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Examination of Glycosylated α_{2u}-Globulin-Containing Medium for Steroid-Binding Activity (40879)[1]

LAURA J. HAARS AND HENRY C. PITOT

Departments of Oncology and Pathology, The McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

Abstract. The function of α_{2u}-globulin, the major urinary protein of adult male rats, is unknown. These studies are an attempt to probe the role that glycosylated α_{2u}-globulin may play in the binding and transport of steroid hormones. Under the conditions of the experiments reported herein, glycosylated α_{2u}-globulin, secreted into the culture medium by primary monolayer cultures of hepatocytes, does not bind dihydrotestosterone, estradiol, or dexamethasone *in vitro*. These results do not preclude the possibility that α_{2u}-globulin binds steroids in other situations or that it has high affinity for other types of molecules.

The urinary output of 20,000-dalton α_{2u}-globulin is controlled by the serum level of testosterone in the male rat (1). Because of this correlation, and in light of the fact that the function of α_{2u}-globulin is unknown, Royce (2) studied the testosterone-binding capacity of purified 20,000-dalton α_{2u}-globulin. He concluded from the association constant of testosterone and α_{2u}-globulin that the 20,000-dalton protein did not play an important role in the transport of testosterone *in vivo*. Lea *et al.* (3) reported subsequently that the dimer of prostatein, a prostatic secretory protein, bound testosterone 15 times more effectively than the monomer. Since α_{2u}-globulin appears to be present in the serum primarily as high molecular weight molecules (4, 5), it was our purpose to determine whether these larger molecules had a greater affinity for testosterone than did the 20,000-dalton urinary protein.

Dexamethasone has been reported by Kurtz *et al.* (5) to induce glycosylated α_{2u}-globulin synthesis, although we found that glycosylated α_{2u}-globulin was synthesized by intact and castrated male rats, as well as intact and ovariectomized female rats, without exogenous dexamethasone administration (unpublished work). In contrast, estrogen has been shown to inhibit the synthesis of the 20,000-dalton protein (6). It was our conjecture that the high molecular weight forms of α_{2u}-globulin may inactivate low levels of estrogens that could potentially inhibit α_{2u}-globulin synthesis, as well as combine with dexamethasone to regulate their own synthesis. We therefore investigated whether or not glycosylated α_{2u}-globulin bound dexamethasone and estradiol. The unavailability of purified glycosylated α_{2u}-globulin precluded a thorough investigation of the steroid-binding capacity of these molecules, but we felt justified in comparing the binding of steroids by glycosylated and nonglycosylated α_{2u}-globulin under a particular set of conditions. Culture medium of hepatocytes from prepubescent male rats was used as a source of α_{2u}-globulin. We have found, under our assay conditions, that glycosylated α_{2u}-globulin does not bind these three steroids. Our results do not preclude the possibility that α_{2u}-globulin may bind steroids in other situations, or that α_{2u}-globulin may play an important role in binding other types of molecules.

Materials and methods. [2,4,6,7-^{3}H(N)]Estradiol (90 Ci/mmole), [6,7-^{3}H(N)]dexamethasone (36 Ci/mmole), and di[1,2-^{3}H(N)]hydrotestosterone (50.6 Ci/mmol) were purchased from New England Nuclear (Boston, Mass.). Dihydrotestosterone (5α-androstan-17β-ol-3-one) and 17β-estradiol were purchased from Research Plus Steroid Laboratories, Inc. (Denville, N.J.). Dexamethasone acetate was

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obtained from Sigma Chemical Company (St. Louis, Mo.).

Hepatocytes were cultured as described previously (4, 7) in Leibovitz L-15 medium supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml), insulin (0.5 μg/ml), glucose (1.5 mg/ml), and Hepes$^+$ buffer (1.8 m*M*) with (LAH)[2] or without (L-15) bovine serum albumin at 2 mg/ml. Spent medium from hepatocyte monolayer cultures in the 4th to the 24th hour of culture served as a source of high molecular weight α_{2u}-globulin. Hepatocytes were isolated from prepubescent male rats to insure that the medium was free of nonglycosylated α_{2u}-globulin. The medium was lyophilized and the residue was resuspended in 20 m*M* Tris buffer (pH 7.9), 1.5 m*M* EDTA, and 1 m*M* DTT (TED buffer) at 30 to 40% of the original volume. Fresh medium, which had not been in contact with hepatocytes, was treated similarly and was used as a control. α_{2u}-Globulin was absorbed from the concentrated spent culture medium with antibody specific for α_{2u}-globulin (4) for 4 hr at 4°C.

Steroid-binding activity was determined by the dextran-coated charcoal assay of Beato and Feigelson (8) as modified by Mierendorf and Mueller (9). Details of the assays are given in the figure legends. Fifty-microliter aliquots of the charcoal-absorbed supernatants were mixed with R.I.A. Solve II (Research Products International, Elk Grove Village, Ill.) and the radioactivity was determined by liquid scintillation counting.

Results and discussion. We approached the question of whether or not glycosylated α_{2u}-globulin bound steroids by first assaying the total spent medium, known to contain α_{2u}-globulin, for steroid-binding activity. As shown in Fig. 1A, spent culture medium demonstrated a low level of dihydrotestosterone (DHT)-binding activity

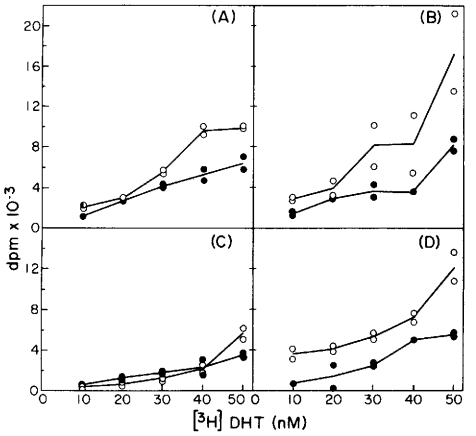

FIG. 1. Dihydrotestosterone binding by hepatocyte culture medium. (A) Various concentrations of di[^{3}H]hydrotestosterone, in the presence (●) or absence (○) of a 1000-fold excess of unlabeled DHT, was mixed with 90 μl of concentrated spent medium and TED buffer to a final volume of 100 μl. The mixture was incubated for 2 hr at 0°C at which time 50 μl of a charcoal–dextran slurry in TED buffer was added. The charcoal was pelleted by centrifugation and the radioactivity in 100 μl of the supernatant was determined by liquid scintillation counting. (B) As in (A), except that 90 μl of anti-α_{2u}-globulin-absorbed medium was substituted for the spent medium. (C, D) As in (A), except that 90 μl of L-15 (C) or LAH (D) medium was substituted for the spent medium.

beyond that which could be attributed directly to nonspecific binding. This result was reproduced in four separate determinations. The DHT was not bound to α_{2u}-globulin, however, since absorption of a large proportion of α_{2u}-globulin from the medium with anti-α_{2u}-globulin did not remove the DHT-binding activity (Fig. 1B). That the binding was in all likelihood due to the albumin content of the medium is shown in Figs. 1C and D. Unconditioned medium lacking albumin (L-15) had no DHT-binding activity whereas the corresponding medium containing albumin (LAH) had binding activity comparable to the spent medium.

Similar results were obtained for the binding of estradiol by molecules in spent medium, L-15, and LAH (Fig. 2). The data suggest that the total estradiol-binding capacity of spent medium and LAH are not significantly different. Under our condi-

[2] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; DHT, dihydrotestosterone; TED buffer, 20 m*M* Tris buffer (pH 7.9), 1.5 m*M* EDTA, 1 m*M* DTT; L-15, Leibovitz L-15 medium with all supplements except albumin; LAH, supplemented L-15 also containing albumin.

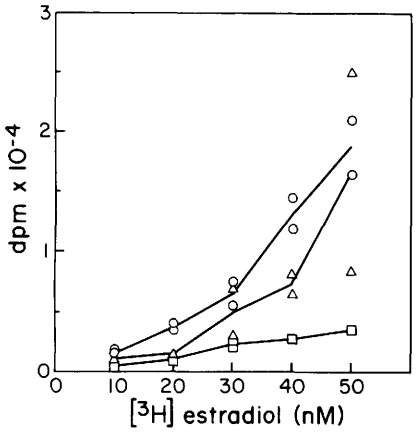

FIG. 2. Estradiol binding by hepatocyte culture medium. [^{3}H]Estradiol from 10 to 50 mM was combined with spent medium (○), L-15 (□), or LAH (△) as described in Fig. 1. Twenty-five microliters of the charcoal–dextran slurry was added, and 50-μl aliquots were used for liquid scintillation counting. Total estradiol-binding by medium components is shown in this figure.

tions, spent medium had no specific dexamethasone-binding activity (Fig. 3). Since this result was reproducible, LAH, L-15, and anti-α_{2u}-globulin-adsorbed medium was not assayed.

In view of the results of Royce (2) as well as the failure of glycosylated α_{2u}-globulin to bind steroids under our assay conditions, we did not attempt to assay the steroid-

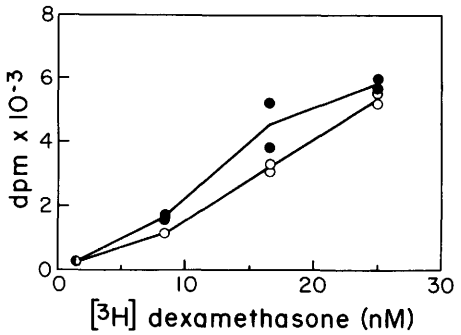

FIG. 3. Dexamethasone binding by hepatocyte culture medium. [^{3}H]-Dexamethasone from 1 to 40 mM, in the presence (●) or absence (○) of a 1000-fold excess of unlabeled dexamethasone acetate, was mixed with 90 μl of spent medium and the assay was conducted as described in Fig. 1. Aliquots for liquid scintillation counting were taken as described in Fig. 2.

binding capacity of 20,000-dalton α_{2u}-globulin.

Albumin is known to bind steroids nonspecifically (10), and we therefore expected it to contribute to the total binding activity in our assay. However, we chose to culture the hepatocytes in the presence of albumin in order to maintain a milieu that resembled plasma *in vivo* more closely (F. Althaus, personal communication). We then subtracted the level of steroid bound to albumin from the amount of steroid bound by the spent medium.

Because of the low level of synthesis of glycosylated α_{2u}-globulin both in the rat and in cultured hepatocytes, it was necessary to allow α_{2u}-globulin to accumulate in the medium for relatively long periods of time. Although we have suggested (4) that some breakdown of the high molecular weight forms occurs in the culture medium with time after secretion, we felt that the 24-hr incubation period utilized for this investigation represented the best compromise for obtaining both reasonable levels of glycosylated α_{2u}-globulin and minimal protein degradation. The amount of α_{2u}-globulin in the medium cannot be determined directly, but an estimate can be made from the incorporation of [^{3}H]leucine of known specific activity into α_{2u}-globulin under the same conditions. The amount of high molecular weight α_{2u}-globulin in 7 ml of spent medium prior to concentration was calculated to be 3 to 5 ng. One assumption in this calculation is that every leucine in the nascent polypeptide is tritiated. This is, of course, not the case and hence this figure represents the lower limit of the α_{2u}-globulin concentration. Even if we assume only a 10-fold dilution of the [^{3}H]leucine with the intracellular leucine pools when calculating the amount of α_{2u}-globulin present in the medium, the amount of labeled steroid specifically bound by α_{2u}-globulin should produce approximately 10,000 dpm in the charcoal-absorbed reaction mixture.

Royce reported earlier (2) that α_{2u}-globulin purified from urine did not have sufficient affinity for androgens to be a significant steroid receptor *in vivo*. Our experiments were designed to assess whether, with the methods available to us for ob-

taining glycosylated α_{2u}-globulin, a thorough investigation of the steroid-binding activity of α_{2u}-globulin could be performed. We concluded that glycosylated α_{2u}-globulin secreted into the culture medium does not, under our assay conditions, bind steroids, and that many technical difficulties involved in obtaining glycosylated α_{2u}-globulin in a purified and concentrated form would have to be overcome before an extensive investigation of the steroid-binding capacity of glycosylated α_{2u}-globulin could be accomplished. However, in view of the ability of many serum proteins to bind certain types of molecules, we propose that α_{2u}-globulin, either glycosylated or nonglycosylated, may function to bind and transport molecules *in vivo*.

The hormonal control of α_{2u}-globulin synthesis (5, 6, 11), as well as several aspects of the structure of the protein molecule (12, 13), has been investigated extensively. α_{2u}-Globulin is the major protein in adult male rat urine (14) and its synthesis represents 1% of total hepatic protein synthesis (15). It is, therefore, a protein of physiological significance. The function of α_{2u}-globulin, however, has not been elucidated. The investigation reported here addresses an important potential function of glycosylated α_{2u}-globulin, that of steroid binding. We feel that this study will contribute to the efforts of many investigators who are studying the functional significance of α_{2u}-globulin.

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