The Binding of Dexamethasone and Triamcinolone Acetonide to Glucocorticoid Receptors in Rat Skeletal Muscle*

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SUMMARY

Specific binding of the synthetic glucocorticoids [1,2,4-\(^{3}H\)]dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregna-1,4-diene-3,20-dione) and [1,2,4-\(^{3}H\)]triamcinolone acetonide (9α-fluoro-11β,10α,17α,21-tetrahydroxy-pregna-1,4-diene-3,20-dione-16,17-acetonide) by the cytoplasmic fraction of rat gastrocnemius muscle was studied. The cytosol binding reaction displayed stereospecificity and high affinity of binding. Biologically active glucocorticoids, administered to adrenalectomized rats or present during the in vitro binding reaction markedly depressed the binding of \([^{3}H]\)dexamethasone and \([^{3}H]\)triamcinolone acetonide. The biologically inactive stereoisomer, epicortisol, had no effect on the binding of the labeled hormones. The binding component displayed high affinity for \([^{3}H]\)dexamethasone and \([^{3}H]\)triamcinolone acetonide \((K_d = 1.9 \times 10^{-11} \text{M}, \text{respectively})\). The number of binding sites was limited \((0.1 \text{ pmol}\)s per mg of cytosol protein\), and Scatchard analysis suggests that only a single class of binding sites exists for both \([^{3}H]\)dexamethasone and \([^{3}H]\)triamcinolone acetonide. Competition studies indicated that the two glucocorticoids interact with the same binding site. The binding macromolecule appears to be a protein since binding is prevented by nagarse treatment and is dependent on the integrity of —SH groups. The glucocorticoid-protein complexes were characterized on 5 to 20% sucrose gradients. Both complexes sedimented at about 1 S in 0.3 M KCl, but only the \([^{3}H]\)triamcinolone acetonide-receptor complex sedimented at 7 S in the absence of salt. Since the specific binding component has the properties of a physiological glucocorticoid receptor, a direct effect of these hormones on skeletal muscle is suggested.

A large body of evidence indicates that an early and essential step in the sequence of events which mediate the biological effects of steroid hormones is the intracellular binding of the hormone to specific receptor molecules (1). Such protein receptors are found in the cytosol fraction of glucocorticoid-responsive tissues, and display selective and high affinity binding of these hormones (2). Their physicochemical properties as well as their implication in the biological response of various target tissues, e.g. liver, thymus, and certain cell lines growing in culture, were thoroughly described in several recent papers (3-7).

Exposure of muscles in vivo to glucocorticoid hormones results in a significant depression of DNA, RNA, and protein synthesis (8, 9), and was recently found to cause activation of myofibrillar proteolytic activity (10). However, information on the sequence of events and mechanism of the antianabolic and catabolic effects of these hormones on muscle tissue is still limited. It is not known whether pronounced muscle wasting brought about by glucocorticoid administration is a result of a direct interaction of the hormone with muscle cells, or a secondary consequence of hormonal action on visceral organs. Such secondary effects could be mediated via changes in the hepatic demand for glucogenic amino acids, causing increased mobilization of these substrates from muscle proteins (11, 12).

Since specific glucocorticoid receptors appear to be necessary for a direct physiological response to these hormones, the presence of such binding components in skeletal muscle might indicate a primary effect of the hormone on that tissue.

Although no binding of cortisol or corticosterone to subcellular fractions of rat skeletal muscle could be demonstrated by DeVenuto et al. (13) using a novel “equilibrium fractionation” technique, a recent paper by Simpson and White (14) reported on the binding of some biologically potent steroids in vivo to rat muscle, and Roth (15) examined the age-related changes in the binding of glucocorticoids to several rat tissues, including muscle. However, in these studies total rather than glucocorticoid-specific binding was measured and no attempt was made to characterize the binding component or the binding reaction itself.

In the present study we examined the specific binding of biologically potent glucocorticoids to rat muscle cytosol. \([^{3}H]\)Dexamethasone\(^{1}\) and \([^{3}H]\)triamcinolone acetonide binding
proteins were found to be present in the supernatant fraction of rat gastrocnemius muscle. These proteins show high affinity and specificity of binding and formed complexes with a sedimentation coefficient of 4 S at high ionic strength. Rat skeletal muscle was also found to contain an $^{3}H$-cortisol binding protein which resembles the corticosteroid binding globulin of rat plasma. The properties of this protein and its interactions with cortisol will be reported elsewhere.

MATERIALS AND METHODS

Chemicals—Cortisol, dexamethasone and progesterone were obtained from Sigma Chemical Co., epicortisol from Steraloids, Inc., and triamcinolone acetonide was a gift from Squibb. Protein standard solution was purchased from Mann Research Laboratories, and crystalline bovine serum albumin from Nutritional Biochemicals Corp. All chemicals and solvents were of reagent grade.

Radiochemicals—[1,2,4-$^{3}H$]Dexamethasone (27 and 29 Ci per mmole) was purchased from New England Nuclear Corp. [1,2,4-$^{3}H$]Triamcinolone acetonide (10.7 Ci per mmole) was obtained from Schwarz-Mann BioResearch.

Animals and Treatments—Male rats, weighing 140 to 160 g were obtained from ARS-Sprague Dawley Co., Madison, Wis., and bilateral adrenalectomy was performed under Nembutal anesthesia. The animals were maintained on 1% NaCl drinking water for 5 days before the experiment. When treated with steroids, the animals were injected subcutaneously with 10 mg per kg body weight, twice daily for 4 days, and killed 24 hours after the last injection. The steroids were dissolved in corn oil.

Preparation of Muscle Cytosol Fraction—The adrenalectomized animals were killed by decapitation, and the gastrocnemius muscles were thoroughly perfused with 10 ml of ice-cold isotonic NaCl injected into the aorta and vena cava at a point midway between the ilio-lumbar vessel and the aortic bifurcation. The muscles were then quickly removed, washed in ice-cold isotonic NaCl and freed of fat and connective tissue. About 1-g samples of muscle were minced with scissors and homogenized in a Potter-Elvehjem homogenizer in 6 volumes of 0.01 M Tris-HCl buffer, pH 7.5, containing 1.0 mM EDTA and 12 mM thioglycerol. The homogenate was centrifuged at 1000 $\times g$ for 10 minutes and the supernatant fraction was used for the binding assay.

Measurement of $^{3}H$-Labeled Steroid Binding—Two milliliters of the 27,000 $\times g$ muscle supernatant fraction were incubated with tritiated steroids (4 to 9 X 10$^{-8}$ M) at 0° for 2 hours or as indicated. Following incubation, the free and macromolecule-bound steroids were separated by activated charcoal, using a modification of the method described by Baxter and Tompkins (16). A 0.1-ml suspension of charcoal (Norit A, 100 mg per ml) treated with 6 N HCl, washed, neutralized and suspended in 0.01 M Tris-HCl buffer (pH 7.5) containing 0.14 M NaCl, 11 mM KCl, 3 mM MgCl$_2$, and 0.4 mM EDTA was added to 0.5-ml fractions of the supernatant fraction after incubation. This suspension was mixed on a Vortex mixer for 15 s and centrifuged at 1000 $\times g$ for 2 min. The supernatant fraction was separated from the charcoal, transferred to another tube, and centrifuged at 1000 $\times g$ for 10 min. Aliquots of 200 $\mu$l of the obtained supernatant fraction were assayed for radioactivity.

The glucocorticoid-specific binding was defined as the binding of [H]$^3$H)dexamethasone or [H]$^3$H)triamcinolone acetonide in the presence of a 1000-fold excess (5 X 10$^{-8}$ M) of nonlabeled epicortisol minus the binding observed in the presence of a similar excess of nonlabeled dexamethasone or triamcinolone acetonide, respectively (7, 17).

Radioactivity was assayed in a Beckman ambient temperature liquid scintillation spectrometer model L5-150. Counting efficiency was determined by the external standard method or by using internal standards. Protein was determined by the method of Lowry et al. (18).

Sucrose Gradient Analysis—The tritiated glucocorticoid-receptor complex was formed by incubating the 27,000 $\times g$ supernatant fraction of muscle homogenate with 5 X 10$^{-8}$ M [H]$^3$H)dexamethasone or [H]$^3$H)triamcinolone acetonide at 0° for 2 hours. After charcoal adsorption of the unbound steroid, 0.5 ml aliquots were layered on 11.5 ml of a linear sucrose gradient (5 to 20%, w/v) in 0.01 M Tris-HCl buffer, pH 7.5, containing 1.0 mM EDTA and 12 mM thioglycerol. In some determinations, 0.3 M KCl was added to the buffer. The binding protein (4.5 S) was crystalline bovine serum albumin (5.0 mg per ml) 0.5 ml of which was added to separate tubes containing the 5 to 20% sucrose gradient. Centrifugation was performed at 2° for 23 hours in a Spinco model L5-50 ultracentrifuge using the SW 41 rotor at 37,000 rpm. Following centrifugation, 10-drop fractions were collected from the bottom of the tubes into counting vials, and assayed for radioactivity in 10 ml of Bray's scintillation fluid (19). Quenching of radioactivity did not vary significantly with the concentration of sucrose or salt in the fractions. The migration of the marker protein was assayed by measuring the absorbance at 280 nm of the fractions collected from the gradients, and subtracting the absorption due to the buffer. Relative sedimentation coefficients were calculated by the method of Martin and Ames (20).

RESULTS

[H]$^3$H)dexamethasone- and [H]$^3$H)triamcinolone Acetonide-binding to Muscle Cytosol—Incubation of a 27,000 $\times g$ supernatant fraction of rat gastrocnemius muscle with 9 X 10$^{-8}$ M [H]$^3$H)dexamethasone or 4 X 10$^{-8}$ M [H]$^3$H)triamcinolone acetonide at 0° results in significant binding of these steroids. The binding is time dependent and was reduced in the presence of a 1000-fold excess of the same nonlabeled steroid. Since epicortisol is an inactive stereoisomer of cortisol (7, 16), it would be reasonable to suppose that it will not compete with [H]$^3$H)dexamethasone or [H]$^3$H)triamcinolone acetonide for specific receptors but might bind to sites which do not play a role in mediating the biological activity of glucocorticoids. On the other hand, an excess of biologically potent steroids should compete with the labeled hormone for both glucocorticoid-specific and nonspecific binding sites. For this reason the binding of the [H]$^3$H)labeled steroids in the presence of nonlabeled epicortisol, minus the binding in the presence of nonlabeled biologically active steroids is taken as an indication of the relative amount of hormone-specific binding components. Fig. 1 depicts the time-dependent specific association of [H]$^3$H)dexamethasone and [H]$^3$H)triamcinolone acetonide with the muscle cytosol binding component. The binding of both hormones follows similar kinetics, approaching a maximum within 120 min, and further incubation over the next 120 min does not appreciably change the degree of specific binding.

[H]$^3$H)dexamethasone- and [H]$^3$H)triamcinolone Acetonide-specific Binding as Function of Steroid Concentration—in Fig. 2 the
amount of the labeled steroids specifically bound by the muscle 27,000 × g cytoplasmic fraction is plotted as a function of the concentration of free [³H]dexamethasone or [³H]triamcinolone acetonide in the incubation mixture. A saturation type binding curve is obtained. To estimate the number of binding sites and the binding affinity, the results were analyzed by the Scatchard technique (21). A plot of the amount of steroid bound against the ratio of bound to free steroid (Fig. 2), inset yielded a straight line for both glucocorticoids. From the slopes of the Scatchard plots the apparent dissociation constants for the reaction: hormone + binding component = bound hormone complex was calculated and found to be 1.9 × 10⁻⁸ M and 1.2 × 10⁻⁸ M for the binding of [³H]dexamethasone and [³H]triamcinolone acetonide, respectively. The concentration of the binding sites, assuming that each site binds one molecule of steroid and that only a single class of specific binding sites exists under the conditions of the assay, is about 0.1 pmole per mg of cytoplasmic protein for each of these steroids. The possibility that the two steroid hormones interact with the same class of binding sites exists under the conditions of the assay, is about 0.1 pmole per mg of cytoplasmic protein for each of these steroids. The possibility that the two steroid hormones interact with the same class of binding sites exists under the conditions of the assay, is about 0.1 pmole per mg of cytoplasmic protein for each of these steroids.

**Protein Nature of Steroid-binding Components of Muscle Cytosol**—To determine the chemical nature of the [³H]dexamethasone- and [³H]triamcinolone acetonide-binding components, muscle cytosol was treated with nagarse. Incubation with this protease destroyed the steroid binding activity (Table I), indicating the protein nature of the hormone-binding macromolecules. The specific binding of [³H]dexamethasone and [³H]triamcinolone acetonide was also found to depend on the integrity of SH groups. When thioglycerol was omitted from the homogenization buffer (see "Materials and Methods"), the subsequent binding of [³H]triamcinolone acetonide to the cytosol fraction was markedly diminished. Furthermore, addition of 0.01 M N-ethylmaleimide or iodoacetic acid to muscle cytosol almost completely abolished [³H]dexamethasone-specific binding (data not shown).

**Effect of digestion with nagarse on specific binding of [³H]dexamethasone and [³H]triamcinolone acetonide to cytosol of rat muscle**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specifically bound steroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>71</td>
</tr>
<tr>
<td>Nagarse</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>4</td>
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</table>

**Sucrose Sedimentation Profiles of Labeled Steroid-Muscle Cytosol Complexes**—Experiments were performed to determine the sedimentation profiles of the glucocorticoid binding components of rat muscle cytosol. The 27,000 × g muscle supernatant fraction was incubated with [³H]dexamethasone or [³H]triamcinolone acetonide at 0°, the unbound steroid was removed by charcoal adsorption and the bound complexes were subjected to 5 to 20% sucrose density gradient centrifugation (Fig. 4). Both steroid-protein complexes had a peak of radioactivity with a sedimentation coefficient of ~4 S in the presence of 0.3 M KCl, whereas only the [³H]triamcinolone acetonide-protein complex displayed an ~7 S value under low ionic strength. It therefore appears that the [³H]triamcinolone acetonide-protein complex is more stable than the [³H]dexamethasone-protein complex in the absence of salt, inasmuch as the latter complex dissociates during prolonged centrifugation.

**Relative Affinity of Various Steroids for [³H]Dexamethasone- and [³H]Triaacinolone Acetonide-binding Sites**—Various nonlabeled steroids were tested for their ability to compete with [³H]dexamethasone or [³H]triamcinolone acetonide for the binding sites.

**TABLE I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[³H]Dexamethasone</th>
<th>[³H]Triaacinolone acetonide</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>71</td>
<td>55</td>
</tr>
<tr>
<td>Nagarse</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>
Effect of nonlabeled steroids on binding of [3H]dexamethasone and [3H]triamcinolone acetonide binding component in sucrose gradients. For details see "Materials and Methods." The arrow indicates the sedimentation peak of bovine serum albumin (BSA). Gradients with KCl, ——; gradients without KCl, -----.

Fig. 4. Sedimentation profiles of the [3H]dexamethasone and [3H]triamcinolone acetonide binding component in sucrose gradients.

**Table II**

<table>
<thead>
<tr>
<th>Nonlabeled steroid</th>
<th>Bound steroid</th>
<th>[3H]Dexamethasone</th>
<th>[3H]Tria. acetonide</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cortisol</td>
<td></td>
<td>94</td>
<td>96 ± 8</td>
</tr>
<tr>
<td>Triamcinol. acetonide</td>
<td></td>
<td>21</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td></td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE II

Effect of nonlabeled steroids on binding of [3H]dexamethasone and [3H]triamcinolone acetonide to cytosol of rat muscle

Aliquots of muscle cytosol (5.0 mg of protein per ml) were incubated with 9 × 10⁻⁸ M [3H]dexamethasone or 4 × 10⁻⁸ M [3H]triamcinolone acetonide in the presence or absence of 5 × 10⁻⁴ M of nonlabeled steroid at °0° for 2 hours. The amount of bound radioactivity was determined after charcoal adsorption and is expressed as percent of that observed in absence of nonlabeled steroids. Nonspecific binding has been subtracted. The 100% value for [3H]dexamethasone and [3H]triamcinolone acetonide binding was 78 and 71 fmol per mg of protein, respectively. Each value is the mean of four determinations.

in muscle cytosol. In these experiments a 1000-fold excess of the nonradioactive competing steroid was used. From the relative effectiveness of the various compounds as competitors for the specific binding sites (Table II) it is evident that the [3H]dexamethasone- and [3H]triamcinolone acetonide-binding sites display the same steroid specificity; whereas the inactive stereoisomer epicortisol did not compete with [3H]dexamethasone or [3H]triamcinolone, other steroids which are biologically active significantly reduced the specific binding of the labeled hormones.

**Effect of Glucocorticoids Administered In Vivo on In Vitro Binding of [3H]Dexamethasone**—In order to assess the relationship between the in vitro binding of [3H]dexamethasone and the in vivo potency of the glucocorticoid hormones, an experiment was conducted in which adrenalectomized rats were treated with various steroids 2 hours before they were killed. Table III shows that the subsequent specific binding of [3H]dexamethasone was reduced markedly by prior treatment with biologically potent glucocorticoids. The biological potency of these hormones on muscle is reflected by their effect on the reduction of muscle mass (22, 23). Under the conditions of the experiment, however, it is difficult to determine whether the reduction in the specific binding of [3H]dexamethasone is a reflection of the increased degree of saturation of the available binding sites by the in vivo administered steroid or an actual reduction in the number of binding sites.

**DISCUSSION**

The binding reaction described in this work was followed in vitro by incubating the 27,000 × g supernatant fraction of rat skeletal muscle with the labeled steroids at °0°. Such a cell-free system has several advantages over the assay system utilized by Simpson and White for the determination of glucocorticoid binding to skeletal muscle (14), since the direct interaction of the hormone with the binding component is measured. The in vitro assay at °0° minimizes possible bio-transformations of the hormone and eliminates changes in binding which might be a result of differences in cellular metabolism, cellular uptake, or binding by extracellular sites.

By utilizing the cell-free system, evidence was obtained for the existence of specific binding sites for glucocorticoid hormones in rat skeletal muscle. The binding reaction displayed saturation kinetics and high affinity binding of [3H]dexamethasone and [3H]triamcinolone acetonide to a cytosol protein. The formed complexes sedimented at about 4 S in sucrose density gradients containing 0.3 M KCl. These properties, as well as the reduction of binding following treatment of the rats with potent glucocorticoid hormones, resemble those described for steroid-specific "receptor" proteins present in the cytosol fraction of various target tissues, including rat liver (3), thymus (4), as well as certain murine lymphoid tumors (24). Other studies* have indicated that in addition to the binding component described in this paper, muscle cytosol contains an [3H]cortisol-binding protein similar to the corticosteroid-binding globulin of rat plasma (25). However, binding of [3H]dexamethasone or [H]triamcinolone acetonide cannot be attributed to the presence of the corticosteroid-binding globulin in muscle cytosol, since that globulin does not bind these synthetic glucocorticoids (5, 25).

In spite of the similarities in the kinetics and specificity of the [3H]dexamethasone- and [3H]triamcinolone acetonide-binding reactions, they differed with respect to their sedimentation

*To be published.
profiles. No sedimentation peak could be found for the [3H]dexamethasone protein complex in sucrose gradients at low ionic strength. This difference might be a result of the interaction of the hormones with different, preexisting or induced, conformational forms of a single binding protein resulting in different stabilities of the formed complexes. Such a possibility was recently suggested by Giannopoulos et al. (26) to explain differences in the binding of cortisol and dexamethasone to nuclear binding sites of rabbit fetal lung.

It is likely that the glucocorticoid-protein complex, as a result of the rather specific binding of glucocorticoid hormones to skeletal muscle cytosol, is involved in the catabolic actions of these hormones on skeletal muscle. The existence of specific intracellular receptors suggests that the interaction of steroids with muscle is of a "primary" type, in which the hormones directly interact with the muscle tissue, and the binding subsequently triggers the catabolic biological response. If this is the mechanism of action one should expect a correlation between hormone binding and catabolic response. The finding of [3H]dexamethasone- and [3H]triamcinolone-specific binding to muscle cytosol is in accord with their high potency in inducing muscle protein catabolism and weight loss (22, 23). Along with the findings on the reduction in binding following in vivo administration of potent glucocorticoids, these results can be taken as an indication for a direct rather than secondary effect of glucocorticoids on muscle.

The observations on glucocorticoid hormones binding to skeletal muscle further imply that a basic common sequence of events mediates the biological response of different tissues to steroid hormones. Since in tissues in which glucocorticoids mediate an essentially anabolic response (liver) a similar binding is known to occur, it is probable that the binding is the initial reaction occurring before any subsequent tissue-specific response, either anabolic or catabolic, is induced by the glucocorticoid hormones.

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REFERENCES