Nuclear Translocation of the Cytoplasmic Glucocorticoid Receptor in the Iris-ciliary Body and Adjacent Corneoscleral Tissue of the Rabbit following Topical Administration of Various Glucocorticoids

A Rapid Screening Method for Glucocorticoid Activity

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The present study demonstrates that the cytoplasmic glucocorticoid receptor in the iris-ciliary body and adjacent corneoscleral tissue translocates to the cell nucleus after topical administration of dexamethasone. This effect was found to be direct: independent of absorption and systemic distribution. Autoradiographic analysis demonstrated that topically applied 3H-dexamethasone specifically localizes to the nucleus of cells of the iris-ciliary body and adjacent corneoscleral tissue. The effect of dexamethasone-21-phosphate on nuclear translocation of the cytosolic glucocorticoid receptor was shown to be dose related. The threshold of this effect occurred following application of 0.001% dexamethasone-21-phosphate with the maximal loss being reached at a steroid concentration of about 0.005%. A study of the time course of nuclear translocation of the cytosolic glucocorticoid receptor after a single topical application of 0.01% decadron showed that 1 hr was required for a maximal effect to occur. By 3 hrs the cytosolic glucocorticoid receptor concentration returns to pretreatment levels. Medrysone, fluorometholone and prednisolone-21-acetate were all found to be less active than dexamethasone-21-phosphate in their ability to cause a decrease in the cytosolic glucocorticoid receptor at all concentrations tested. The dosages required for 50% activity are as follows: decadron 0.003%, prednisolone-21-acetate 0.02%, fluorometholone 0.03%, and medrysone > 1%. Control experiments have indicated that the differences in the activities of the steroids are not due to vehicle or to aqueous solubility. The present study suggests that nuclear translocation of the cytosolic glucocorticoid receptor is an early and necessary event in steroid-induced elevation in intraocular pressure. The nuclear translocation assay described in the present report may be useful as a rapid screening method for studying the dose relationships of new steroids and analogues in elevating intraocular pressure. Invest Ophthalmol Vis Sci 24:147–152, 1983

Since the original description of steroid-induced glaucoma1–3 there has been considerable interest in the relationship between glucocorticoids and intraocular pressure (IOP). Research in this area, however, was hampered by the absence of a suitable experimental model. In the rabbit, elevation of IOP with...
glucocorticoids was initially found to be variable. The observation that young rabbits (8-week-old New Zealand albino) are uniformly sensitive to this glucocorticoid effect indicates that this experimental animal is indeed a useful test system.

It is well established that steroid hormones, including glucocorticoids, exert their biologic action by binding to receptor molecules in the cytoplasm of specific target cells. This steroid receptor complex is then translocated to the cell nucleus where it alters the expression of the genome leading to the hormone response. We have demonstrated previously the presence of glucocorticoid receptors in the iris-ciliary body and adjacent corneoscleral tissue of the rabbit by biochemical and autoradiographic techniques and have shown that these cytoplasmic glucocorticoid receptor translocates from the cell nucleus following systemic administration of active glucocorticoids. Since the increase in IOP is usually associated with topical administration of glucocorticoids, it was necessary to demonstrate that the glucocorticoid receptors in the cells of the outflow pathway are accessible to this mode of administration before a relationship between these receptors and IOP elevation could be established.

The present report shows that following topical administration of glucocorticoids the glucocorticoid receptor translocates from the cytoplasm to the cell nucleus. This essential event in hormone expression can be detected within 40 min of topical ocular administration of the steroid and can provide a rapid assessment of the biologic activity of a glucocorticoid.

Materials and Methods

Animals

New Zealand albino rabbits of both sexes, 1.5 to 2.0 kg in weight, were used for all experiments. Twenty-five microliters of test solutions containing various dilutions of a variety of steroids were dropped onto the cornea of each eye using an Eppendorf pipette. The lids were held shut for 1 min after each application. Sixty minutes later, or at other time intervals as indicated, the animals were killed by intravenous injection of air or by cervical fracture.

Preparation of Cytosol

After death, the eyes were enucleated and rinsed in cold balanced salt solution (BSS) (1.45 \times 10^{-2} \text{ M Tris}, 4.26 \times 10^{-1} \text{ M NaCl}, 5 \times 10^{-4} \text{ M MgCl}_2, 5 \times 10^{-6} \text{ M CaCl}_2 and 0.1\% glucose, pH 7.6). The iris-ciliary body and adjacent corneoscleral tissue was then removed, freed of adhering vitreous, and placed in cold BSS. The tissue was washed three times with this solution (50 ml for 20 min each time) at 0 C to remove any unbound steroid. For each determination, four pairs of iris-ciliary body were pooled. The tissue was minced and homogenized in 1.0 ml buffer (0.25 M sucrose, 0.01 M Tris, 10^{-3} \text{ M MgCl}_2, 5 \times 10^{-4} \text{ M dithiothreitol and 5} \times 10^{-4} \text{ M spermine, pH 7.9}) in a glass homogenizer, kept cold in an ice slurry, using five strokes of a motor-driven Teflon pestle. The resulting homogenate was centrifuged for 50 min at 140,000 \times g at 5 C. The supernatant fraction (cytosol) was assayed immediately for glucocorticoid receptor activity. Typically, the cytosol contained about 5 mg protein per ml, as determined by the method of Lowry using BSA as standard.

Measurement of Glucocorticoid Receptor Concentration

\(^3\text{H}-\text{dexamethasone (Sp. act 20 to 50 Ci/mmol) was purchased from Amersham Searle or New England Nuclear Corp. and stored in ethanol at}\) –20 C. Periodic checks for purity of the labeled steroid were carried out as described previously. Suitable aliquots were dispensed into borosilicate tubes, evaporated to dryness in vacuo, and cooled in an ice bath. A 400-\mu l aliquot of cytosol was added to each tube, resulting in a final concentration of \(^3\text{H}-\text{dexamethasone of} 10^{-7} \text{ M. We have previously shown that this concentration saturates the glucocorticoid receptor.}^8 \text{ One of the tubes also contained a 200-fold excess of nonlabeled dexamethasone (purchased from Steraloids Inc.)} \text{ to determine the nonspecifically bound steroid. After incubating at 0 C for 20 hrs, duplicate aliquots from each tube were each applied to a small column of Sephadex G-50 and eluted at 4 C to separate the bound from the free} \(^3\text{H}-\text{dexamethasone.}^8 \text{ The fractions corresponding to the bound} \(^3\text{H}-\text{dexamethasone were counted with 10-ml econofluor (New England Nuclear) in a scintillation counter (}^3\text{H efficiency 40\%). The amount of} \(^3\text{H}-\text{dexamethasone bound in the presence of an excess of nonlabeled dexamethasone is subtracted from the total bound} \(^3\text{H}-\text{dexamethasone to give the specifically bound steroid. The concentration of the glucocorticoid receptor is expressed in femtomoles dexamethasone specifically bound per milligram of cytosolic protein.}

Preparation of Steroids

The following commercially available preparations were used: Decadron (0.1% dexamethasone-21-phosphate) (Merck, Sharpe and Dohme), Pred-Forte (1% prednisolone-21-acetate), medrysone (1% 11β-hydroxy-6α-methyl progesterone), fluorometholone (0.1% 9α-fluoro-11β,17-dihydroxy-6α-methyl progesterone) (all from Allergan), and Inflamase Forte (1% prednisolone-21-phosphate) (Smith, Miller and Patch). Suitable dilutions of these commercial preparations were prepared in physiologic saline (0.9% NaCl).
In order to control for possible effects of different vehicles, dexamethasone-21-phosphate, prednisolone-21-acetate, medrysone, and fluorometholone were obtained in crystalline form from the respective companies and suspended in physiologic saline by homogenization in a glass homogenizer with a Teflon pestle. This resulted in a fine suspension of the steroids that has been shown to minimize corneal irritation and permit a rapid dissolution in the tear film. Dexamethasone powder (free alcohol) was obtained (Steraloids) and prepared in saline as above. These suspensions were prepared at a concentration of 0.01%.

Autoradiography of $^3$H-Dexamethasone

Twenty-five microliters of saline containing 6.25 μCi of $^3$H-dexamethasone were applied topically to each eye of two New Zealand albino rabbits. One of the animals was treated topically with 32 μg (340-fold molar excess) of nonlabeled dexamethasone (Decadron) in 50 μl of saline to each eye 10 min prior to $^3$H-dexamethasone. Approximately 1 hr later the animals were killed with intravenous sodium pentobarbital, and the eyes were enucleated. The tissue samples, without washing, were processed for dry mount autoradiography as we described previously.

Results

Dose Response of Topically Applied Dexamethasone-21-phosphate on the Decrease of the Glucocorticoid Receptor in the Cytosol

The concentration of glucocorticoid receptor in the cytosol of the rabbit iris-ciliary body following a single topical ocular application of various concentrations of Decadron is seen in Figure 1. The threshold of loss of receptor from the cytosol was found at 0.001% Decadron, with a maximal loss being reached at a Decadron concentration of about 0.005%. Further increases in concentration of Decadron to 0.01% or to 0.1% did not appreciably reduce the concentration of receptor in the cytosol (approximately 80 femtomoles per mg protein). In our previous study following intravenous injection of cortisol, we found that the loss of glucocorticoid receptor from the cytoplasm is associated with a concomitant gain in the nucleus. In addition, as in the present study, the cytoplasmic glucocorticoid receptor could not be reduced below approximately 75 femtomoles per mg cytosol protein even with large doses of intravenous cortisol.

In order to determine whether the effect of topical Decadron is mediated by absorption and systemic distribution of the steroid to the iris-ciliary body, eight rabbits were each treated with 0.01% Decadron to one eye and physiologic saline to the other. Cytosol was prepared separately from the Decadron-treated eyes and the saline controls. Table 1 shows the results of this experiment. As can be seen, the Decadron-treated eyes show a reduction of the glucocorticoid receptor in the cytosol to 97 femtomoles per mg protein, while the saline-treated eyes maintained a high level of cytosolic receptor (347 femtomoles per mg protein). Thus, the application of 0.01% Decadron to one eye does not affect the concentration of glucocorticoid receptor in the cytosol to 97 femtomoles per mg protein, while the saline-treated eyes maintained a high level of cytosolic receptor (347 femtomoles per mg protein). Therefore, the application of 0.01% Decadron to one eye does not affect the concentration of glucocorticoid receptor in the cytosol of the iris-ciliary body in the other eye, indicating that at this concentration the steroid is acting directly and not through absorption and systemic distribution.

To exclude the possibility that the decrease in cytosolic glucocorticoid receptor seen was due to the topically applied, nonlabeled Decadron persisting in the cytosolic preparation, cytosols that were prepared from eyes separately treated with 0.01% Decadron or saline were mixed. The cytosols from the two groups were assayed separately and after mixing (1:1). As can be seen in Table 1, the mixed cytosol preparation had a value approximately midway between the Decadron-treated and the control. This indicates that there was no nonlabeled Decadron contaminating the cytosol preparations, which could interfere with the mea-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytosolic glucocorticoid receptor specifically bound (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Saline</td>
<td>347</td>
</tr>
<tr>
<td>B. Decadron (0.01%)</td>
<td>97</td>
</tr>
<tr>
<td>Mixture of cytosol from A &amp; B</td>
<td>197</td>
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Table 1. Effect of topical application of decadron to one eye of the rabbit on nuclear translocation of the cytosolic glucocorticoid receptor

Fig. 1. Dose response of topically applied dexamethasone-21-phosphate (Decadron) on the concentration of cytosolic glucocorticoid receptor. Solutions were prepared by dilution of the commercially available ophthalmic preparation (0.1%).
Fig. 2. Time course of the concentration of glucocorticoid receptor in the cytosol following single application of 0.01% dexamethasone-21-phosphate (Decadron).

Figure 2 shows the amount of receptor present in the cytosol at various times after a single topical application of 0.01% Decadron. A significant decrease in the cytosolic glucocorticoid receptor concentration is seen at 40 min with a maximal effect occurring at 1 hr and persisting for an additional hour. By 3 hrs the cytosolic receptor concentration has returned to pretreatment levels. It is not clear whether this increase is due to the receptor migrating back from the nucleus to the cytoplasm or to synthesis of new receptor protein.

Table 2. Concentration of glucocorticoid receptor in the cytoplasm following topical application of 0.01% solution of a variety of steroids

<table>
<thead>
<tr>
<th>Test solution*</th>
<th>Cytosolic glucocorticoid receptor specifically bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline control</td>
<td>252</td>
</tr>
<tr>
<td>Fluorometholone</td>
<td>255</td>
</tr>
<tr>
<td>Medrysone</td>
<td>222</td>
</tr>
<tr>
<td>Prednisolone-21-acetate</td>
<td>228</td>
</tr>
<tr>
<td>Prednisolone-21-phosphate</td>
<td>193</td>
</tr>
<tr>
<td>Dexamethasone-21-phosphate</td>
<td>120</td>
</tr>
<tr>
<td>Dexamethasone (free alcohol)</td>
<td>48</td>
</tr>
</tbody>
</table>

* All of the solutions were prepared from the crystalline steroids in physiological saline except for prednisolone-21-phosphate that was a 100-fold dilution of Inflamase Forte.

P values were determined by a student's t-test.

Dose Response of Topically Applied Medrysone, Fluorometholone and Prednisolone-21-Acetate on the Decrease in the Glucocorticoid Receptor in the Cytosol

As can be seen in Figure 3, medrysone and fluorometholone are less active than Decadron (data from Fig. 1) at all concentrations in their ability to decrease the concentration of the cytosolic glucocorticoid receptor. Prednisolone-21-acetate is less active than Decadron at 0.01% but similar at 0.1%. Medrysone has little activity even when used in a concentration of 1%. Thus, the approximate concentration of steroid required in this assay for 50% activity is as follows: dexamethasone-21-phosphate 0.003%, prednisolone-21-acetate 0.02%, fluorometholone 0.03%, and medrysone > 1%.

The ability of the crystalline steroids, prepared at concentrations of 0.01% in physiologic saline, to cause a decrease in concentration of glucocorticoid receptor is shown in Table 2. Dexamethasone and dexamethasone-21-phosphate are both very active. Medrysone, fluorometholone and prednisolone-21-acetate and phosphate are all virtually inactive (not significantly different from saline). This indicates that the differences in activity seen in Figure 3 are due to the steroids themselves rather than to the different vehicles.

Nuclear Localization of $^3$H-Dexamethasone Following Topical Application by Autoradiography

The distribution of silver grains after topical application of $^3$H-dexamethasone was similar to that previously reported by us following systemic injection of the labeled steroid. Figure 4A is an autoradiograph showing concentration of grains over the nuclei of cells of the outflow pathway following topical application of $^3$H-dexamethasone. Application of an excess
Fig. 4. Autoradiography of the outflow pathway of a rabbit eye following topical administration of \( ^3 \text{H}-\text{dexamethasone} \). A, \( ^3 \text{H}-\text{dexamethasone} \) alone. Six months exposure to the emulsion (hematoxylin-eosin, \( \times 1750 \)). Note the localization of the silver grains to the nuclei of the cells of the outflow pathway (arrows). B, \( ^3 \text{H}-\text{dexamethasone} \) following pretreatment with an excess of nonlabeled dexamethasone-21-phosphate. Six months exposure to the emulsion (hematoxylin-eosin, \( \times 1750 \)). Note the lack of localization of the silver grains to the nuclei of the outflow pathway cells (arrows).

Discussion

The present study demonstrates that the cytoplasmic glucocorticoid receptor in the iris-ciliary body and adjacent corneoscleral tissue translocates to the cell nucleus after topical administration of dexamethasone similar to that found by us after intravenous injection of cortisol. Moreover, topical administration was found to have a direct effect on this tissue independent of absorption and systemic distribution of the steroid. Autoradiographic analysis demonstrated that the topically applied \( ^3 \text{H}-\text{dexamethasone} \) specifically localizes to the nucleus of cells of the iris-ciliary body and adjacent corneoscleral tissue. This distribution was identical to that seen by us following intravenous injection of the labeled steroid. Thus, the glucocorticoid target cells are accessible to topical as well as to systemic steroids.

The effect of dexamethasone-21-phosphate on nuclear translocation of the cytosolic glucocorticoid receptor was shown to be dose related with the threshold of loss of receptor from the cytoplasm occurring at a steroid concentration of about 0.001%. The maximal loss of cytosolic receptor was reached at a dexamethasone-21-phosphate concentration of about 0.005%. Further increases in concentration of the steroid did not appreciably reduce the concentration of receptor in the cytosol. This dose-related response in nuclear translocation of the glucocorticoid receptor parallels the dose-related rise in IOP seen in susceptible individuals following topical administration of dexamethasone-21-phosphate.

A study of the time course of nuclear translocation of the cytosolic glucocorticoid receptor after a single topical application of 0.01% Decadron showed that 1 hr was required for a maximal effect to occur. We have previously shown that intravenously injected cortisol produced a maximal effect in less than 30 min.

Medrysone, fluorometholone, and prednisolone-21-acetate were all found to be less active than dexamethasone-21-phosphate in their ability to decrease the concentration of the cytosolic glucocorticoid receptor at all concentrations tested. This effect was not related to the vehicle since the same pattern was seen when saline solutions in the crystalline steroids were used (Table 2). Aqueous solubility was also not a factor since both dexamethasone (free alcohol) and dexamethasone-21-phosphate were active and both prednisolone-21-acetate and prednisolone-21-phosphate were inactive at the same concentrations (Table 2). The phosphate preparations were completely soluble at the concentration used, while the other preparations were in the form of suspensions. In addition, relative corneal penetration cannot be a significant determinant in our results since dexamethasone (free
alcohol) and prednisolone-21-acetate have been shown to penetrate the cornea to a far greater extent than their respective phosphate esters when similar concentrations of aqueous suspensions/solutions were applied topically to the intact noninflamed cornea. The finding that triamcinolone and tetrahydrotriamcinolone, which differ markedly in their ability to raise IOP, penetrate the cornea to the same extent also supports this conclusion. These data indicate that the differences in activities of the various steroids in the nuclear translocation assay is related to the steroid molecule itself and is less a function of vehicle, aqueous solubility, or relative corneal penetration. The molecular basis for the lower activity of medrysone, fluorometholone, and prednisolone relative to dexamethasone is not known. It may be due to more rapid metabolic inactivation or to lower affinity of the steroids for the glucocorticoid receptor. In addition, medrysone, fluorometholone, and prednisolone may bind to the glucocorticoid receptor in the cytoplasm and not translocate to the nucleus. However, since these steroids all are capable of elevating IOP and, thus, have some glucocorticoid agonist activity, it is likely that the loss from the cytoplasm is indeed due to nuclear translocation.

The biologic effect of glucocorticoids in virtually all tissues is mediated by nuclear translocation of the cytosolic glucocorticoid receptor. The dose response for this activity (Fig. 1) closely resembles that found for increases in IOP in sensitive patients. Further, those steroids such as medrysone, fluorometholone, and prednisolone, which are less active in causing nuclear translocation of the cytosolic glucocorticoid receptor (Fig. 3, Table 2), have been shown to be less active in raising IOP. These findings suggest that nuclear translocation of the cytosolic glucocorticoid receptor in this ocular tissue is an early and necessary event in steroid-induced elevation in IOP. The nuclear translocation assay described in the present report may be useful as a rapid screening method for studying the dose relationships of new steroids and analogues in elevating IOP.

Key words: glucocorticoid receptor, iris-ciliary body, nuclear translocation, dexamethasone, medrysone, fluorometholone, prednisolone, autoradiography.

Acknowledgment

The authors thank Ms. Auvra Kartub for her assistance in preparation of the manuscript.

References