Binding of dihydrotestosterone to a nuclear-envelope fraction from the male rat liver

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Intact nuclear 'ghosts' containing small amounts of DNA were obtained from rat liver. Incubation of radiolabelled dihydrotestosterone with isolated nuclear-envelope fraction from male rat liver resulted in specific binding of the dihydrotestosterone to the membranes. Optimal binding occurred at 20°C after 20 h incubation. Storage for 2 weeks at –80°C resulted in little loss of specific binding. Scatchard analysis revealed a class of binding sites with a K_D of 23.2 nM. Pronase and heat treatment destroyed the binding site. Androgens and glucocorticoids competed for labelled dihydrotestosterone binding to the ghosts, whereas oestrogens did not compete. Castration 24 h before preparation of ghosts did not alter the binding site, and a similar class of binding sites was identified on female rat liver nuclear envelopes.

Most steroid hormones act by the same intracellular mechanism (Gorski & Gannon, 1976; Chan & O'Malley, 1976*a,b*). After binding to a cytosolic receptor protein present only in target tissues, the steroid–receptor complex translocates to the nucleus, where its interaction at the genome results in altered cellular function. For continued hormone action, the steroid and the receptor are recycled into the cytoplasm, or degradation of the receptor occurs and more receptor is synthesized.

Clearly the steroid–receptor complex must interact with the nuclear envelope during translocation into the nucleus and possibly during its recycling into the cytoplasm. This interaction may be a point where control of steroid-hormone action is exerted. We wish to determine the molecular mechanisms by which steroids traverse the nuclear envelope, and have established that a nuclear-envelope fraction isolated after heparin treatment of purified nuclei from the rat ventral prostate contains a saturable high-affinity binding site which is specific for androgens and which is not present in nuclei prepared from 24 h-castrated animals (Lefebvre & Novosad, 1980). We report here the characteristics of a dihydrotestosterone-binding site on a male rat liver nuclear-envelope fraction prepared by the method of Harris & Milne (1974).

Materials and methods

Animals

Adult male and female rats (250–350 g) were purchased from Woodlyn Laboratories, Guelph, Ontario, Canada, and maintained on a diet of Wayne Lab Blox (Allied Mills, Chicago, IL, U.S.A.) and water *ad libitum*. The rats were killed by decapitation and the livers quickly removed, rinsed in 10 mM-Tris/HCl, pH 7.4, containing 2 mM-MgCl₂ and 0.25 M-sucrose (homogenization buffer), stripped of connective tissue, placed on ice and weighed.

Castration of male rats, where indicated, was performed via the scrotal route under diethyl ether anaesthesia.

Preparation of nuclear envelopes

Purified nuclear envelopes were obtained by slight modifications of the method of Harris & Milne (1974). All procedures were performed at 4°C, unless stated otherwise. Purified nuclei were obtained from the livers after homogenization in the homogenization buffer with a Polytron (Brinkmann Instruments, Rexdale, Ontario, Canada) and sedimentation of the 7000 g nuclear pellet through 2.2 M-sucrose in 10 mM-Tris/HCl, pH 7.4, containing 2 mM-MgCl₂, at 100 000 g for 1.5 h in a Beckman

L2-65B ultracentrifuge (SW27 rotor, r_{av} 11.6 cm). The nuclear pellets were then washed twice at 3000 *g* for 5 min in the homogenization buffer. The nuclei obtained by this method were relatively free of cytoplasmic tags as monitored by phase-contrast microscopy, and the combined pellets of purified nuclei were taken as the starting material for the isolation of nuclear envelope.

The nuclei were suspended in 1 mM-NaHCO₃, pH 7.2 (40 ml/20 g starting wt. of tissue), by syringing through a large-bore needle and allowed to equilibrate for 5 min before centrifuging at 33 000 *g* for 5 min in a Sorvall RC-2B centrifuge (SS-34 rotor, r_{av} 4.25 cm). Each pellet was again resuspended in a small volume of 1 mM-NaHCO₃ by syringing before being adjusted to the former volume and re-centrifuged as above after a further equilibration period of 5 min. The gelatinous swollen pellet was dispersed by the addition of 1 mM-NaHCO₃ containing deoxyribonuclease I (Sigma Chemical Co., St. Louis, MO, U.S.A.; type DN-100) to give 40 ml of suspension/20 g of starting material with an enzyme concentration of 10 µg/ml. The suspension was then incubated at room temperature (20°C) for 20 min. The nuclear envelopes were washed twice in the same volume of 1 mM-NaHCO₃, pH 7.2, by centrifugation for 5 min. Each pellet was resuspended in 2 ml of 1 mM-NaHCO₃ and centrifuged at 500 *g* for 5 min. The supernatant was layered over a discontinuous sucrose gradient made up of 10 ml of 2.0 M-sucrose, 10 ml of 1.8 M-sucrose, 10 ml of 1.5 M-sucrose and 6 ml of 0.25 M-sucrose (all solutions made up in 10 mM-Tris/HCl, pH 7.4). The gradient was then centrifuged for 90 min at 100 000 *g* in a Beckman L2-65B ultracentrifuge (SW27 rotor, r_{av} 11.6). The major band of purified nuclear envelope was removed from the gradients with a Pasteur pipette and washed twice in 10 mM-Tris/HCl, pH 7.4, at 33 000 *g* for 5 min.

Assay of dihydrotestosterone binding to purified nuclear envelopes from rat liver

Nuclear envelopes purified from the rat liver (200–400 µg of protein/assay tube) were incubated for the times and at the temperatures specified in a final volume of 0.25 ml of 10 mM-Tris/HCl, pH 7.4, containing radioactive dihydrotestosterone. To a duplicate tube, 100-fold excess of unlabelled dihydrotestosterone was also added to determine non-specific binding. The reaction was stopped by centrifugation in a Microfuge B (Beckman Instruments, Edmonton, Alberta, Canada) and the supernatant was removed with a Pasteur pipette. The top of the pellet was washed twice with 0.25 ml of 10 mM-Tris/HCl, pH 7.4. The pellet was extracted with 0.20 ml of 100% ethanol. Bound steroids were extracted from the pellet by incubation in 0.20 ml of

100% ethanol for 30 min at room temperature (20°C) and, after centrifugation for 1 min in the Microfuge B, 0.15 ml of the supernatant was removed and placed in a scintillation vial. After the ethanol had evaporated, 5 ml of toluene containing 0.3% (w/v) 2,5-diphenyloxazole (Fisher Scientific Co., Fair Lawn, NJ, U.S.A.) and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene (Nuclear Chicago, Des Plaines, IL, U.S.A.) was added and the radioactivity was assayed in a liquid-scintillation spectrophotometer. Specific binding was defined as that binding obtained by subtracting the radioactivity in the tube containing 100-fold excess of unlabelled steroid (non-specific binding) from that tube containing only labelled steroid (total binding).

Biochemical analyses

Protein content of nuclear-envelope fractions was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. DNA was measured by the method of Thomas & Farquhar (1978), with calf thymus DNA as standard. Phospholipid content was measured after extraction of nuclear-envelope lipids by the method of Garbus *et al.* (1963), and measurement of organic phosphate was by the method of Bartlett (1959).

Androgens bound to the liver nuclear envelope after incubation of the nuclear envelope with the radioactive dihydrotestosterone were analysed as follows. The washed pellet was resuspended in 1.0 ml of 10 mM-Tris/HCl, pH 7.4 and transferred to a glass tube to which 5 ml of ethyl acetate was added. After vortex-mixing, the non-aqueous phase was removed. The aqueous phase was washed twice with ethyl acetate and the three extractions were combined. Anhydrous Na₂SO₄ (1 g) was added to the extracts and this suspension was left overnight. The Na₂SO₄ was removed by filtration. The extracts were evaporated to dryness. Standards of dihydrotestosterone and testosterone were added in chloroform. T.l.c. was performed on 0.4 mm-thick silica-gel-coated plates and run twice in cyclohexane/ethyl acetate/acetic acid (36:24:0.07, by vol.). Dihydrotestosterone was detected by exposure to iodine vapour; testosterone was detected under u.v. Portions of the gel (1 cm) were scraped and assayed for radioactivity.

Chemicals

5α-[1,2,4,5,6,7,16,17-³H]Dihydrotestosterone (190–200 Ci/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A. All other unlabelled steroids were from Steraloids, Wilton, NH, U.S.A. Pronase (Type V Protease) was obtained from Sigma.

Results

Characterization of the male rat liver nuclear-envelope preparation

The rat liver nuclear-envelope fraction used in the studies described was prepared as described by Harris & Milne (1974), with only slight modifications, to accommodate large quantities of starting material. The major band of purified nuclear envelope usually formed at the 1.5 M/1.8 M-sucrose interface after the discontinuous-sucrose-gradient ultracentrifugation, but occasionally was positioned at the 1.8 M/2.0 M-sucrose interface. Phase-contrast examination of the final membrane material obtained after removal of the band from either interface revealed the presence of intact nuclear 'ghosts' as described by Harris (1978) and sheets of torn envelope. The yield of nuclear-envelope material was 0.76 ± 0.35 mg of protein/rat (29 preparations) or 0.075 ± 0.025 mg of protein/g of tissue (29 preparations). The DNA content of the nuclear-envelope preparation was 0.03 mg/mg of protein and the phospholipid content was 0.32 mg/mg of protein.

Characterization of androgen binding to the rat liver nuclear-envelope fraction

Conditions for optimal binding of dihydrotestosterone to the male rat liver nuclear-envelope fraction were determined. Fig. 1 shows that optimal binding was obtained after incubations of 15 nM-dihydrotestosterone with the nuclear-envelope frac-

Fig. 1. Effect of time and temperature on the binding of dihydrotestosterone to male rat liver nuclear envelopes. Nuclear envelopes (290 μ g of protein/assay tube) were incubated in triplicate with 15.5 nM-[^{3} H]dihydrotestosterone in the presence and absence of 100-fold excess of unlabelled dihydrotestosterone at 4°C (●) or at 22°C (□) for 0–24 h. Specific binding was calculated from the difference in binding of ^{3} H-labelled steroid in the presence and absence of unlabelled steroid.

tions (0.300 mg of protein/assay tube) for 20 h at 20°C. Fig. 2 demonstrates that under these conditions a linear relationship exists between binding and increasing membrane-protein concentrations to at least 400 μ g of membrane protein/assay tube.

Both Figs. 1 and 2 show that there is considerable non-specific binding of dihydrotestosterone to the membrane preparation. The amount of non-specific binding varied from preparation to preparation, but always represented 75–90% of the total binding.

Storage of the membrane at -80°C for 14 days resulted in a loss of 34% of the specific binding measured under optimal conditions, compared with the binding observed with the fresh preparation (Fig. 3). The loss of binding occurred at a fairly steady rate until day 10 of storage. However, storage at -15°C resulted in a 60% loss in 24 h (Fig. 3). For this reason, membrane preparations were routinely stored in liquid N_2 .

A Scatchard analysis of the binding of dihydrotestosterone to the male rat liver nuclear-envelope fraction was carried out. Fig. 4(a) shows that there was considerable non-specific binding, but that the K_D for specific binding could be calculated as 23.2 nM with an apparent number of binding sites of 578 fmol/mg of protein. As yet, more than one class of binding sites cannot be ruled out, especially in view of the fact that there is considerable non-specific binding. In addition, the amount of endogenously bound androgens is unknown at present.

After proteolytic digestion (1 mg/ml; 37°C,

Fig. 2. Effect of increasing concentrations of nuclear-envelope protein on the binding of dihydrotestosterone to male rat liver nuclear envelopes.

Nuclear envelopes (145–600 μ g of protein/assay tube) were incubated in triplicate with 16.02 nM-[^{3} H]dihydrotestosterone in the presence (●) and absence (■) of 100-fold excess of unlabelled dihydrotestosterone. Specific binding (▲) was calculated from the difference in binding of ^{3} H-labelled steroid in the presence and absence of unlabelled steroid.

30 min) of a nuclear-envelope fraction which had been previously incubated with dihydrotestosterone, 84.6% of the specific binding was lost. Heat denaturation (90°C, 30 min) resulted in a loss of

Fig. 3. Effect of storage at -15°C or -80°C on the binding of dihydrotestosterone to male rat liver nuclear envelopes

Nuclear envelopes prepared on day 0 were stored in 0.15 ml batches at either -15°C (●) or -80°C (□). On the days indicated samples were removed from storage, thawed at room temperature and incubated in triplicate for 22 h at 20°C with 15.2 nM [^3H]dihydrotestosterone in the presence and absence of 100-fold excess of unlabelled dihydrotestosterone. Specific uptake was calculated from the difference in binding of [^3H]steroid in the presence and absence of unlabelled steroid.

36.5% of the specific binding. This evidence is consistent with the conclusion that the specific dihydrotestosterone-binding sites associated with the rat liver nuclear-envelope fraction are protein in nature.

To investigate the physiological significance of these dihydrotestosterone-binding sites, Scatchard analyses (not shown) of dihydrotestosterone binding to liver nuclear-envelope fractions obtained from male rats castrated 24 h previously and from female rats were compared with Scatchard analyses of liver nuclear-envelope fractions obtained from intact male rats. Table 1 shows that the K_D values for the binding of dihydrotestosterone to all three preparations are similar. The number of binding sites in the male rat preparations is similar, whereas the female rat liver nuclear-envelope preparations may have slightly fewer sites.

Steroid specificity of the dihydrotestosterone binding to the male rat liver nuclear-envelope preparation was examined by competition experi-

Table 1. Scatchard analyses of binding of dihydrotestosterone to liver nuclear-envelope fractions obtained from intact male rats, castrated male rats and female rats. Scatchard analyses were performed as described in the legend to Fig. 4.

Rat liver nuclear-envelope preparation	K_D (nM)	No. of binding sites (fmol/mg of protein)
1. Intact male	23.2	578
2. Castrated male	37.7	637
3. Female	27.2	340

Fig. 4. Saturation analyses of [^3H]dihydrotestosterone binding to male rat liver nuclear envelopes

Nuclear envelopes ($333\mu\text{g}$ of protein/assay tube) were incubated for 22 h at 20°C with increasing amounts of [^3H]dihydrotestosterone (0.9–65.0 nM) with (□, to measure non-specific binding) and without (■, to measure total binding) unlabelled dihydrotestosterone (DHT; 100-fold excess). Specific binding was calculated from the difference in retained radioactivity in the presence and absence of unlabelled steroid (Fig. 4a) and plotted according to Scatchard (Fig. 4b). The line of best fit was determined by linear regression analysis and the correlation coefficient was -0.94 ($P < 0.01$).

Fig. 5. *Steroid specificity of total dihydrotestosterone binding to male rat liver nuclear envelope*

Male rat liver nuclear-envelope preparations were incubated for 22 h at 20°C in the presence of [³H]dihydrotestosterone (15 nM) with unlabelled steroids as competitors. Bound radioactivity was determined; 100% represents the binding in the presence of 15 nM-[³H]dihydrotestosterone alone. (Values are means of two determinations.) Abbreviations: E₂, oestradiol; DES, diethylstilboestrol; T, testosterone; DHT, dihydrotestosterone (unlabelled); DOC, deoxycorticosterone; DEX, dexamethasone.

ments in which 1-fold, 10-fold or 100-fold unlabelled competitor steroid was added to nuclear-envelope incubations with radiolabelled dihydrotestosterone (Fig. 5). Testosterone, also an androgen, was not as effective a competitor for 15 nM-[³H]dihydrotestosterone at 1500 nM as dihydrotestosterone. The oestrogens oestradiol and diethylstilboestrol competed less effectively for this site than did the androgens. However, the glucocorticoids dexamethasone and deoxycorticosterone competed as well as the androgens for the binding of labelled dihydrotestosterone.

Analysis of bound steroid revealed that after incubation of the membrane fraction with 15 nM radiolabelled dihydrotestosterone, 75% of the recovered label remained unchanged, whereas 5% was recovered as testosterone. In the presence of 100-fold unlabelled dihydrotestosterone, 55% of the radioactivity remained as dihydrotestosterone, whereas 23% was recovered as testosterone.

Discussion

An understanding of steroid-hormone action necessitates consideration of the process by which the steroid-receptor complex crosses the nuclear

envelope before it interacts with the genome and possibly afterward as it is recycled into the cytoplasm during continued action of the hormone. The translocation of some proteins across the nuclear envelope appears to occur via the nuclear pores (Gurdon, 1970; Paine & Feldherr, 1972), but there is a size limitation (Gurdon, 1970). Whether translocation of the steroid-receptor complex occurs via the pores or not, the transport of such a complex across the nuclear membranes probably requires a specific series of interactions with components of the nuclear envelope. We have begun investigations on this aspect of steroid-hormone action in the androgen target tissue, the rat ventral prostate, by characterizing interactions of androgens with a nuclear-envelope fraction obtained from the rat ventral prostate by heparin treatment of purified nuclei (Lefebvre & Novosad, 1980). However, this nuclear-envelope fraction did not contain nuclear pores. We wished to investigate interactions of steroids with a more intact preparation of the nuclear envelope and decided to use the nuclear 'ghost' preparation of Harris & Milne (1974). While modifying this procedure to obtain ghosts from the rat ventral prostate, we investigated interactions of androgens with nuclear ghosts prepared from the male rat liver. Although the rat liver is not generally considered a primary target for sex hormones, sex steroids can affect certain hepatic functions in a manner consistent with the accepted model for the action of steroid hormones on their target tissues (Kurtz *et al.*, 1976), and androgen and oestrogen receptors in rat liver cytosol have been reported (Milin & Roy, 1973; Van Beurden-Lamers *et al.*, 1974).

The preparation of rat liver nuclear envelopes used in this study (Harris & Milne, 1974) results in a preparation of relatively intact nuclear ghosts which retain the nuclear inner membrane and nuclear outer membrane with morphologically well-preserved nuclear-pore complexes (Harris, 1978). In our hands intact ghosts as monitored by phase-contrast microscopy were obtained, and the DNA content of the preparation compared with that reported by Harris (Milne *et al.*, 1978). The phospholipid content, on the other hand, is somewhat higher (0.32 versus 0.20 mg/mg of protein). The RNA content of our preparation was not determined, but has been found to be approx. 0.09 mg/mg of protein (Milne *et al.*, 1978). The density of the rat liver nuclear-envelope preparation was variable. Agutter & Gleed (1980) have reported that the density of sheep liver nuclear-envelope preparations varied according to the concentrations of nuclei during the lysis stage.

The experiments described in the present paper identify specific steroid-binding sites associated with the nuclear envelope. The measurement of steroid-binding sites involved incubation *in vitro* of the

isolated nuclear-envelope fraction with labelled steroid. These studies therefore do not distinguish between binding of the labelled steroid to membrane sites previously occupied by endogenous unlabelled steroid and binding to unoccupied sites, nor do they distinguish between binding of steroid to a receptor of cytoplasmic origin which is in turn bound to a membrane site and to a site which does not involve receptors.

Scatchard analysis of the binding of dihydrotestosterone to the intact male rat liver indicated a class of binding sites having a relatively high affinity (K_D 23.2 nM), although this is a lower affinity than that calculated for the class of binding sites investigated on the rat ventral-prostate nuclear-envelope fraction (K_D 8.4 nM). The binding sites also differed in that they were not altered appreciably by castration. Indeed, sites with a similar K_D and number were identified in female rat liver.

Competition experiments revealed that the other biologically active androgen, testosterone, competed somewhat less effectively than dihydrotestosterone for this site. Furthermore, steroid analysis showed that in the presence of 100-fold excess of unlabelled dihydrotestosterone more of the steroid is converted into testosterone. This conversion involves the 5 α -reductase, which has been reported on the nuclear envelope (Moore & Wilson, 1972), which would be active at the higher concentration of steroid. As testosterone binds as avidly to the site, this conversion would probably not significantly alter the amount of binding. Oestrogens did not compete well, but glucocorticoids competed as well as dihydrotestosterone. It would be of interest now to characterize further glucocorticoid binding to this preparation, as liver is recognized as a primary target tissue for glucocorticoids.

In summary, a binding site for androgens displaying high affinity and saturability has been identified on the liver nuclear envelope. Oestrogens do not compete for binding of androgens to this site, whereas glucocorticoids are effective competitors. As this class of binding sites is present in 24-h-castrated male rats, intact male rats and females, the

physiological role of the sites is not clear, but may be involved in translocation of androgens and/or glucocorticoids into or out of the nucleus.

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