Nuclear translocation of the cytoplasmic glucocorticoid receptor in the iris–ciliary body of the rabbit. A. Louis Southren, Gary G. Gordon, Helena S. Yeh, Michael W. Dunn, and Bernard I. Weinstein.

The cytoplasmic glucocorticoid receptor of the iris–ciliary body of the rabbit has been shown to translocate to the cell nucleus within 30 min of an injection of cortisol. Over the next 2 1/2 hr the amount of receptor returns to the control value. The threshold of the loss of receptor from the cytosol was found at 0.04 mg of cortisol per kilogram body weight, with a maximal loss being reached at a cortisol dose of 0.5 mg/kg B.W. The inactive glucocorticoid binds with many of the biochemical properties of a glucocorticoid receptor. The present study demonstrates that this binding protein moves from the cytoplasm to the nucleus in response to physiological doses of active glucocorticoids.

Materials and methods

Experimental animal. New Zealand white rabbits, 2.5 to 3.5 kg, were used for all experiments. The steroids were suspended in 2 to 3 ml of physiological saline and injected into an ear vein. At various intervals the animals were sacrificed by injection of a bolus of air. The eyes were enucleated, and the iris-ciliary body was removed, freed of adhering vitreous, and placed in a cold balanced salt solution (1.45 x 10⁻²M Tris, 1.26 x 10⁻⁴M KCl, 10⁻³M MgCl₂, 5 x 10⁻⁴M CaCl₂, and 0.01% glucose, pH 7.6). The tissue was washed three times for 1 to 1.5 hr with this solution at 0°C to remove any unbound steroid.

Fractionation of tissue into nuclear and cytoplasmic components. All experiments involving cytoplasmic and nuclei fractionation were carried out with fresh, nonfrozen tissue. After washing, the tissue was placed in a cold Tris-buffered sucrose (0.25M sucrose, 0.01M Tris, 10⁻³M MgCl₂, 5 x 10⁻⁴M dithiothreitol, and 5 x 10⁻⁴M spermine, pH 7.9) for 5 min, drained, finely minced, and disrupted in 1 to 1.5 ml of this buffer in a motor-driven homogenizer with a Teflon pestle. Five strokes at a low speed were used to homogenize the tissue. The homogenate was centrifuged for 10 min at 1000 x g, and the supernatant was centrifuged for 50 min at 100,000 x g to yield a clear supernatant fraction, the cytosol. The 1000 x g pellet, which contained, in addition to the cell nuclei, connective tissue and cell debris, was rehomogenized in 7 ml of the same buffer, filtered through Miracloth (Chioopee Mills, New York, N. Y.), sedimented at 1000 x g for 10 min, and then resuspended with the homogenizer in 3.5 ml of tricine buffer (2 x 10⁻¹²M tricine, 2 x 10⁻⁴M dithiothreitol, and 5 x 10⁻⁴M spermine, pH 7.9). The resulting preparations of nuclei and cytosol were analyzed for DNA content by the diphenylamine reaction and for protein by the method of Lowry et al.

In a typical experiment, six pairs of iris-ciliary body were homogenized in 1.5 ml of buffer and yielded a preparation of cytosol containing 6 mg of protein and less than 0.02 mg of DNA (in 1.2 ml) and a preparation of partially purified nuclei containing 1 mg of DNA and 3.2 mg of protein (in 3.5 ml). In some experiments frozen iris-ciliary body was homogenized in either tricine buffer or Tris-buffered sucrose and then centrifuged for 50 min at 100,000 x g. The supernatant in this instance is referred to as high-speed supernatant rather than cytosol since the nuclei may have been broken.

In vitro incubation of nuclei and cytosol with steroids. Stock solutions of steroids* purchased from Steraloids, Inc., were prepared in absolute ethanol and stored at -40°C. 1,2-³H-dexamethasone (sp.act. 24 Ci/mmol) was purchased from Amersham/Searle Corp.; 1,2-³H-cortisol (sp.act. 44.3 Ci/mmol) and 1,2-³H-corticosterone (sp.act. 47.5 Ci/mmol) were purchased from New England Nuclear Corp. Periodic checks for purity were carried out as described previously. When

*Generic names of steroids: corticosterone = 11β, 21-dihydroxy-preg-4-ene-3,20-dione; cortisol = 11β, 21-trihydroxy-preg-4-ene-3,20-dione; dexamethasone = 1,4-pregnadien-9-fluoro-16α-methyl-11β, 17α, 21-tri-3,20-dione; dexamethasone = 5α-androstan-17β-ol-3-one; estradiol = 1,3,5(10)-estratrien-3,17β-diol; progesterone = preg-4-ene-3,20-dione; tetrahydrocortisol = 3α, 11β, 17, 21-tetrahydro-5α-pregnan-20-one.

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needed, aliquots of the steroids were diluted in ethanol, dispensed into borosilicate tubes, evaporated to dryness, and cooled in an ice bath. Solutions of cytosol or nuclei were added to these tubes for incubation at 0°C. Preparations of cytosol were incubated with 1,2-3H-dexamethasone at 10^{-7}M. A duplicate aliquot containing a large excess of nonlabeled dexamethasone (2 x 10^{-5}M) was prepared to determine the nonspecific binding. At specified times, aliquots were analyzed by gel filtration with Sephadex G-50 to determine the amount of bound and free 3H-dexamethasone. Four 0.5 ml aliquots of the nuclei preparation were incubated with 5 x 10^{-6}M 3H-dexamethasone. Two of these aliquots also contained an excess of nonlabeled dexamethasone (10^{-5}M) to determine the nonspecific binding. The incubation was at 0°C for 24 hr with occasional mixing (vortex).

Fig. 1. A, Time course of specific binding of 3H-glucocorticoids to extracts of the iris-ciliary body. High-speed supernatant fraction prepared in tricine buffer was incubated at 0°C with 5 x 10^{-8}M 3H-dexamethasone, 3H-corticosterone, or 3H-cortisol; cytosol prepared in Tris-buffered sucrose also was incubated at 0°C with 5 x 10^{-8}M 3H-dexamethasone. At the indicated times 0.2 ml aliquots were removed and analyzed for specifically bound steroid. B, Time course of exchange of tritiated steroids bound to receptor with unbound dexamethasone. Extracts were prepared and incubated as described for A. After 4 hr, portions of each incubate were transferred to tubes containing nonlabeled dexamethasone (final concentration 10^{-5}M), and the incubation continued at 0°C. At the indicated times 0.2 ml aliquots were removed and analyzed for specifically bound steroid. The results are expressed as amount of steroid specifically exchanged relative to a control incubation.

Fig. 2. Time course of glucocorticoid receptor translocation after a single injection of nonlabeled cortisol. For each experimental point four rabbits were injected with cortisol (0.3 mg/kg B.W. as Solu-Cortef) and sacrificed at the indicated times. The cytosol was prepared and incubated with 10^{-7}M 3H-dexamethasone at 0°C overnight, and the specific binding was determined as described in Materials and methods.

Fig. 3. Dose response of glucocorticoid receptor translocation. Animals were sacrificed 30 min after injection of nonlabeled cortisol (as Solu-Cortef) at the indicated dose. Each point represents an independent experiment utilizing pooled iris-ciliary body from four animals. The cytosol was prepared and incubated with 10^{-7}M 3H-dexamethasone at 0°C overnight, and the specific binding was determined as described in Materials and methods.
during the first and last 4 hr. The nuclei containing bound dexamethasone were separated from the free hormone by sedimentation at 1000 × g for 10 min. After two washings with 3 ml of tricine buffer containing 10^{-5}M nonlabeled dexamethasone, 1 ml of methanol was added to each tube containing the washed nuclei, and the mixture was incubated at 20° for 1 hr. The suspension was transferred to scintillation vials containing 10 ml of Econofluor (New England Nuclear) and counted in an Isocap 300 scintillation counter (H efficiency 40%). Data for cytosol and nuclei are reported as specific binding, i.e., after subtraction of the nonspecifically bound steroid. Quantification of the glucocorticoid receptor is expressed as femtomoles dexamethasone specifically bound per milligram of protein for cytosol and per milligram of DNA for nuclei.

**Results and discussion**

*In vitro time course of dexamethasone binding and exchange.* As seen in Fig. 1, A, dexamethasone bound specifically to its receptor, reaching a maximum within 4 hr at 0°. Over the next several days there was a gradual decrease in the amount of steroid which was specifically bound. This seemed to be independent of buffer and salt concentration. The natural glucocorticoids, cortisol and corticosterone, bound to the same extent as dexamethasone after 4 hr of in vitro incubation but decreased rapidly thereafter. This indicates that dexamethasone stabilized the receptor to a greater extent than either cortisol or corticosterone.

In order to assay the total amount of receptor present, it was necessary to establish conditions whereby receptors bound to injected and endogenous steroids would change during an in vitro incubation with labeled dexamethasone. Fig. 1, B, illustrates that dexamethasone once bound to the receptor did not exchange readily with additional steroid. Only 50% or less of the bound 3H-dexamethasone exchanged with an excess of unlabeled dexamethasone after 24 hr at 0°. Not shown are experiments at higher temperatures where inactivation of the receptor was greatly accelerated without any increase in exchange. Bound cortisol and corticosterone, however, were exchanged to a much greater extent with non-bound dexamethasone, reaching completion after 1 and 2 days, respectively. Thus, in order to measure the total amount of receptor present, cortisol was used as the injected glucocorticoid. That is, even though the receptor had become bound to the injected cortisol in vivo, it could be readily exchanged during subsequent in vitro incubations with dexamethasone.

**Time course of the translocation of cytosolic receptor to the nucleus after in vivo injection of cortisol.** In Fig. 2 is shown the amount of receptor present in the cytosol after a single injection of cortisol (0.3 mg/kg B.W. as Solu-Cortef (The Upjohn Co.)). The amount of receptor decreased to approximately 33% within ½ hr. Over the next 2½ hr the amount of receptor in the cytosol gradually increased to the preinjection value. This increase may have been due to the receptor migrating back from the nucleus to the cytoplasm, although the possibility of synthesis of new receptor protein is not excluded. Table I shows the results of several experiments where the amount of receptor in the nucleus and cytoplasm was measured ½ hr after an injection either of cortisol or saline. As can be seen, cortisol resulted in a significant (p < 0.05) increase in the amount of receptor in the cell nucleus coinciding with the loss (p < 0.01) from the cytoplasm. The amount present in the nuclei, however, may be an underestimate because all the sites bound to the injected steroid may not have been exchanged under in vitro conditions. By contrast, cortisol bound to the cytoplasmic receptor appears to exchange readily under these conditions (Fig. 1, B).

**Dose response to in vivo injection of cortisol.** In Fig. 3 is shown the amount of receptor present in the cytosol of the iris–ciliary body ½ hr after injection of various dosages of cortisol (as Solu-Cortef). The threshold of the loss of receptor from the cytosol was found at 0.04 mg of cortisol per kilogram body weight, with a maximal loss being reached at a cortisol dose of 0.5 mg/kg. Increasing the dose to 3.0 mg/kg did not further reduce the amount of receptor. The 50% response was reached at a dose of cortisol, 0.1 mg/kg.

**Table I. Glucocorticoid receptor in cell nucleus and cytosol from iris–ciliary body of the rabbit following a single injection of cortisol**

<table>
<thead>
<tr>
<th>Glucocorticoid receptors</th>
<th>Cytosol (fmol/mg of protein)</th>
<th>Nuclei (fmol/mg of DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>380 ± 62</td>
<td>0.75 ± 0.11</td>
</tr>
<tr>
<td>(n = 4)</td>
<td></td>
<td>(n = 2)</td>
</tr>
<tr>
<td>Cortisol†</td>
<td>127 ± 11</td>
<td>1.2 ± 0.15</td>
</tr>
<tr>
<td>(n = 3)</td>
<td></td>
<td>(n = 3)</td>
</tr>
<tr>
<td>p &lt; 0.01</td>
<td></td>
<td>p &lt; 0.05</td>
</tr>
</tbody>
</table>

†The values are expressed as the mean ± S.D.; n = number of experiments. Each experiment utilized a pool of iris–ciliary body from six animals treated in an identical manner.
Fig. 4. Specificity of glucocorticoid receptor translocation. Approximate equimolar amounts of the indicated nonlabeled steroids were injected 30 min prior to sacrifice: cortisol 5 mg/kg B.W., tetrahydrocortisol 5 mg/kg B.W., dihydrotestosterone 4 mg/kg B.W., estradiol 3.8 mg/kg B.W., and progesterone 5 mg/kg B.W. The cytosol was prepared and incubated with $10^{-7}$M $^3$H-dexamethasone at $0^\circ$ overnight, and the specific binding was determined as described in Materials and methods. Each experiment consisted of a pool of iris-ciliary body from four animals. The vertical brackets designate the range of values for several experiments.

Table II. Amount of glucocorticoid receptor in various extracts* from the iris-ciliary body of the rabbit

<table>
<thead>
<tr>
<th>Extract</th>
<th>Treatment</th>
<th>Receptor concentration (fmol dexamethasone specifically binding of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. High-speed supernatant</td>
<td>None</td>
<td>268</td>
</tr>
<tr>
<td>B. Cytosol</td>
<td>4 mg/kg cortisol (as Solu-Cortef)†</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>A and B (calculated average)</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>A and B (1:1 mixture)</td>
<td>158</td>
</tr>
<tr>
<td>C. High-speed supernatant</td>
<td>None</td>
<td>222</td>
</tr>
<tr>
<td>D. Cytosol</td>
<td>5 mg/kg cortisol†</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>C and D (calculated average)</td>
<td>166</td>
</tr>
<tr>
<td></td>
<td>C and D (1:1 mixture)</td>
<td>150</td>
</tr>
</tbody>
</table>

*For each treatment the iris-ciliary body from 4 to 6 animals were pooled and homogenized as described in Materials and Methods.†Animals injected 30 min prior to sacrifice.

Fig. 4 shows the effect of various steroids on the amount of glucocorticoid receptor present in the cytosol. As can be seen, cortisol significantly lowered the amount of receptor in the cytosol, whereas the inactive glucocorticoid, tetrahydrocortisol, and the major sex steroids, dihydrotestosterone, estradiol, and progesterone, had little or no effect. There was no significant sex difference in the response to estradiol. It is noteworthy that progesterone was without effect in lowering the cytoplasmic receptor concentration, since at high concentrations it can compete in vitro with dexamethasone for binding to this receptor.2

In view of the large doses of injected cortisol used for some of the experiments shown in Figs. 3 and 4, it was necessary to determine whether the decrease in the amount of receptor observed was due to the presence of nonspecifically bound cortisol in the tissue extracts. To test this possibility, mixing experiments were carried out, and the results are shown in Table II. They indicate that the cytosol preparations obtained from animals in-
jected with large doses of cortisol did not interfere with the binding of 3H-dexamethasone to receptor present in the high-speed supernatant obtained from uninjected animals. Mising both extracts before assaying yielded a value virtually identical to the mean of the two values when assayed separately. If there had been free nonlabeled cortisol in the cytosol preparations, it would have lowered the amount of receptor assayed in the control extracts. Therefore the decrease in specific binding seen in these cytosols after injection of high doses of nonlabeled cortisol was due to a decrease in the amount of receptor present rather than to contamination with the injected steroid.

These data indicate that physiological doses of active glucocorticoids cause translocation of the glucocorticoid receptor molecule from the cytoplasm into the cell nucleus. This receptor appears to migrate to the cell nucleus in a manner similar to that reported for the glucocorticoid receptor of the thymus and the estrogen receptor of the uterus. The time course of this response is similar to that found in these other steroid-sensitive tissues and is consistent with the accepted mechanism whereby these hormones regulate differential gene expression in the nucleus.

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REFERENCES


A neuron of the inner nuclear layer (INL) with some processes extending to the outer plexiform layer (OPL) and others to the inner plexiform layer (IPL) was discovered near the posterior pole of the mouse retina. The neuron's location and appearance are similar to the interplexiform cells of several other species. The relatively recent recognition of this cell type along with its characteristic of infrequent staining by the Golgi technique makes the extent of its distribution among species uncertain. With each demonstration in a new species, the existence of the interplexiform cell as a sixth neuronal element of all vertebrate retinas becomes more assured.

For many years the neuronal complement of the vertebrate retina has been thought to consist of five basic types, each with several distinct subtypes. Recently, however, several authors have reported the existence of a sixth type, the nucleus of which is located in the inner nuclear layer (INL). This cell has been named the interplexiform cell because it has processes which ramify in the outer plexiform layer (OPL) as well as processes which branch in the inner plexiform layer (IPL). In this respect it is like a bipolar cell, and yet the appearance of Golgi-stained interplexiform cells is quite different from similarly stained bipolar cells.

Interplexiform cells have been demonstrated by fluorescence microscopy in teleost fish and new world monkeys and by Golgi staining techniques in cat, rhesus and squirrel monkey, and dolphin. Boycott et al. further point out that Cajal had described similar cells in dog and, perhaps, frog. This paper reports the existence of a cell type in the mouse, revealed by Golgi stain, which closely resembles the published pictures of interplexiform cells.

Materials and methods. Mice of the C57BL/6 strain were killed by cervical dislocation; the eyes were then enucleated and hemisected. The resulting eye cups were cycled once through the Golgi-Kopsch procedure, allowing 7 days each in the glutaraldehyde-dichromate and silver nitrate solutions. Rinsing in distilled water and dehydration through alcohols and propylene oxide were followed by embedding in Epon. The blocks were