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Physiopathological Role of Bald-Scalp Cytosolic Proteins*

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The physiopathological role of androgen binding proteins in male pattern baldness (MPB) has been studied by using tritiated dihydrotestosterone (DHT) and methyltrienolone (R 1881) as ligands. DHT binding in bald scalp from subjects suffering from MPB is high (53 ± 12 fmol/mg protein) in cytosol obtained from bald areas, being undetectable in hairy areas from the same subject. Since methyltrienolone does not bind in bald scalp cytosol, there must be no specific DHT receptor in this material. Several kinetic and molecular parameters of DHT binding in bald scalp cytosol and serum were similar in both samples. Only the association rate constant (k_{+1}) was significantly higher in serum (8.8×10^6 M^{-1} min^{-1}) than in cytosol (3.08×10^6 M^{-1} min^{-1}). DHT binding in serum as well as the evaluation of plasma contamination in the skin samples (by nephelometric analysis) strongly suggests that DHT binding in skin cytosol is merely due to the presence of contaminating SHBG but it does not explain the lack of DHT binding in non bald areas. Thus, the possibility arises of there being a specific mechanism for the uptake of the plasmatic testosterone SHBG-complex taking place only in the hypertrophic sebaceous gland as well as the existence of active T metabolites other than DHT, probably 3β-androstanediol.

Key words: Male pattern baldness, Cytosolic proteins, Androgen binding.

The direct influence of androgens on the sebaceous gland and their role as supporters of male pattern baldness (MPB), were demonstrated by HAMILTON (6, 7). In MPB a genetic predisposition to the follicular atrophy and to the sebaceous gland hypertrophy is generally accepted; such disturbances are mainly located in the frontoparietal areas of the scalp. This localization has permitted the successful technique of hair autotransplantation from the hairy-occipital donor area to the alopecic frontal one (13). Towards the end of the nineteen-sixties, several authors demonstrated the metabolic conver-

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sion of testosterone (T) into dihydrotestosterone (DHT) by the action of 5-α-reductase (5AR) in several tissues, especially in those androgen-dependent (prostate, testis, seminal vesicles, etc.) but also in other tissues, including skin. These findings, along with the existence of specific DHT binding proteins, demonstrated that DHT rather than T was the active form of the androgenic hormone in most tissues. Thus, in MPB, an excess of DHT could be responsible for the maintenance of alopecia, at least as one of the multiple factors probably involved.

The human bald scalp is an interesting model, not only for the clarification of the etiology of baldness itself, but also because the study of the physiopathology of the androgenic actions can be made from a general point of view.

Very little is known about the androgen receptor in the skin, and much care is necessary in the interpretation of the results obtained, as happens in other tissues, because of the presence in the cytosolic fraction of a specific plasma sex hormone binding globulin (SHBG) which binds androgens with similar affinity but higher capacity as compared to that of the androgen receptor (9). To avoid this problem, synthetic steroids have been widely used for the study of specific steroidal receptors. For the androgenic receptor research, the synthetic radiolabelled androgen metyltrienolone (R 1881) is a useful ligand (1) because it does not bind to the SHBG.

Our aim was to demonstrate the presence of DHT receptor in the bald and non-bald scalp areas of patients affected by MPB, and eventually to find significant differences between both areas.

The results of DHT and R 1881 binding on both the cytosolic fraction of hypertrophic sebaceous glands from MPB and serum are presented here. Several molecular and kinetic parameters concerning DHT binding were also done on both samples.

Materials and Methods

Specimens. — Scalp biopsies were obtained from patients affected by MPB, who had been subjected to hair autotransplantation. Pieces were obtained with an Orentreich punch (n.° 4.25). Some control and comparative experiments were also carried out in human and bull prostate. Human prostate was obtained from patients with benign adenoma; bull prostate was purchased from the slaughterhouse. In all cases, tissues were rinsed with saline and kept frozen at −70°C until processed (not longer than three months). Serum of four bald patients and other healthy controls was also used in some binding experiments.

Chemicals. — 5-α-dihydro (1-α-2-α-(n)-^{3}H) testosterone (60 Ci/mmol) (^{3}H-DHT) was purchased from the Radiochemical Centre (Amersham) as well as (1, 2, 6, 7-^{3}H) testosterone (103 ci/mmol) (^{3}H-T). (17—α methyl— ^{3}H) R 1881 (87 Ci/mmol) and unlabelled R 1881 were obtained from New England Nuclear. Unlabelled steroids were purchased from Sigma. DEAE-Sephacel and Concanavalin-4B-Sepharose (Con-A), were purchased from Pharmacia Fine Chemicals. The purity of the labelled steroids was checked by thin layer chromatography. All the other reagents were of analytical grade.

Analysis of DHT binding. — a) General method: We used the method described by WILSON and FRENCH (20) modified. Scalp and prostate biopsies were minced and homogenized in a Sorvall Omni-Mixer with buffer in a 1:4 ratio (w/v). The buffer employed was: Tris-HCl 20 mM, EDTA 1.5 mM, pH 7.4. Na_2MoO_4 10 mM was added when R 1881 binding was studied. Homogenates were filtered through a gauze and centrifuged at 105,000 × *g* for 65 min in a Beckman L5-65 ultracentrifuge, with a

fixed-angle Type R-40 rotor. Supernatants were carefully removed by aspiration to avoid contamination with fat particles. The cytosol obtained was used immediately.

In serum experiments, native serum was diluted 1 to 8. Aliquots were taken for protein determination by Lowry assay. Both cytosol (1.5-2.5 mg protein/ml) and serum (8-10 mg protein/ml) were treated with mercaptoethanol (1 mM final concentration), and 500 μl were incubated at 4°C with labelled steroid in the presence or absence of a 1,000 fold excess of unlabelled steroid. Some serum experiments were also performed in a range of protein concentration from 0.9 to 39 mg/ml. For the R 1881 binding assay, triamcinolone (1,000 fold) was added in order to avoid the R 1881 binding into the progesterone receptor. At the end of the incubation period, 500 μl of charcoal suspension (Norit A 1% in 0.1% solution of Dextran T-70 in Tris-HCl 10 mM, EDTA 1 mM buffer at pH 7.5) were added to each incubation tube, which were then shaken for 20 min in the cold room and afterwards centrifuged at 2,000 × g for 20 min in a cool centrifuge. Charcoal supernatant fractions (400 μl) were transferred to a scintillation plastic vial containing commercial scintillator (Unisolve, Koch-Light). CPM were obtained in a Packard Tri-Carb 3255 spectrometer and transformed in DPM by the analysis of the counting efficiency. Specific binding was calculated by the difference of radioactivity between samples containing only labelled steroid and those also containing unlabelled steroid. Each experimental point was done in duplicate.

b) Association binding assay. Cytosol or serum (500 μl) were incubated with 25 nM ^{3}H-DHT in absence or presence of 25 μM DHT. Radioactivity of bound steroid was determined at different periods of time (ranging from 5 min to 24 hours).

c) Dissociation binding assay. Cytosol or serum, prepared as described above, was incubated with ^{3}H-DHT 25 nM for 60 min at 4°C; three tubes also contained a 1,000-fold excess of unlabelled DHT. The total specific binding in those three tubes was calculated. A 1,000-fold excess of unlabelled DHT was added to the rest of the tubes, and the incubation was continued for several periods of time (1 to 8 min). Bound radioactivity at the end of each period of time was determined using charcoal adsorption assay.

d) Determination of kinetic constants. All the kinetic parameters described in this paper have been measured at 4°C. Equilibrium dissociation constant was calculated from the Scatchard plot of saturation data. Dissociation rate constant (k_{-1}) was calculated from half time of the dissociation reaction. Association rate constant (k_{+1}) was determined from the reversible second order equation:

$$\ln\left[\frac{B_{max}\left[(F) - B_t \cdot \frac{B_{max}}{n}\right]}{F\,(B_{max} - B_t)}\right] = k_{+1}\,t\left[\left[(F)\frac{n}{B_{max}}\right] - B_{max}\right]$$

where: B_{max} is the maximum binding capacity; F is the molar free DHT concentration; B_t is the binding at the time «t»; n = maximum number of binding sites; t is the time in minutes (4).

Sucrose density gradients centrifugation of DHT-protein complexes. — Both cytosol and serum were partially purified with DEAE-Sephacel. The starting sample was loaded into a DEAE-Sephacel minicolumn (5 × 70 mm) equilibrated with buffer Tris-HCl 20 mM, EDTA 1,5 mM, pH 7.4. The column was then washed with two bed volumes of the same buffer.

Retained proteins were then eluted with 0.7 M NaCl in the same buffer. Fractions eluted from DEAE-Sephacel with NaCl were incubated with ^{3}H-DHT 25 nM in the presence or absence of a 1,000-fold excess of unlabelled steroid for 18 hours at 4°C. Labelled fractions were incubated with charcoal suspension for 20 min at 4°C to remove free steroid immediately before its application to the gradient.

Aliquots were layered on 3.8 ml linear 5 to 20% (w/v) sucrose gradients containing 10% glycerol, Tris-HCl 20 mM, EDTA 1.5 mM, pH 7.4. Gradients were checked by the addition of potassium dichromate to the sucrose solution and reading the different fractions at 370 nm.

Gradients were centrifuged at 4°C in a Beckman L5-65 ultracentrifuge using a SW 60 type rotor for 18 hours at 55,000 rpm. At the end of this time, 200 μl fractions were collected from the top of the tube and radioactivity was measured.

Tubes containing standards of human serum albumin (S 4.6) and globulin (S 7.12) were also run at the same time as the samples.

Protein was determined by the Lowry assay.

Con-A Sepharose fractionation of the cytosol and human serum. — To obtain Con-A-binding material, the method described by NISULA *et al.* (12) was used, with some modifications. Cytosol or serum (4 ml) were incubated with 25 mg of Con-A-4B-Sepharose (dry weight equivalence) in Tris-HCl 20 mM, EDTA 1.5 mM, pH 7.4 buffer, in a final volume of 7 ml for one hour at room temperature and continuously stirred. At the end of the incubation period, this mixture was poured into a small glass column (70 × 5 mm) stoppered with glass-wool. The material not retained was immediately collected and 5 ml of buffer were added to rinse the excess of this non retained material.

The Con-A Sepharose was taken out of the glass column and again poured into a small beaker with 3 ml 0.4 M glucopyranoside in Tris-HCl buffer. The mixture was stirred for 1 hour at room temperature and at the end of this period, it was poured into a small glass column. The Con-A binding proteins which were now detached by the glucopyranoside, were collected from the column. Both, Con-A retained proteins and non retained proteins, were assayed for DHT specific binding, as described previously.

Determination of plasma contamination. — IgG, IgA and IgM were measured in scalp cytosol and in serum immunonephelometry, using an automated immunochemistry system (Auto ICS) Beckman. The system consists of a nephelometer to measure the rate of light scatter formation resulting from an immunoprecipitin reaction. In some cases, both samples were obtained from the same subject.

Results

DHT binding. — Figure 1 shows specific ^{3}H-DHT binding to cytosol in the bald area from 20 patients and the corresponding Scatchard plot. A linear relationship indicates the presence of only a single class of high affinity binding sites, with a Kd value of $4.23 \pm 0.11 \times 10^{-9}$ M, and a maximum number of binding sites $n = 53 \pm 12$ fmol/mg protein.

There was no ^{3}H-DHT specific binding in the assays with cytosol from hairy-occipital donor area. These results were obtained in 8 patients using ^{3}H-DHT only at 25 nM concentration because of the difficulty of obtaining enough tissue to perform a complete curve.

The controls in human prostate from 10 patients revealed a number of binding sites $n = 56.5 \pm 13$ fmol/mg protein, with a Kd value of $2.46 \pm 0.13 \times 10^{-9}$ M, calculated through the Scatchard plot (not shown).

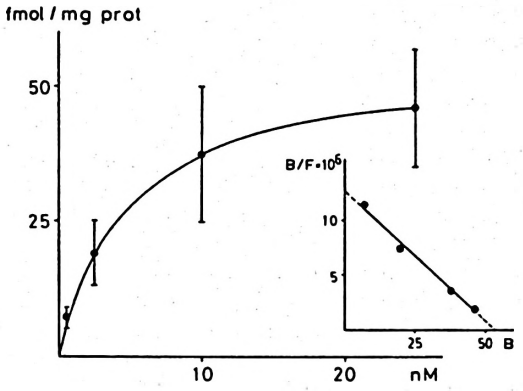

Fig. 1. *DHT binding in bald scalp cytosol.* Cytosol was incubated with ^{3}H-DHT as described in methods. Direct plot of DHT binding, mean values from 20 subjects. Ordinate: DHT specific binding; abscissa: molar concentration. Insert: Scatchard plot of the same results, ordinate: DHT bound to free ratio; abscissa: fmol of DHT bound/mg protein. Results ± S.D.

In serum, values for Kd and maximum binding sites were, respectively, 3.7 ± 0.18 × 10^{-9} M and 206 ± 18 fmol/mg protein. Those results were obtained in four bald patients (205 ± 21 fmol/mg protein) and 16 healthy controls (206 ± 17 fmol/mg protein). No statistical differences were observed between both groups.

DHT specific binding in serum follows a linear pattern in the range of protein concentrations used.

R 1881 specific binding. — There was no R 1881 binding in the bald and non-bald scalp areas of patients affected by MPB. Controls in 5 bull and 6 human prostates revealed a number of binding sites of 15.4 ± 1.9 and 21.9 ± 2.4 fmol/mg protein, respectively. No binding of R 1881 was observed in serum.

Testosterone-binding. — T binds to serum and cytosol to a lesser extent than does DHT. Taking the DHT binding as 100%, T binding to human serum represents only 16.32% ± 8.01 of that observed with DHT. This percentage observed in scalp cytosol is 19.13% ± 7.15.

Time-course of the binding of ^{3}H-DHT to cytosol. — The binding increases gradually with time until 30 min, and remains constant thereafter, with a slight decrease at the end of the incubation period.

The binding of ^{3}H-DHT to human serum gradually increases with time until 60 min, and remains constant until 24 hours.

Data from cytosol and serum were analyzed using Wilcoxon's test for the significance of differences between scalp cytosol and human serum. Significance was achieved at $p < 0.001$ (table I).

Dissociation of the ^{3}H-DHT-protein complex. — Dissociation follows first order reaction kinetics.

In human serum, dissociation also fol-

Table I. *Kinetic constants determined for the binding reactions of bald scalp cytosol and serum with ^{3}H-DHT.*

k_{+1}: association rate constant. $t_{1/2}$: half time of dissociation.

	$t_{1/2}$ (min)	k_{-1} (min^{-1})	k_{+1} ($min^{-1} M^{-1}$)
BALD SCALP CYTOSOL	5.3 ± 1.38 n = 9	0.141 ± 0.038 n = 9	(3.08 ± 1.32) 10^6 n = 6
SERUM	6.9 ± 3.15 n = 19	0.115 ± 0.043 n = 19	(8.80 ± 3.34) 10^6 n = 14

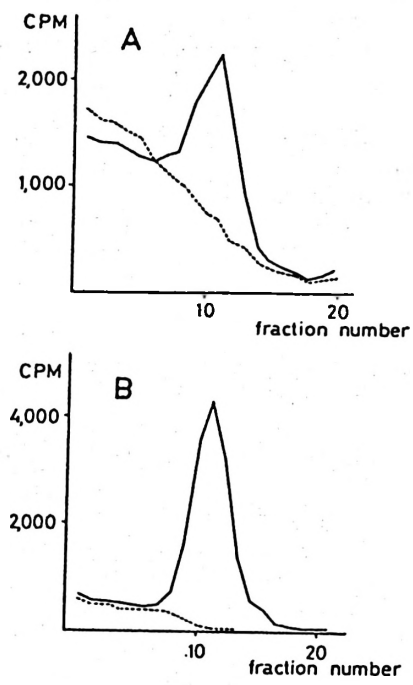

Fig. 2. *Sedimentation coefficient (S) of DHT-binding protein.*
DEAE-Sephacel purified cytosol or serum were incubated with 25 nM ^{3}H-DHT in absence (———) or in presence (— — —) of 25 μM DHT, for an 18 hour period. Free steroid was eliminated through charcoal adsorption and labelled proteins were layered (final protein concentration around 5 mg/ml) onto a linear sucrose gradient (see methods for details). Fraction 1 corresponds to the top of the gradient. A: bald scalp cytosol. B: serum.

lows first order kinetics. The differences observed between serum and cytosol were not significant (table I).

Sucrose density gradient centrifugation of DHT-protein complexes. — DEAE-Sephacel purification of both cytosol and serum allows the recovery of about 90% of specific binding in the NaCl eluted fraction. In the present experiments a 5-fold concentration for cytosol proteins was obtained, which was useful for the sucrose gradient. Such a concentration was not necessary for the serum proteins. Figure 2 shows the results for cytosol and serum, in both cases a peak for DHT-binding was obtained in the 4.6 S region.

Concanavalin A-4B-Sepharose fractionation. — No DHT binding was observed in the fraction which was not retained by the Con-A, either in serum or in cytosol. All the DHT binding material is retained by the Con-A. Glucopyranoside eluted material is able to bind DHT but to a lesser extent than crude preparations are. Thus, cytosol-Con-A binding proteins exhibit only 35.36 ± 7.5% of the original binding capacity; in serum, this value is 31.63 ± 6.05%. Glucopyranoside-incubated controls show the same binding capacity for DHT as do crude serum cytosol preparations.

Plasma contamination. — Table II shows the amount of immunoglobulins

Table II. *IgG, IgA and IgM were determined in four bald subjects, both in serum and in skin scalp cytosol.*
% refers to the percentatge of cytosol to serum.

	CYTOSOL			SERUM			%		
PATIENT	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM
1	80.2	24.7	11.0	9390	2230	1910	0.85	0.10	0.58
2	73.1	19.7	10.0	7230	1620	2460	1.01	1.21	0.40
3	28.2	10.0	10.0	6750	1200	1180	0.42	0.80	0.80
4	78.7	20.8	21.0	8180	1900	6830	0.96	1.10	0.30

present in the cytosol of bald scalp areas from 4 patients and their respective serums, as well as the calculated contamination percentages. As can be seen, those percentages are generally less than 1%. The amount of immunoglobulins determined in the cytosol of other patients also remained below 1% of the normal values expected in serum.

Discussion

Our results clearly demonstrate the high DHT binding in the bald region in MPB, in comparison with the absence of steroid binding in non bald areas. In fact, the number of binding sites for DHT in the bald areas is high in relation to the results obtained in other tissues (2, 5, 8).

The results obtained in human prostate using R 1881 as ligand, are in agreement with other studies previously reported (3, 16, 17). The kinetics obtained in human prostate using ^{3}H-DHT and ^{3}H-R 1881 also agree with other studies previously reported (10).

Since no specific R 1881 binding could be detected in skin cytosol it can be concluded that ^{3}H-DHT binding in cytosol samples is not due to an androgen receptor. Moreover, from the results obtained in serum a SHBG concentration of 15 nmol/l (DHT binding capacity) can be calculated. As serum contamination in bald scalp cytosol ranges about 1% (nephelometric determinations), a DHT binding capacity of cytosol of some 75 fmol/mg protein (taking a mean value of 2 mg protein/ml) can be supposed. As a binding capacity has been found in bald scalp cytosol of 50 fmol DHT/mg protein, it can be concluded that this binding is merely due to the plasma contamination.

In kinetic studies the only significant difference observed between both specimens lies in the rate constant for association. This process appears to be faster in serum than in scalp cytosol. Since, to the best of our knowledge, this kind of test has not been undertaken by other authors, the present results obtained with scalp cytosol cannot be compared. The results obtained in human serum are in agreement with those reported by DANZO *et al.* and HASSON *et al.* (11): thus, they found 4.5 and 5.2 min respectively, for the $t_{1/2}$ for dissociation in rabbit serum, using DHT as a marker. When testosterone is used as specific marker, a $t_{1/2}$ of 12.8 min is found in human serum (15).

The values obtained in all cases for the sedimentation coefficient S = 4.6 correspond with SHBG fraction and agree with previously reported results (14).

In our conditions, T binds to serum and cytosol to a lesser extent than does DHT, and these results are also identical in both specimens. In scalp cytosol T binding represents 19.13% of DHT binding and in serum this value amounts to 16.32%. These values are lower than those reported by VERMEULEN and VERDONCK (19), who found values for T binding in human serum of 30% of those obtained with DHT; nevertheless, their value refers to only one determination and, in any case, it would be in agreement with the highest value of those obtained here.

Concerning the results obtained with human serum with Con-A Sepharose, those obtained are in agreement with those reported (12). The glucopyranoside eluted material binds DHT in the same manner in both serum and cytosol.

All these similarities lead to the conclusion that protein responsible for specific DHT binding observed in scalp cytosol is the same as that present in serum, namely SHBG. The different K_{+1} may be due to a small modification of the protein during its entry into the scalp tissue. The greater protein concentration in our serum samples does not modify the specific

DHT binding under the equilibrium conditions of our experimentation, but it could explain this higher value for k_{+1} observed in serum.

In other experiments (not shown here) carried out in plucked anagen follicles, no specific binding was observed either with DHT or with R 1881. In fact, if the binding results presented here had taken place in the follicular cells rather than in sebaceous gland, higher binding in non-bald areas would have been observed, but this is not the case. Hence it can be accepted that the DHT binding observed must take place in the sebaceous gland.

However, if this specific binding is only due to a simple contamination process, the lack of DHT binding in the non alopecic areas, cannot be explained, since the blood flow in it is similar or even greater than in alopecic areas. On the other hand, Ig determinations are not specific enough to evaluate the plasmatic contamination, since Ig are also produced locally. Thus, a specific DHT uptake mechanism in the hypertrophic sebaceous gland cannot be discarded, leading us to pose the following question: what is the exact role of the uptake of the circulating SHBG in the sebaceous gland if no specific DHT receptor has been revealed? In this sense, it is necessary to account that DHT is converted into 3-α-and 3-β-androstanediols by means of a 3-hydroxy-steroid-dehydrogenase system. In our previous studies (not shown here) a high conversion of DHT into androstanediols in bald scalp homogenates has been observed. On the other hand TAKAYASU *et al.* (18) have also found a high conversion of DHT into 3-β-androstanediol in several tissues which have high sebaceous activity. It can then be hypothesized that the excessive presence of SHBG, probably carrying circulating T, is the first step in the stablishment of the sebaceous hypertrophy and androstanediol but not that DHT is the active T metabolite in bald scalp cytosol.

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Resumen

Para valorar el papel fisiopatológico de las proteínas fijadoras de andrógenos en la alopecia seborreica masculina (ASM), se han realizado diversos estudios utilizando dihidrotestosterona (DHT) y metiltrienolona (R 1881) tritiadas. La fracción soluble (FS) de cuero cabelludo de pacientes con ASM presenta una elevada capacidad de fijación de DHT, del orden de 53 ± 12 fmol/mg de proteína en las zonas alopécicas con hipertrofia sebácea, siendo nula en las zonas no alopécicas. La no fijación de R 1881 en FS excluye la existencia de un verdadero receptor de DHT. La comparación de diversos parámetros cinéticos y moleculares de la fijación de DHT en suero y FS demuestran una diferencia significativa en los valores de k_{+1}; en cambio, no existen diferencias entre ambas muestras en lo que se refiere al resto de los parámetros estudiados. La fijación de DHT obtenida en suero y la contaminación plasmática de las muestras de piel, determinada por nefelometría de inmunoglobulinas, sugieren que la fijación de DHT puede deberse a una simple contaminación por la proteína plasmática fijadora de esteroides (SHBG) pero no explica la ausencia de fijación de DHT en piel no alopécica. Por todo ello, se sugiere la posibilidad de una captación específica de SHBG cargada de testosterona por parte de la glándula sebácea hipertrófica y la existencia de metabolitos activos distintos de la DHT, probablemente el beta-androstanodiol.

Palabras clave: Alopecía seborreica, Proteínas solubles, Fijación de andrógenos.

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