Dexamethasone Induces Specific Proteins in Human Trabecular Meshwork Cells

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Previous studies have demonstrated the presence of high-affinity, glucocorticoid-specific receptors in explants of human outflow tissue and in cultured trabecular meshwork. Glucocorticoid-induced responses of scleral fibroblasts and trabecular meshwork cells were evaluated in this study. Incubation of human trabecular meshwork (HTM) and scleral fibroblast (HS) cells with $10^{-7}$ M dexamethasone (DEX) results in a 60% inhibition of prostaglandin production. The effects of glucocorticoid treatment on cellular and secreted proteins using $[^{35}S]$ methionine incorporation were evaluated. Treatment of HTM cells cultured from two normal individuals with DEX induced the expression of $[^{35}S]$ methionine-labelled cellular proteins of 35, 65 and 70 kD, and secreted proteins of 40, 90 and 100 kD. Under the same experimental conditions, a 70 kD molecular weight cellular protein was induced in the HS cells. There were no apparent DEX-induced alterations in HS-secreted proteins. Since a functional common response to glucocorticoid treatment in both HS and HTM cells was inhibition of prostaglandin production, the dexamethasone-induced expression of the 70 kD protein in these cells may be related to this effect. Further studies are required to elucidate specific roles of the steroid-induced proteins in the effects of glucocorticoids on HTM and HS cells. Invest Ophthalmol Vis Sci 30:1843–1847, 1989

The trabecular meshwork is thought to play a key role in the regulation of intraocular pressure (IOP), and altered trabecular meshwork morphology and function have been described in primary open-angle glaucoma (POAG). Previous studies have demonstrated the presence of high-affinity glucocorticoid-specific receptors in cultured trabecular meshwork cells,1 and other cellular components of the human anterior segment.2 In view of the clinical observations of glucocorticoid sensitivity in POAG patients resulting in a pronounced rise in IOP,3,4 a more precise knowledge of the action(s) of glucocorticoids on the trabecular meshwork is required. Glucocorticoids have been reported to inhibit prostaglandin (PG) production in cultured human trabecular meshwork (HTM) cells in a manner that requires new mRNA and new protein synthesis.5-7 Since some prostaglandins can reduce intraocular pressure (IOP)8,9 and prolonged topical application of glucocorticoids can increase IOP,3-5 a possible mechanism of glucocorticoid increase of IOP is via inhibition of release of hypotensive PGs from various cells in the eye. Glucocorticoids have also been shown to alter the synthesis of collagen and glycosaminoglycans in trabecular meshwork cells in explants of human outflow tissue.10 In order to identify glucocorticoid-responsive proteins, which may be related to the biological effects of these steroids, we examined the effects of a 16 hr treatment with $10^{-7}$ M dexamethasone on the expression of $[^{35}S]$ methionine-labelled cellular and secreted proteins from human trabecular meshwork (HTM) and scleral fibroblast (HS) cells using two-dimensional polyacrylamide gel electrophoresis (2-D PAGE).

Materials and Methods

Tissue Culture

Trabecular meshwork and scleral fibroblasts were grown from explants obtained from autopsy eyes. The two cell types were distinguished on the basis of the morphologic and ultrastructural features originally described by Polansky et al.,11 as described in our previous communication.7 The cells used in this study were from the fourth to six passage and were routinely grown in Dulbecco’s Modified Eagle Media (DME) containing 10% fetal bovine serum (FBS).
Two-Dimensional Polyacrylamide Gel Electrophoresis of Cellular and Secreted Proteins

Confluent HS or HTM cells were incubated 16 hr in methionine-free DME containing 1% dialyzed fetal bovine serum (FBS) and 50 μCi/ml [35S] methionine (1096 Ci/mmol), with or without (control) 10^-7 M dexamethasone (DEX). The incubation media were collected, phenylmethylsulfonyl fluoride (PMSF) was added (final concentration 50 μM), and media were dialyzed against 10 mM ammonium acetate, pH 8.0, (8 hr) followed by lyophilization. The cells were washed three times with phosphate-buffered saline (PBS), and lysed in first dimension isoelectric focusing (IEF) sample buffer (9 M urea, 2% NP-40, 2% β-mercaptoethanol and 2% ampholines, pH 3.5–10). The lyophilized media proteins were re-suspended in the IEF sample buffer. Quantification of [35S] methionine incorporation into cellular and secreted proteins was performed by precipitation of samples with 10% trichloroacetic acid (TCA) followed by collection of the precipitated proteins on Whatman GFC filters. After washing, filters were placed in scintillation vials, 4 ml of Liquiscint scintillation fluid added, and radioactivity determined. An aliquot of each sample equivalent to 5 x 10^6 cpm (35S-methionine) was run in the first dimension of 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE) performed as described by O'Farrell. Gels were always run in pairs of control versus DEX-treated samples. The first dimension IEF gel was fused to a second dimension 10–20% acrylamide gradient slab gel using a 0.5% agarose solution. After electrophoresis, the slab gel was fixed in 50% methanol, 10% acetic acid, and soaked in Fluoro-Hance. The gels were dried and exposed to Kodak XAR-5 film for 72 hr at -85°C. The steroid treatments were carried out at least three times in each cell line used and in two HTM cell lines cultured from different patients. Pairs of autoradiograms were compared by viewing on a light box and differences identified. After initial identification of spot changes, the autoradiograms were analyzed by a second unbiased observer. Relative spot density was assessed by comparison to an invariant control spot of similar intensity on the autoradiogram.

Radioimmunoassay of Released Prostaglandins

HTM and HS cells were incubated 16 hr in DME media containing 1% dialyzed FBS, with or without 10^-7 M DEX. The media were removed, the cells washed three times with 1 ml of 37°C Dulbecco's Phosphate Buffered Saline (PBS) then preincubated 15 min in 1 ml 37°C PBS. This buffer was removed and the cells incubated with A23187 (5 μg/ml in PBS) for 15 min at 37°C. This solution was then removed and assayed for prostaglandin (PG)E2 and 6-keto PGF₁α, as previously described. 7

Materials

[35S] Methionine, [3H] PGE₂ and [3H] 6-keto PGF₁α were purchased from Amersham (Clearbrook, IL). Dexamethasone was from Steraloids (Wilton, NH). Liquiscint scintillation fluid was from National Diagnostics (Manville, NJ). All SDS-PAGE materials were from Bio-Rad (Richmond, CA). All cell culture materials were purchased from GIBCO (Grand Island, NY). Fluoro-Hance was from RPI (Mt. Prospect, IL). Antiserum against PGE₂ and 6-keto PGF₁α were from Advanced Magnetics (Cambridge, MA).

Results

[35S] Methionine Incorporation into Cellular and Secreted Proteins

Figures 1–4 show representative gels prepared from control and dexamethasone-treated HTM and HS
cell lysates and culture media. Proteins identified by a number are those that demonstrated a reproducible increase in density in all experiments. Within an individual experiment, there was sometimes an increased density of other cellular or secreted proteins, but these proteins were not consistently increased in all experiments. The entire gel is shown in each Figure, although the 97.4 kD standard is given only in Figure 3 due to a difference in the commercial source of Mr standards that were used.

Comparison of patterns of HTM and HS cellular proteins obtained using 2-D PAGE showed that these two cell types exhibited several differences in the pattern of $[^{35}S]$ methionine-labelled proteins (Figs. 1, 2). For example, the HS cells demonstrated a group of $[^{35}S]$ methionine-labelled high molecular weight proteins (>68 kD) not seen in the HTM cells (Figs. 1, 2).

When DEX was present during the $[^{35}S]$ methionine labelling period with the HTM cells there was an increase in the synthesis of three proteins (labelled 1, 2 and 3) with apparent Mr of 70, 65 and 35 kD respectively. Increased synthesis of these three proteins was evident in both HTM cell lines and in all experiments. There was no significant change in the amount of $[^{35}S]$ methionine incorporation into the TCA-precipitable proteins present in the cell lysates or media of either HTM or HS cells (Table 1) after treatment with DEX.

Incubation of the HS cells with DEX during the $[^{35}S]$ methionine labelling period resulted in the apparent induction of one protein (4) with an apparent Mr of 70 kD (Fig. 2). Based on the relative positions in the 2-D SDS-PAGE gels, DEX apparently induced one common or similar protein, with an Mr of 70 kD and a pl between 4 and 5 in both HTM and HS cells.

Both cell types exhibited a number of $[^{35}S]$ methionine-labelled proteins in the media, protein patterns that were markedly different between the two cell types (Figs. 3, 4). HTM cells secreted a limited number of anionic $[^{35}S]$-labelled proteins in the culture media, whereas the HS cells appeared to secrete many more anionic proteins. A consistent feature of the autoradiograms prepared from HS media proteins was a “blurred” appearance. This may reflect considerable charge heterogeneity of the different proteins as well as possible proteolysis. Charge heterogeneity is indicated by the horizontal “streaking” of the protein spots. Proteolytic degradation of secreted products is
suggested by the vertical "streaking" of protein spots. In this study PMSF was added to inhibit serine protease activity, but there may have been other types of proteases secreted by the HS cells which degraded some of the secreted proteins. Since the major objective of this study was to identify glucocorticoid responsive proteins in HTM cells, we did not pursue the effects of different protease inhibitors on the protein secretion pattern of HS cells.

Dexamethasone treatment of HTM cells increased the intensity of labelling of two media proteins of approximately 100 kD (5) and 90 kD (6) and a group of proteins with apparent molecular weights of approximately 40 kD (7) (Fig. 3). In contrast, DEX did not appear to alter the intensity or pattern of [35S] methionine-labelled media proteins of the HS cells (Fig. 4).

Prostaglandin Release

As an index of glucocorticoid action on the HTM and HS cells, we measured A23187 (5 μg/ml)-stimulated prostaglandin release. After a 16 hr incubation with DEX, A23187-stimulated prostaglandin release was reduced to a similar degree (approximately 60%) in both cell types (Table 2).

Discussion

The trabecular meshwork normally functions as a labyrinth of interconnecting trabecular spaces through which the aqueous humor exits from the eye. However, in the trabecular meshwork of eyes of POAG patients, various morphological alterations have been described, including sclerosis and thickening of the trabeculum, and altered collagen and basal lamina components. Although the precise etiology of POAG remains unknown, a number of studies have suggested a role for glucocorticoids.

In an attempt to identify cellular or secreted proteins whose synthesis is regulated by glucocorticoids, in this study we have examined cultured trabecular meshwork and scleral fibroblast cell [35S] methionine-labelled proteins. Possible mechanism(s) of glucocorticoid-induced alterations in IOP may include altered production of substances that influence IOP, and altered synthesis of extracellular matrix components that could obstruct the trabecular spaces. In the current study, we evaluated the effects of an acute (16 hr) treatment of cultured trabecular meshwork and scleral fibroblasts with DEX on the ability of these cells to secrete substances known to influence IOP (prostaglandins), and on the synthesis of cellular and secreted proteins.

Incubation of HTM and HS cells with 10^-7 M DEX resulted in a similar degree of inhibition of prostaglandin release. Parallel studies of [35S] methionine incorporation into TCA-precipitable proteins of HTM and HS cells and media revealed that DEX treatment increased the intensity of labelling of two media proteins of approximately 100 kD (5) and 90 kD (6) and a group of proteins with apparent molecular weights of approximately 40 kD (7) (Fig. 3). In contrast, DEX did not appear to alter the intensity of media proteins of the HS cells (Fig. 4).

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Table 1. [35S] Methionine incorporation into TCA-precipitable proteins of HTM and HS cells and media

<table>
<thead>
<tr>
<th>Cells (n)</th>
<th>Incubation</th>
<th>Lysozyme (cpm X 10^7)*</th>
<th>Media (cpm X 10^6)</th>
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<tbody>
<tr>
<td>HTM (3)</td>
<td>Control</td>
<td>3.11 ± 0.99</td>
<td>1.33 ± 0.07</td>
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<tr>
<td>Dexamethasone</td>
<td>3.23 ± 0.96</td>
<td>1.01 ± 0.63</td>
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<tr>
<td>HS (3)</td>
<td>Control</td>
<td>2.61 ± 1.51</td>
<td>1.13 ± 0.50</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>3.16 ± 1.80</td>
<td>1.23 ± 0.61</td>
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* Data are expressed as the mean plus or minus the standard error of the mean. Number of experiments is in parentheses.

Table 2. Effects of dexamethasone on A23187-stimulated prostaglandin release

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Incubation</th>
<th>PGE_2 (ng/well)</th>
<th>6-keto PGF_2alpha (ng/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTM</td>
<td>Control</td>
<td>11.5 ± 0.6</td>
<td>Not determined</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>4.3 ± 0.2*</td>
<td></td>
<td>Not determined</td>
</tr>
<tr>
<td>HS</td>
<td>Control</td>
<td>11.4 ± 0.1</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>3.3 ± 0.1*</td>
<td>0.02 ± 0.01*</td>
<td></td>
</tr>
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Confluent cells were incubated 16 hr in DME/1% dialyzed FBS without (control) or with 10^-7 M dexamethasone. A23187-stimulated prostaglandin release was then determined as described in Methods.

* Significantly different from control, P < 0.05 (student t-test, non-paired samples).
nine-labelled protein synthesis were performed to determine if the synthesis of a similar protein might be induced or increased by DEX in both cell types. The 2-D PAGE protein patterns of cellular and secreted proteins of HTM versus HS cells were reproducible and specific to the cell type; indeed, this protein "fingerprint" might be a useful additional criterion for the identification of HTM cells. While dexamethasone treatment of HTM cells resulted in the induction or increased synthesis of three cellular proteins, with Mr of 35, 65 and 70 kD, only one reproducible and detectable change occurred in the cellular [35S] methionine-labelled proteins of the HS cells, an apparent induction of a 70 kD protein. Dexamethasone induced or increased the secretion of two anionic proteins (90 kD and 100 kD) from HTM cells, but apparently did not alter the secretion of any 35S-methionine-labelled proteins from the HS cells. Polansky and coworkers reported that treatment of cultured HTM cells with glucocorticoid for 3 weeks induced several cellular proteins, having sizes of approximately 40 to 60 kD as analyzed by 1-D PAGE. These proteins are smaller than those increased or induced by 16 hr treatment with DEX in this study, and may be different proteins. This suggests that HTM cells may synthesize different proteins in response to short- or long-term treatment with DEX or, alternatively, that the smaller proteins detected after long-term treatment are modified products of larger proteins initially induced by DEX treatment.

Since glucocorticoids are thought to initiate most of their biological effects through changes in the transcriptional activity of genes coding for specific proteins, 2-D PAGE analysis has been a widely used technique to identify glucocorticoid responsive genes. The pattern of proteins synthesized by cells and seen on 2-D PAGE analysis is generally thought to reflect levels of specific mRNAs coding for each polypeptide. However, post-translational modifications (eg, phosphorylation, glycosylation, ADP ribosylation) can result in charge heterogeneity, yielding multiple spots or altered locations of spots on gels. An additional consideration is that glucocorticoids could influence the translation of certain mRNAs.

With these limitations in mind, the results of the current study indicate that two of the glucocorticoid-responsive proteins of HTM cells are unique to these cells and one is common to both HTM and HS cells. A common biological effect of glucocorticoids in both cell types was the inhibition of prostaglandin synthesis. The induced cellular protein of Mr 70 kD may be related to the biological effect of steroids. The identity and function of all the glucocorticoid-responsive proteins remain to be determined.

Key words: trabecular meshwork, glucocorticoids, glaucoma

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References