°C. Sedimentation constant was determined by the method of Martin and Ames[14] using bovine serum albumin (4.6S) and γ-globulin (6.9S) as standards.

Determinations protein

Protein was also determined by the method of Lowry *et al.*[15] with bovine serum albumin as a standard.

RESULTS

Specific binding of [^{3}H]DHT to the cytosol of bovine ovarian capsule

After the incubation of [^{3}H]DHT with a cytosol of bovine ovarian capsule, and the following gelfiltration of the mixture on Sephadex G-50, an excess amount of unlabeled DHT was found to significantly depress the total binding of [^{3}H]DHT, indicating that the presence of cytoplasmic androgen-binding macromolecule in the capsule. Figure 1 shows the progress curve for specific binding of [^{3}H]DHT to the cytosol of bovine capsule. The maximum binding was observed at 2 h-incubation and found to be stable for 4 h examined. Thus, the incubation was done for 2 h-period in subsequent experiments.

Properties of androgen-binding macromolecule in the ovarian capsule

Table 1 shows the displacement of [^{3}H]DHT from androgen binding sites by excess concentrations of other steroids. A 200-fold excess of unlabeled DHT

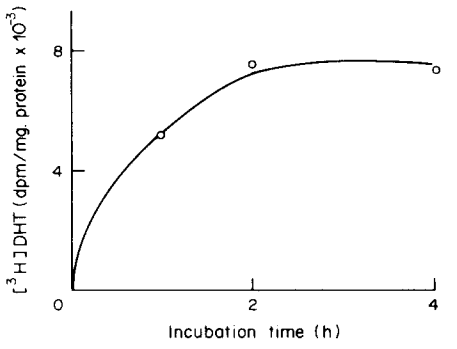

Fig. 1. Time course of specific binding of [^{3}H]DHT to the cytoplasmic macromolecule in bovine ovarian capsule. The cytosol was incubated for various periods at 4°C with 5.0 nM [^{3}H]DHT in the presence or absence of a 200-fold excess of unlabeled DHT, and then filtered on Sephadex G-50. The specific binding was calculated from the difference of bound radioactivity between presence and absence of unlabeled DHT. Other experimental conditions are described in the text.

was found to significantly inhibit the specific binding of [^{3}H]DHT. Testosterone and R1881 also show strong inhibitory effect. Although both oestradiol-17β and progesterone were noteworthy inhibitors of androgen binding, it is known that in human testis [16] and skin [17], an excess of these steroids in the range of 200-fold competitively inhibits binding of androgen to the receptor.

A typical saturation curve for the binding of [^{3}H]DHT to the macromolecule in the ovarian capsule is shown in Fig. 2. Specific androgen binding, as measured by the gel filtration assay, was saturable at low steroid concentration, indicating a limited number of binding sites. An equilibrium dissociation constant of 7.4 nM was obtained by Scatchard analysis. The amount of specific androgen binding averaged 58.8 fmol/mg protein in the 105,000 *g* supernatant.

Figure 3 shows the sedimentation pattern of the

Table 1. Competition of various steroids for the specific binding of [^{3}H]DHT to the cytoplasmic macromolecule in bovine ovarian capsule

Unlabeled steroid	Competition (%)*
5α-Dihydrotestosterone	100
Testosterone	87 ± 5
17α-Methyltrienolone (R1881)	95 ± 11
Dehydroepiandrosterone	1 ± 2
Dehydroepiandrosterone sulfate	8 ± 13
Androstendiol	40 ± 3
Androstenedione	59 ± 5
Oestradiol-17β	89 ± 16
Estriol	39 ± 6
Progesterone	82 ± 17
Cortisol	2 ± 3

The cytosol was incubated for 2 h at 4°C with 5.0 nM [^{3}H]DHT in the presence or absence of a 200-fold excess of unlabeled steroid. Binding was measured by using filtration on Sephadex G-50. Other detailed conditions are described in the text. *The competitive inhibitory effect obtained by 200-fold unlabeled DHT was defined as 100%. Data are expressed as mean ± SD of three samples.

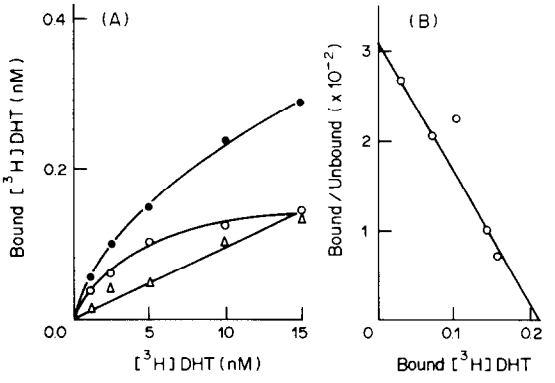

Fig. 2. (A) Saturation curve of [^{3}H]DHT binding to the androgen-binding macromolecule in the bovine ovarin capsule. The cytosol was incubated for 2 h at 4°C with the increasing amount of [^{3}H]DHT in the presence of a 200-fold excess of unlabeled DHT. Other experimental conditions are described in the text. (●), total binding; (○), specific binding; (△), non-specific binding. (B) Scatchard analysis for the cytoplasmic androgen-binding macromolecule in the ovarian capsule.

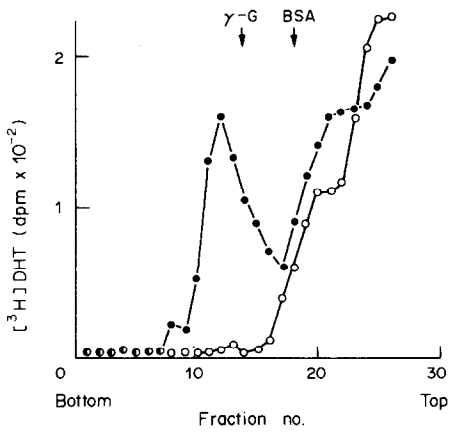

Fig. 3. Sucrose density gradient analysis of the androgen-binding macromolecule in the bovine ovarian capsule. The cytosol was labeled with [^{3}H]DHT in the presence (○) or absence (●) of unlabeled DHT and freed from unbound [^{3}H]DHT as described in Fig. 1, and then laid on 4 ml of 5–20% (w/v) sucrose density gradient in buffer A/10% (v/v) glycerol. Other experimental conditions are described in the text. γ-G, γ-globulin (6.9S); BSA, bovine serum albumin (4.6S).

androgen-binding macromolecule. A single radiolabeled peak sedimenting at 9S was observed and this peak was completely depressed by an excess amount of unlabeled DHT. When sodium molybdate was removed from the buffer A, the amount of the [^{3}H]DHT–macromolecule complex recovered was significantly less than that recovered in buffer A, and the complex was found to be sedimented at lower density (data not shown).

To examine the effect of molybdate on the stability of [^{3}H]DHT–macromolecule complex, the thermal stability of the complex was observed in the presence or absence of 10 mM sodium molybdate. When the molybdate was present, neither incubation at 4°C nor at 25°C affected the stability of the complex (Fig. 4A). In the absence of molybdate, however, the 9S form completely disappeared at 25°C. The effect of ionic strength on the stability of [^{3}H]DHT–macromolecule complex was also examined. Neutral salt decreased the 9S peak and a new peak at 3.5S appeared simultaneously (Fig. 5), indicating the dissociation of 9S form into smaller units.

DISCUSSION

The cytoplasmic DHT-binding macromolecule in the bovine ovarian capsule sediments as a 9S moiety and the [^{3}H]DHT binding is inhibited by R1881, a

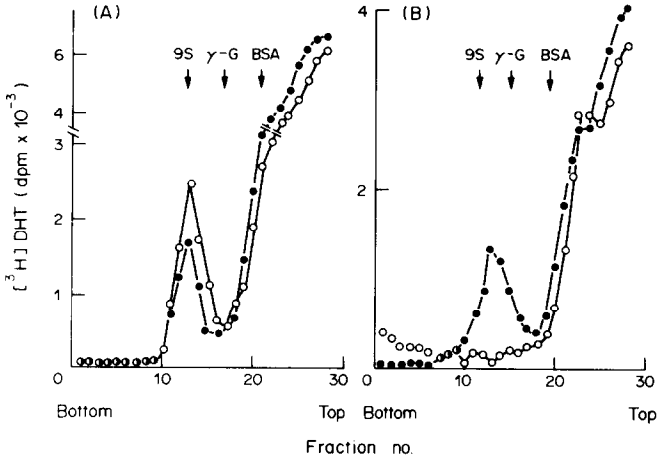

Fig. 4. Effect of incubation temperature on the sedimentation pattern of androgen-binding macromolecule in the sucrose density gradient analysis. The cytosol was incubated with 5.0 nM [^{3}H]DHT at 4 or 25°C, and then filtered on Sephadex G-50 to remove the unbound [^{3}H]DHT. The labeled cytosol was analyzed in a sucrose density gradient as described in Fig. 2. (A), With sodium molybdate (10 mM) during all procedures. (B), Without molybdate. Incubation temperature: 4°C (●) and 25°C (○).

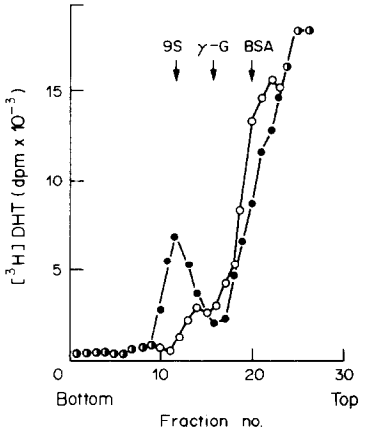

Fig. 5. Effect of neutral salt on the sedimentation pattern of androgen-binding macromolecule in the sucrose density gradient. The cytosol was labeled with [^{3}H]DHT at 4°C, treated with the filtration on Sephadex G-50 as described in Fig. 1, and then applied to the sucrose density gradient analysis. Two portions were prepared under presence (○) and absence (●) of 0.5 M KCl during all procedures.

ligand that is specific for androgen receptors [18–20]. Furthermore, bovine AR is stabilized by sodium molybdate, and is changed in its molecular form by high ionic strength (i.e. under low salt conditions, the AR is present predominantly in the 9S form, while under high salt conditions, it is recovered as a 3.5S species). These properties of AR in the bovine ovarian capsule correspond to those of AR in human prostate [21] and human skin fibroblasts [22].

The decrease in sedimentation coefficient observed at high ionic strength suggests that the AR is composed of several smaller units and that it is easily dissociated. Similar observations have been described for other steroid hormone receptors [23, 24]. It is known that molybdate stabilizes the 9S form of AR, and that in the absence of molybdate, AR dissociates in a temperature-dependent manner. Murayama and Fukai have reported similar phenomena with the estrogen receptor in bovine uterus and have discussed the correlation between the changes in molecular form and translocation of cytoplasmic AR to the nucleus [25].

Previously, we have reported that patients with PCO have enlarged ovaries and thickening of the ovarian capsule [5], as well as a high level of circulating plasma androgen [6–9]. It is also known that testosterone induces PCO-like changes in ovary of rhesus monkeys [11] and that androgen accelerates collagen biosynthesis in human skin fibroblasts, *in vitro* [26]. These observations suggest that androgens may play a direct role in causing thickening of the ovarian capsule. In this work we have demonstrated the presence of a DHT-binding protein in ovarian capsule cytosol that has the steroid-binding, sedimentation and stability properties of an androgen receptor.

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