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THE ROLE OF TRANSCORTIN IN GLUCOCORTICOID MEDIATED ENZYME INDUCTION: TYROSINE AMINOTRANSFERASE INDUCTION IN HEPATOMA TISSUE CULTURE CELLS

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SUMMARY

Transcortin from rats selectively binds cortisol but not the synthetic glucocorticoid dexamethasone. Both steroids are potent inducers of the enzyme tyrosine aminotransferase in hepatoma tissue culture cells *in vitro* under serum free conditions. Transcortin is shown to block selectively enzyme induction by cortisol but not dexamethasone. The inhibitory effect of added transcortin can be abolished by adding sufficient cortisol to overwhelm the transcortin binding capacity. It is also shown that transcortin can inhibit the entry of radiolabelled cortisol, but not dexamethasone, into hepatoma cells *in vitro*. This probably is the mechanism which inhibits enzyme induction by cortisol. In addition a rapid and reproducible technique for estimating transcortin binding capacity is described.

INTRODUCTION

The role of transcortin in mediating glucocorticoid effects is controversial. There is a considerable body of evidence which suggests that glucocorticoid bound to transcortin (CBG)‡ is biologically unavailable to cells [1–4]. On the other hand, some recent evidence obtained in a variety of systems has suggested that CBG bound steroid is biologically active [5–8] and in one study that it may be essential for glucocorticoid effect. The major objection to these studies is that most of them were done *in vivo* under circumstances in which metabolism of steroid and/or CBG by other tissues and other non specific effects such as interactions of other hormones could not be controlled. In addition the important control provided by a glucocorticoid which does not associate with CBG has not been provided in previous work. Therefore we undertook an investigation of the role of CBG in glucocorticoid action by studying the effects of glucocorticoids and CBG in a more readily controlled tissue culture system. The induction of tyrosine transaminase (E.C. 2.6.1.5) (TAT) by various glucocorticoids in hepatoma tissue culture (HTC) cells has been well characterized [9]. Furthermore these cells do not metabolize dexamethasone [10]. From the wild-type HTC cells, we have developed a line of HTC cells which grow and respond to glucocorticoids under serum free conditions in a manner virtually identical to the parental strain [11]. In this relatively uncomplicated system we have been able to show that glucocorticoid, apparently bound to CBG, is essentially unavailable for enzyme induction.

‡ Abbreviations used in this paper: CBG—corticosteroid binding globulin; PBS—phosphate buffered saline pH 7·4; TAT—tyrosine aminotransferase (E.C. 2.6.1.5); HTC—hepatoma tissue culture; ESA refers to TAT specific activity expressed as nm/mol product/min/mg of protein.

METHODS

Tissue culture

Two kinds of HTC cells were used throughout. Wild-type HTC cells (HTC^+) were grown in monolayer or suspension culture using standard techniques [12]. A subclone of these cells, HTC-SF1, was derived which showed essentially normal growth and inductive responses to glucocorticoid when grown or induced in serum free conditions [4].

Enzyme and protein determination

TAT enzyme activity was determined using a modi-

fication of the Diamondstone method [13]. Enzyme specific activity (ESA) is given as nm/mol product formed/min/mg protein. Protein determinations were performed by the Lowry technique [14].

Transcortin

Partially purified transcortin was prepared from Sprague Dawley rats and from human sera (obtained from outdated pooled blood bank plasma) using the technique of Westphal [15].

Transcortin binding assays

Transcortin binding activity was performed using a modification of the DEAE cellulose filter paper technique for measuring cytoplasmic glucocorticoid binding proteins [16] in which samples to be assayed were incubated in the presence of various concentrations of ^{3}H-Dexamethasone (Amersham 22 Ci/mmol) or ^{3}H-Cortisol (Amersham 48 Ci/mmol) and in the presence or absence of a 1000-fold excess of the appropriate unlabelled steroid. Samples were incubated in phosphate buffered saline pH 7·4 (PBS) at 0–2 C for 2 h. After incubation the samples were diluted 20-fold in Buffer I (20 mM Tris–HCl pH 7·5, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0·25 M sucrose) and collected by filtration through DEAE cellulose filter papers at a rate of about 10 ml/min. Samples were washed with 40 ml more of Buffer I. Filters were air dried and counted in 1 ml of NCS (Amersham) and 10 ml of Liquifluor–toluene in a liquid scintillation counter (^{3}H efficiency 20%). This technique permitted assay of as little as 20 μl of a 50-fold dilution of serum for binding activity.

Induction experiments. To cells growing logarithmically in monolayers replenished with fresh media, appropriate concentrations of steroid in PBS were added along with the appropriate addition of serum or transcortin fraction. Following 24 h of sterile incubation in a 5% CO_2 incubator at 37°C, the cells were harvested, washed and assayed for TAT as described above.

Distribution of steroid. Cells growing logarithmically in culture were harvested, washed three times in the ice cold PBS and suspended in serum-free medium at a density of 2–3 million/ml. The medium in which the cells were suspended was made $1{\cdot}3 \times 10^{-8}$ M with respect to radiolabelled cortisol or dexamethasone and then divided equally into three 25 ml Ehrlenmeyer flasks. Serum or media were rapidly added to the flasks and the stoppered flasks gently shaken in a gyrorotatory waterbath at 37°C. After 30 min the cells were chilled by diluting with 5 vol. iced PBS and washed three times in PBS and collected by centrifugation at 800 *g* for 1 min. The cell buttons were suspended in a small volume of water, disrupted by sonication for 10 sec in a Bronwill sonicator (setting 15) and aliquots assayed for protein and radioactivity by solubilizing in 8 vol. of NCS and 10 ml of Liquifluor–toluene as described above.

RESULTS

The binding of cortisol and dexamethasone to CBG preparations of various origins is shown in Fig. 1. As can be seen, this assay technique (which has not previously been applied to CBG) delineates high affinity limited capacity binding of cortisol by CBG. There is essentially no binding of dexamethasone to CBG from any of the sources employed at the concentrations of dexamethasone we used. It is of interest that we [17] and others [18] have shown that the cytoplasmic steroid binding protein which has been strongly implicated in steroid action binds both dexamethasone and cortisol with very high affinity. It is of note that the partially purified CBG gives an almost identical binding curve in terms of affinity to that seen with the unfractionated sera.

Thus, we reasoned that if CBG binding of glucocorticoid rendered it unavailable to the cell we would observe differences between cortisol and dexamethasone induction of TAT in cells when transcortin was present but that induction by either steroid would be similar when medium lacking a source of transcortin was used. In Fig. 2a induction curves for HTC-SF1 cells in increasing concentrations of steroid are shown for cells induced under serum-free conditions. These curves are essentially identical for cortisol and dexamethasone except for the slight but highly reproducible increased potency of dexamethasone previously described [19]. This is consistent with the higher

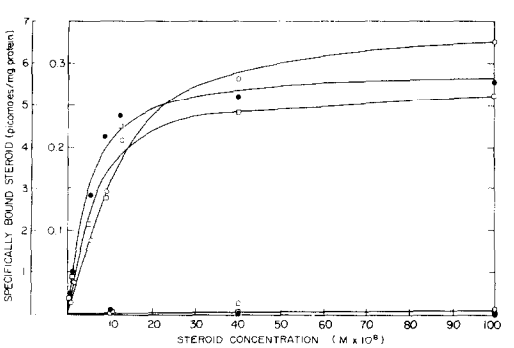

Fig. 1. The binding of cortisol (upper curves) or dexamethasone (lower line) to partially purified human CBG (○—○) left hand ordinate, serum from adrenalectomized rats diluted 1:50 (●—●) and fetal calf serum diluted 1:20 (□—□). Techniques are given in "Methods". Points are means of duplicate determinations.

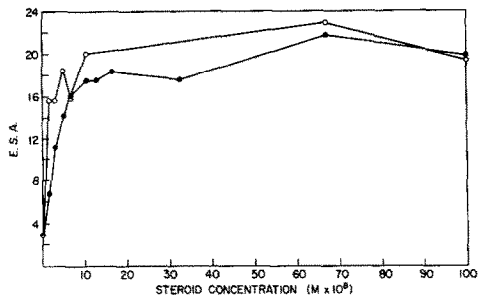

Fig. 2a. Induction of tyrosine aminotransferase by increasing concentrations of cortisol (●—●) or dexamethasone (○—○) induced under serum free conditions. Each point represents the mean of at least a duplicate determination of a separate culture. Enzyme specific activity (ESA) for transaminase is given as nmol product formed/min/mg protein.

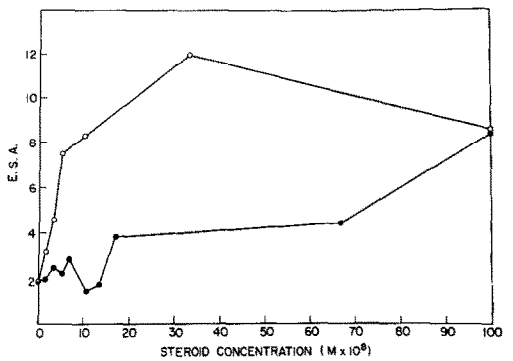

Fig. 2b. Induction of tyrosine aminotransferase by increasing concentrations of cortisol (●—●) or dexamethasone (○—○). All inductions were in the presence of 4% dialyzed serum from adrenalectomized rats. Each point represents the mean of at least a duplicate determination.

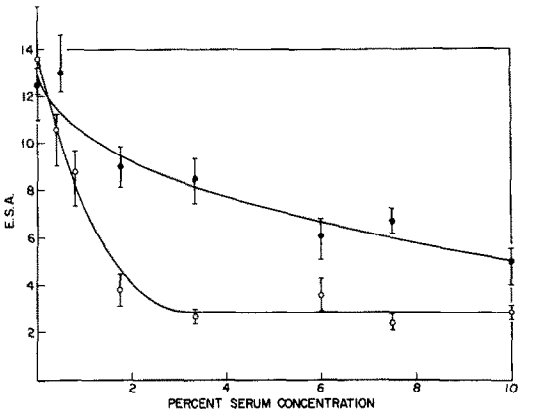

Fig. 3a. Effect of increasing concentration of sera from adrenalectomized rats on tyrosine aminotransferase induction by 3×10^{-8} M cortisol (○—○) or dexamethasone (●—●). Vertical bars represent range of quadruplicate determinations.

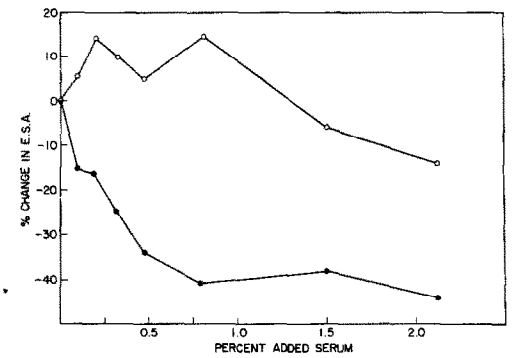

Fig. 3b. Per cent of control induction achieved under serum-free conditions achieved with increasing concentration of serum from adrenalectomized rats; either 3×10^{-8} M cortisol (●—●) or dexamethasone (○—○) was used. Quadruplicate determinations were performed for each point shown.

affinity of cytoplasmic steroid receptor proteins for dexamethasone which has been previously shown [17]. In Fig. 2b similar induction curves are shown except that the media now contain in addition 4% dialyzed serum from adrenalectomized rats used as a source of transcortin. As can be seen, serum from adrenalectomized rats virtually completely blocks induction by cortisol but not by dexamethasone. Cortisol induction is restored at the highest concentration of cortisol, a concentration calculated to be enough to saturate the CBG binding of cortisol. These results strongly suggest a physiologic inactivation of cortisol when bound to CBG. As can be seen in the figure, maximum induction of the cells with added serum from adrenalectomized rats is only about one half of that noted under serum-free conditions. Under phase microscopy the cells incubated in media containing rat serum appeared vacuolated and it was felt likely that the decreased induction of all cells, in rat serum whether cortisol or dexamethasone treated, was due to a non-specific toxic effect of the added serum. To be certain that this toxicity was not the source of the differences between cortisol and dexamethasone induction noted in Fig. 2b, additional experiments were performed.

Cells were incubated in the presence of 3×10^{-8} M cortisol or dexamethasone in increasing concentrations of serum as shown in Fig. 3a. This concentration of steroid was chosen because as Fig. 2a shows, a small change in available steroid concentration in this range results in a large variation in induced TAT levels. The decrease in induction of TAT by dexamethasone (Fig. 3a upper curve) with increasing serum concentrations is consistent with a toxic

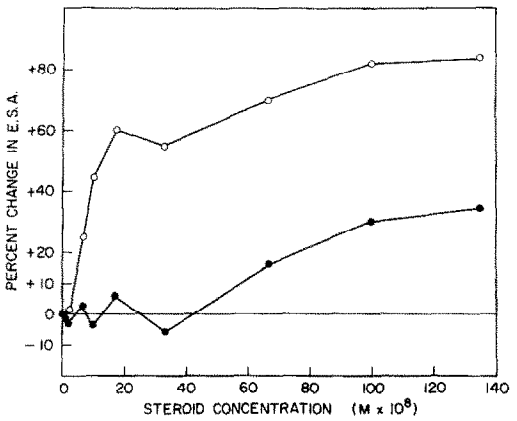

Fig. 4. Effect of 20% fetal calf serum on induction of tyrosine aminotransferase by either cortisol (●—●) or dexamethasone (○—○) in HTC$^+$ cells. Unattached solid symbols represents maximal induction achieved by cortisol (■) without serum. Each point represents quadruplicate determinations.

effect of the serum. Compare this with the marked decrease in induction by cortisol seen in the lower curve, presumably reflecting CBG binding of cortisol. Similar results from another experiment expressed differently are shown in Fig. 3b where cortisol induction is obviously affected far more than dexamethasone by serum addition.

Further support for the significant role of transcortin is shown in Fig. 4 where instead of serum from adrenalectomized rats the non-toxic fetal calf serum in which HTC$^+$ cells are normally grown is used as a source of transcortin. Here, results similar to those obtained in Fig. 2 are obtained and the extent of maximal induction by dexamethasone is unaffected by the addition of serum.

If CBG were acting to prevent enzyme induction by binding extracellular steroid, it should be possible to detect diminished entry of cortisol, but not dexamethasone, into cells incubated in media containing CBG. As shown in Table 1, cells incubated with radioactive steroid in 4% adrenalectomized rat serum or 5% fetal calf serum show a marked diminution of cortisol associated with cells as compared to cells plus steroid incubated in serum-free medium. This difference in steroid distribution between cells in serum and serum-free media is not apparent when dexamethasone is the steroid in the incubation instead of cortisol. At the concentration of steroid used ($1{\cdot}3 \times 10^{-8}$ M) most steroid is bound to specific cytoplasmic or nuclear sites [17]. Thus it appears likely that CBG acts by binding cortisol extracellularly reducing the concentration of free steroid outside the cell.

DISCUSSION

Our results are consistent with those of Slaunwhite *et al.* [3], Kawai and Yates [1] and Blecher [4] who have shown that certain glucocorticoids bound to CBG were unable to stimulate glycogen deposition in the liver, block histamine provoked ACTH release, and decrease glycolysis in fat cells. All of these results would suggest that cortisol bound to CBG is physiologically inactive. Our results are not in harmony with those of Rosner [17] who demonstrated that highly purified CBG associated with cortisol could induce TAT in liver *in vivo* and cause peripheral lymphocyte depletion. Though great pains were taken in the latter study to show that a physiologically insignificant amount of unbound cortisol was available *in vitro*, it was impossible to prove that there was no extensive dissociation of steroid–CBG complexes or metabolism of steroid and/or CBG *in vivo*, where the induction was demonstrated.

Keller *et al.* noted differences in inducibility of the enzyme alanine transaminase between liver and pancreas when various glucocorticoids were used. In situations in which CBG was elevated (estrogen treatment) only the liver enzyme was inducible by cortisol whereas dexamethasone always induced both [6]. They did not attempt to overwhelm the CBG binding capacity with excess cortisol administration. The affini-

Table 1. The effect of serum on the entry of cortisol or dexamethasone into HTC cells

Addition	[^{3}H]-Cortisol	[^{3}H]-Dexamethasone
Buffer	30	31
5% Fetal calf serum	12·5 (58%)	30 (3·2%)
4% Adrenalectomized rat serum	16 (47%)	25 (19%)

Numbers represent the mean of duplicate determination expressed as c.p.m./mg cellular protein. The per cent difference from control values is given in parentheses. Experimental technique is given in "Methods".

ties which have been reported for several cytoplasmic glucocorticoid receptor molecules [17, 18] are similar to those reported for CBG [15]. It is possible that small differences in affinity of different cellular receptors might allow them to become saturated with glucocorticoid to a different extent for a given concentration of free cortisol. Extent of binding to cytoplasmic receptor correlates well with observed responses to hormone [17, 18]. Thus, CBG could modulate the organism's response to glucocorticoids if its affinity for a given steroid molecule were close to those of different cytoplasmic receptors. Chader has reported a possible role for CBG in glutamine synthetase induction. It was found that glucocorticoid bound to CBG was still able to induce increased enzyme synthesis in chick retina [5]. However, the affinity of retinal cytoplasmic receptors for glucocorticoid is probably greater than CBG [20] and thus in this case CBG is unable to block cortisol induction. Further characterization of cytoplasmic receptors from a variety of sources will do a great deal to clarify this situation.

More difficult to interpret are the results of Werthamer and Amaral who reported that for leukemic lymphocytes CBG was required for cortisol to exert an inhibitory effect on RNA synthesis but not on protein synthesis [8]. These authors have recently suggested that intracytoplasmic receptors are actually CBG [21] a result which is not in keeping with much information from other sources concerning significant differences in steroid specificity, sedimentation and thermolability [17, 18] as well as our own results in cells grown in serum-free medium *in vitro* which are easily shown to have a glucocorticoid receptor molecule which binds both cortisol and dexamethasone [11].

In summary, we have shown that CBG can decrease the amount of cortisol which can associate with HTC cells and presumably through this mechanism inhibit glucocorticoid effect. We feel that this phenomenon may represent an unsuccessful competition of cytoplasmic receptor for cortisol. Furthermore we describe a new application of the DEAE cellulose filter paper assay for the rapid determination of CBG.

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