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ANDROGEN BINDING TO CYTOSOL AND NUCLEI OF HAMSTER SEBACEOUS GLANDS

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SUMMARY

Specific dihydrotestosterone (DHT) binding was demonstrated in the cytosol of hamster sebaceous glands. When the cytosol was incubated with [^{3}H]-DHT and subjected to sucrose gradient centrifugation, a sharp peak of radioactivity was observed in the 7–8*S* region. This binding showed a high affinity for DHT ($K_D = 1.5 \times 10^{-9}$ M). Testosterone was also bound in that region, if to a much lesser extent. Competition among various kinds of excess cold steroids revealed their binding affinities in the decreasing order, as follows: DHT, cyproterone acetate, testosterone, 17β-estradiol, 5α-androstane-3α, 17β-diol, progesterone, androstenedione, cortisol, and androsterone. For the demonstration of intranuclear binding, the homogenate was incubated with [^{3}H]-DHT and the extracts from crude nuclei were filtered through Sephadex G-100. Three peaks of radioactivity were observed. The second peak, which approximated the elution of chymotrypsinogen A, appeared to represent the specific binding to the androgen from the following results: saturated at 7×10^{-9} M of DHT, completely abolished by an excess amount of unlabeled DHT and decreased by more than 75% by 100-fold excess of cyproterone acetate. Incubation with [^{3}H]-testosterone did not result in the formation of the second peak. The formation of this [^{3}H]-DHT–macromolecule complex in the nuclei required cytoplasmic factors.

INTRODUCTION

It is the current belief that the steroid–receptor complex plays a key role in implementing the biological action of androgens in target organs such as prostate and epididymis [1–5]. Sebaceous glands are also responsive to androgens [6, 7]. Using hamster costovertebral glands, which consist of several large sebaceous glands [8], we previously demonstrated that they convert testosterone into dihydrotestosterone (DHT) [9] and the latter is bound to proteins in the cytosol and the nuclei after the administration of labeled testosterone [10, 11]. In addition, some reports have shown the presence of receptor proteins for androgens in both the cytosol and the nuclei of some specialized sebaceous glands [10, 12, 13], although their characteristics have yet to be clarified.

This paper reports on some new properties of androgen-binding components in hamster sebaceous glands and confirms their characteristics previously reported.

EXPERIMENTAL

Labeling of cytosol

Costovertebral glands were excised from adult male Syrian hamsters which had been castrated 16 h earlier. Six hamsters were used in each experiment. The following procedures were carried out at 0–4°C. Subcutaneous tissues were removed from these glands (500–600 mg), which were then homogenized in an all-glass homogenizer with 4 ml of 20 mM Tris–HCl buffer, pH 7.4, containing 1.5 mM EDTA. The homogenate was filtered through two layers of nylon nets. The cytosol fraction was obtained by centrifugation at 105,000 *g* for 1 h. Subsequent treatment of cytosol fraction with a Zeineh's microconcentrator (Biomed Instrument Co.), which consists of dialysis membranes and retains high molecular weight solutes, resulted in two- to four-fold higher protein concentrations. The concentrated cytosol fraction was incubated with labeled steroids for 3 h at 3°C, mixed with a half volume of 0.5% charcoal–0.05% dextran, and shaken for 10 min; 0.2 ml of the supernatant was layered on the top of linear 5–20% sucrose gradients (4.8 ml) containing 20 mM Tris–HCl, pH 7.4 and 1.5 mM EDTA. The solution was centrifugated in a Hitachi 55P ultracentrifuge for 18 h at 38,000 rev./min. Fractions were collected from the bottom of the tubes. Determination of sedimentation constants for the DHT-binding macromolecules was made by the method of Martin and Ames [14]. Bovine serum albumin ($S_{20,w} = 4.6$), yeast alcohol dehydrogenase ($S_{20,w} = 7.4$), and catalase ($S_{20,w} = 11.3$) were used as standards.

Intranuclear binding. Costovertebral glands were homogenized in the same manner as described above, except for the use of 0.32 M sucrose containing 1.5 mM EDTA and 20 mM Tris–HCl, pH 7.4. The homogenate was incubated with labeled steroids for

The following trivial names are used: dihydrotestosterone=17β-hydroxy-5α-androstan-3-one; cyproterone acetate=1,2α-methylene-6-chloro-4,6-pregnadien-17α-ol-3,20-dione 17α-acetate: Sch 13521=α,α,α-trifluoro-2-methyl-4′-nitro-m-propionotoluidide.

1.5 h at 0°C, and it underwent further incubation for 30 min at 25°C. After these incubation procedures, crude nuclear fraction was obtained by centrifugation at 600 *g* for 10 min at 3°C; washed once with 3 ml of the 0.25 M sucrose solution containing 1 mM $CaCl_2$, 0.02 M Tris–HCl, pH 7.4, and 0.25% Triton X-100; and once again washed with the same sucrose solution without Triton. Nuclei were extracted with 0.4 M KCl solution by a modification of the method of Bruchovsky and Wilson[15] as previously delineated by us [11]. For gel-filtration with Sephadex G-100, a column of 2.5 × 37 cm in size was used, and elution was performed with 0.02 M Tris–HCl buffer, pH 7.4, containing 0.4 M KCl and 5.0×10^{-5} M EDTA. In some experiments, the homogenates were sonicated for 20 s, sedimented before incubation in a refrigerated centrifuge at 600 *g* for 10 min, and cell nuclei were further purified by the method of Maggio *et al.*[16] as depicted in our previous report [11]. Nuclei were incubated with the 600 *g* supernatant which had been prelabeled by the incubation with [^{3}H]-DHT (1×10^{-8} M) for 1.5 h at 0°C. In addition, with a view to knowing whether DHT was metabolized during the incubation described above, steroids were extracted from the cytosol and nuclear fraction with chloroform–methanol (2:1, v/v) and analyzed by thin-layer chromatography as described previously [11]. Radiochemical purity of DHT and 5α-androstane-3α,17β-diol was confirmed by recrystallization to constant S.A. New England Nuclear, Boston, was the supplier of [1,2-^{3}H]-5α-dihydrotestosterone ([^{3}H]-DHT) (S.A. 49 Ci/mmol), [1,2,6,7-^{3}H]-testosterone (S.A. 85 Ci/mmol) and [6,7-^{3}H]-17β-estradiol (S.A. 48 Ci/mmol).

RESULTS

I. Cytosol receptor

When cytosol (1.5–2.5 mg protein/ml) was incubated with [^{3}H]-DHT without concentrating, it often failed to show a distinct peak, but instead only a small shoulder was obtained in the 7–10*S* region by sucrose density gradient centrifugation. On the other hand, when a concentrated cytosol fraction usually containing 4–6 mg protein/ml was used, a sharp peak was consistently seen at 7–8*S* (Fig. 1). Therefore, cytosol was concentrated before incubation in the following experiment. A small 4–5*S* shoulder was often observed. This shoulder may, at least partly, represent contamination of the serum albumin, since hamster serum incubated with [^{3}H]-DHT showed a sharp peak in the 4–5*S* region, but no peak in the 7–8*S*.

Binding affinity and specificity. The cytosol was incubated with various concentrations of [^{3}H]-DHT. From Scatchard plots [19] of the data (Fig. 2), the dissociation constant (K_D) was calculated to be about 1.5×10^{-9} M.

In order to test the specificity of androgen binding to the 7–8*S* component, we incubated cytosol with [^{3}H]-testosterone and [^{3}H]-17β-estradiol. The test

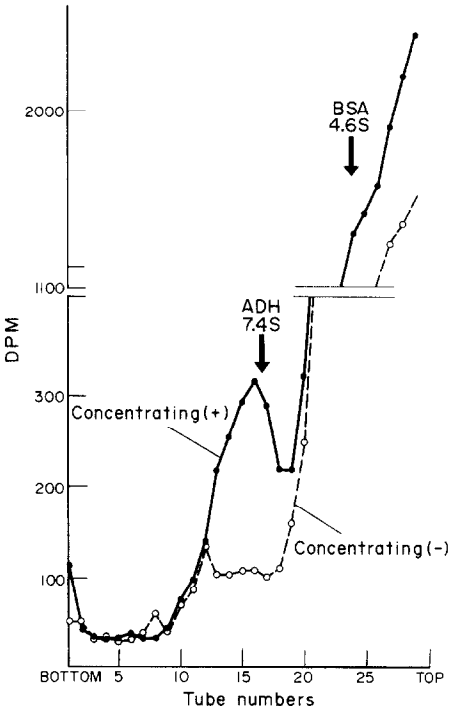

Fig. 1. Sedimentation of DHT–receptor complex in the cytosol fraction by sucrose gradient centrifugation. After incubation with 1.4×10^{-8} M of [^{3}H]-DHT, the cytosol fraction was treated with charcoal–dextran, layered on a sucrose gradient (5–20%, linear) and centrifuged. ●——● The cytosol was concentrated before incubation; 4.22 mg protein/ml. O---O: Not concentrated; 2.10 mg protein/ml. BSA: Bovine serum albumin. ADH: Alcohol dehydrogenase.

revealed that testosterone is bound to a much lesser extent (20% of DHT), and that 17β-estradiol has no affinity for 7–8*S* component at 1×10^{-8} M. Besides, various steroids and anti-androgens as shown in

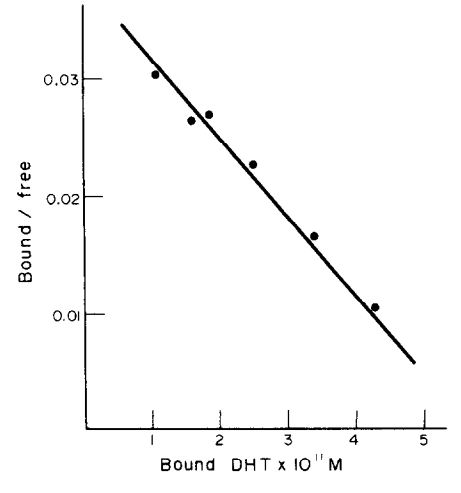

Fig. 2. Scatchard plots of [^{3}H]-DHT binding to the cytosol of hamster sebaceous glands. The cytosol (4.1–7.0 mg protein/ml) was incubated with [^{3}H]-DHT (0.4×10^{-9}–2.0×10^{-9} M), and analyzed by sucrose density gradient centrifugation.

Table 1 were incubated with cytosol containing 1×10^{-8} M of [^{3}H]-DHT. The depression by 2×10^{-7} M of DHT was almost complete, and testosterone at the same concentration also strongly inhibited the binding. 17β-Estradiol moderately decreased the binding, whereas other steroids thus far tested did not affect the binding significantly. Cyproterone acetate remarkably inhibited the binding, whereas Sch 13521, a potent antiandrogen [20], hardly curbed it even at 100-fold excess.

Binding of various hormones to skin cytosol. Sex hormones are known to affect almost every tissue. In this respect, we investigated whether adjacent dorsal skin also possesses similar binding components for DHT, testosterone, and 17β-estradiol. Employed for the investigation was the skin cytosol (3.75 mg protein/ml) incubated with each of these radioactive steroids at 1×10^{-8} M. Consequently, however, the sucrose gradient centrifugation evidenced that none of these hormones was bound to the 7–8*S* component.

II. Intranuclear binding

Elution pattern of nuclear extract on Sephadex G-100. When the nuclear extract prepared from the labeled homogenate was filtrated through Sephadex G-100, three peaks of radioactivity appeared (Fig. 3). The first peak was in the void volume, the second one corresponded to the elution of chymotrypsinogen A, and the third one accounted for the free radioactive steroid. Thus, the first and the second peaks were considered to be bound to macromolecules. The filtration through Sephadex G-100 of the cytoplasmic fraction (600 ***g***, supernatant) gave only two peaks, one eluted with the void volume and the other was the free steroid. This pattern was always observable either in the presence or in the absence of 0.4 M KCl. Therefore, it may be said at least that the second peak in the elutes of nuclear extracts was not due to mere contamination of the cytoplasmic fraction. Besides,

Table 1. Effects of various non-radioactive steroids and anti-androgens on [^{3}H]-DHT binding in the cytosol fraction

Non-radioactive steroid	Conc. (μM)	% Decrease in binding
DHT	0.2	>90
Testosterone	0.2	66
17β-Estradiol	0.1	37
Progesterone	0.1	16
5α-Androstane-3α,17β-Diol	0.1	0
Cyproterone acetate	0.1	71
Sch 13521	1	20
Androstenedione	1	14
Androsterone	1	3
Cortisol	1	10

The cytosol fraction (3.8–5.7 mg protein/ml) was incubated with [^{3}H]-DHT ($0.9–1.2 \times 10^{-8}$ M) in the presence of a non-radioactive steroid or anti-androgen and subjected to sucrose gradient centrifugation. Radioactivity peaks migrating to 7–8*S* were compared.

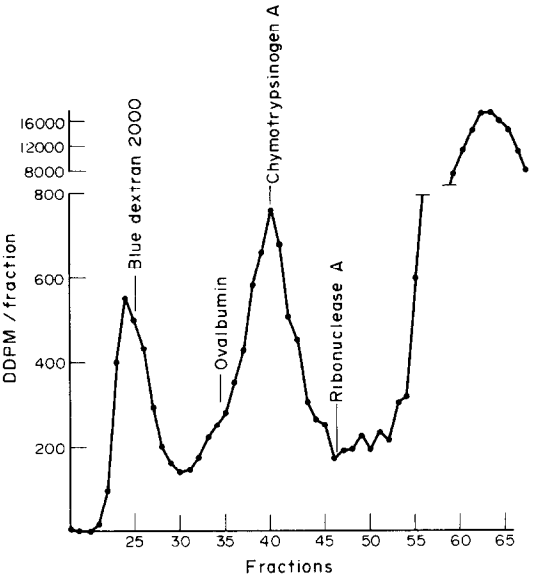

Fig. 3. Gel-filtration of nuclear extracts on a Sephadex G-100 column. The bed volume was 179 ml. The flow rate was 15–20 ml/h, ascending, and 3 ml fractions were collected.

we studied whether DHT metabolism occurred, since the homogenate was incubated with [^{3}H]-DHT at 25°C for 30 min. More than 50% of the radioactivity was recovered as unchanged DHT both in the cytosol and nuclear fraction at the end of the incubation. The major metabolite was 5α-androstane-3α,17β-diol in both fractions (21% in the cysosol and 12% in the nuclear fraction). Therefore, an ample amount of DHT was available for binding during the incubation.

Binding specificity. First, the homogenate was incubated with [^{3}H]-DHT at six different concentrations in the range from 0.13 to 2.3×10^{-8} M in order to examine whether the binding was saturable or not. The second peak was saturated at 0.7×10^{-8} M of DHT, whereas the radioactivity of the first peak increased linearly in proportion to the substrate concentration until a level of 2.3×10^{-8} M was obtained. In addition, inclusion of 100-fold excess of unlabeled DHT in the incubation decreased the second peak to an undetectable level, but the first peak only to 40%. These results indicate that the second peak is saturable with DHT, while the first peak has a much larger capacity for the steroid.

Second, the effect of antiandrogens on the binding was studied (Table 2). When the homogenate was incubated with 1×10^{-8} M of [^{3}H]-DHT in the presence of 1×10^{-6} M of cyproterone acetate, the radioactivity of the second peak was decreased to less than 25% of the control on a protein basis, whereas that of the first peak only to 50%. Sch 13521 at 1×10^{-6} M did not significantly affect the radioactivity of either peak as in the case of cytosol. Besides, incubation with 1×10^{-8} M of [^{3}H]-testosterone did not result in the formation of the second peak, but yielding the first and third ones only.

Table 2. Effects of antiandrogens on the formation of [^{3}H]-DHT–macromolecule complex by cell nuclei

	Radioactivity (d.p.m./mg protein)	
	Peak I	Peak II
Experiment 1		
Control	901	1920
Cyproterone acetate	481	405
Experiment 2		
Control	870	835
Sch 13521	807	850

The homogenates were incubated with 1×10^{-8} M of [^{3}H]-DHT for 1.5 h at 0°C and further for 30 min at 25°C. Crude nuclei were isolated by centrifugation at 600 *g* for 10 min, and the extract from the nuclei was filtered through Sephadex G-100. Added to the homogenates was 1×10^{-6} M of cyproterone acetate or Sch 13521, if used.

These results shown above indicate that the second peak represents the specific binding of DHT, whereas the binding in the first peak is probably nonspecific.

Temperature dependency of the intranuclear binding. The cytoplasmic fraction (600 *g*, supernatant) previously exposed to 1×10^{-8} M of [^{3}H]-DHT was incubated with the purified nuclei at 0°C or 25°C for 30 min. The radioactivity of the second peak was nearly three times higher at 25°C than at 0°C. Similar effects of temperature on DHT retention were reported before [3, 21]. When the whole homogenate was incubated with [^{3}H]-DHT or crude nuclear fraction was used instead of purified nuclei, such difference of incorporation by temperature could not be observed.

Requirement of cytoplasmic factors on the intranuclear binding (Table 3). When purified nuclei were incubated with the previously labeled cytoplasmic fraction of the sebaceous gland, the nuclear extract showed a significant radioactivity of the second peak in the Sephadex gel-filtration. On the other hand, incubation of the nuclei alone with [^{3}H]-DHT or liver cytoplasmic fraction did not bring about the specific binding characterized by the second peak. The radioactivity of the first peak did not significantly change by the presence or absence of the cytoplasmic fraction. These findings suggested a possible involvement of the cytoplasmic factors in the specific intranuclear binding, as in the prostate [22]. When crude nuclei were used instead of purified nuclei, even incubation with [^{3}H]-DHT alone led to the formation of the second peak.

Table 3. Retention of [^{3}H]-DHT–macromolecule complex by cell nuclei

Sources of preparation (Cytoplasm)	(Nuclei)	Radioactivity associated with peak II (d.p.m./mg DNA)
Sebaceous gland	Sebaceous gland	1120
None	Sebaceous gland	0
Liver	Sebaceous gland	56
Sebaceous gland	Liver	72

The cytoplasmic fraction (600 *g* supernatant) prepared from either sebaceous glands or liver (1 g wet weight each) was incubated with 1×10^{-8} M of [^{3}H]-DHT at 0°C for 1.5 h, mixed with purified nuclei and kept at 25°C for 30 min. Alternatively, the nuclei of sebaceous glands were incubated with a buffer containing [^{3}H]-DHT at 0°C for 30 min and further at 25°C for 30 min. Nuclei were reisolated by centrifugation at 600 *g*, 10 min and the extract from them were analyzed by Sephadex G-100 column.

DISCUSSION

It is difficult to obtain even from large specialized sebaceous glands a sufficient quantity of the material required for the androgen-receptor assay. Concentration of the cytosol fraction with a dialysis membrane made it easier to detect androgen binding with a relatively small amount of the sebaceous glands. Although Eppenberger *et al.*[23] demonstrated the presence of androgen-binding components in the rat dorsal skin, we could not find them in the adjacent dorsal skin of hamsters. Therefore, the 7–8*S* cytosol receptors are not contained in an appreciable amount in the other components of the skin such as hair follicles, epidermis, and connective tissues, but appear to be specific to sebaceous glands.

Generally, these characteristics of the cytosol receptors of hamster sebaceous glands shown in the present study are similar to those of the rat prostate. The dissociation constant is the same order of magnitude as that found for the DHT receptors [24, 25, 26]. The competition data reported here are in fairly good agreement with those for the prostate [26]. The major discrepancy between the present data and previous reports on sebaceous glands is in the relative strength of competition by testosterone, which was proved not to compete with [^{3}H]-DHT in the binding to cytosol [10].

The elution pattern of the nuclear extracts on Sephadex G-100 well coincides with the findings of our previous *in vivo* study [11]. The both experiments suggest that the second peak represents specific binding to the androgen. The rat prostate reportedly shows a similar pattern of Sephadex G-200 [27]. The radioactivities both of the first and the second peaks varied moderately from experiment to experiment. This is probably ascribable to rather inconstant results of homogenization of skin tissues which are extremely tough. In addition to homogenization, sonication was required in order to recover a sufficient amount of purified nuclei. Purification decreased the binding ability to nearly one third of that of the crude nuclei when compared on a protein basis.

When the whole homogenate or crude nuclear fraction was used instead of purified nuclei, the specific intranuclear binding was similar at 0°C or 25°C. Adachi[10] also showed the intranuclear binding at 0°C using hamster sebaceous glands. The result suggests

that DHT could be incorporated into the nuclei even at 0°C, if some factors in the cytoplasm are contaminated with nuclei.

Sch 13521 showed little inhibitory effects on androgen binding by cytosol and nuclear components even at 100-fold excess. These outcomes are somewhat surprising, since the previous work [20] demonstrated that this drug owes its antiandrogenic effect to an interference with intracellular binding. This discrepancy is not due to the ineffectiveness of the drug on sebaceous glands, because topical application of it conspicuously reduced the size of hamster sebaceous glands [28].

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