Inhibition of Dexamethasone-Induced Cytoskeletal Changes in Cultured Human Trabecular Meshwork Cells by Tetrahydrocortisol

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Purpose. To determine the cellular mechanism of action of the intraocular pressure (IOP) lowering steroid tetrahydrocortisol (THF).

Methods. Tetrahydrocortisol was evaluated for glucocorticoid antagonist activity using in vitro and in vivo assays. Systemically administered THF was evaluated for its ability to inhibit dexamethasone-induced body weight loss and systemic hypertension in rats. In vitro receptor antagonism was tested using the supernatant fraction of IM9 cells as the source of soluble glucocorticoid receptor in \(^{3}H\)-dexamethasone displacement binding assays. In addition, six different primary human trabecular meshwork (TM) cell lines were cultured for 0 to 14 days in the absence or presence of dexamethasone (\(10^{-7}\) M) and/or THF (\(10^{-8}\) to \(10^{-6}\) M). The effects of these steroids on the TM cytoskeleton were determined by epifluorescent microscopy and by transmission electron microscopy.

Results. Tetrahydrocortisol was unable to inhibit the dexamethasone (DEX)-induced systemic hypertension and decrease in body mass in rats and was unable to displace \(^{3}H\)-DEX from the soluble human glucocorticoid receptor. However, THF inhibited the DEX-induced formation of cross-linked actin networks in cultured human TM cells in a progressive and dose-dependent manner (IC\(_{50}\) = \(5.7 \times 10^{-7}\) M). Dexamethasone caused changes in the TM cell microtubules that were reversed partially by concomitant treatment with THF. Tetrahydrocortisol alone appeared to increase microfilament bundling in TM cells.

Conclusions. Tetrahydrocortisol was not a glucocorticoid antagonist at the level of the classical glucocorticoid receptor and did not appear to antagonize systemically mediated glucocorticoid activity in the rat. Tetrahydrocortisol inhibited DEX-induced changes in the TM microfilaments and microtubules. These results may explain partially the IOP lowering activity of THF because glucocorticoid-mediated changes in the TM cytoskeleton have been proposed to be involved in the generation of ocular hypertension. Invest Ophthalmol Vis Sci. 1996;35:805–813.

Topical ocular or systemic administration of glucocorticoids can lead to the development of ocular hypertension in susceptible persons,\(^1\)–\(^4\) and if glucocorticoid administration is continued, open angle glaucoma\(^5\)–\(^6\) that in many ways mimics primary open angle glaucoma will develop in many of these steroid responders.\(^7\) Glucocorticoid-induced ocular hypertension has been shown to be caused by increased resistance to aqueous humor outflow\(^1\)–\(^4\) and is associated with biochemical\(^8\)–\(^9\) and ultrastructural changes\(^10\)–\(^12\) in the trabecular meshwork (TM). Glucocorticoid-induced ocular hypertension also can be generated in rabbits,\(^13\)–\(^14\) cats,\(^15\)–\(^16\) and monkeys.\(^17\) A number of studies have reported glucocorticoid-mediated changes in cultured TM cells, including altered gene and protein expression,\(^18\)–\(^19\) altered deposition of TM extracellular matrix molecules,\(^20\)–\(^21\) decreased extracellular proteinase activities,\(^22\)–\(^23\) TM cell and nucleus enlargement,\(^24\)–\(^26\) reorganization of TM cytoskeletal elements,\(^24\)–\(^25\) inhibition of TM cell functions,\(^18\)–\(^25\)–\(^27\) and activation of the endoplasmic reticulum and Golgi apparatus in TM cells.\(^24\)

The ability of glucocorticoids to induce ocular hy-
pertension has inspired the search for steroids that may have the opposite effect and, thus, lower IOP.38-31 One of these ocular hypotensive steroids is tetrahydrocortisol (THF), which has been reported to lower the intraocular pressure (IOP) of dexamethasone (DEX)-induced ocular hypertensive rabbits on topical administration.32 Tetrahydrocortisol is a natural component of steroid metabolism and is the major metabolite of cortisol, the endogenous glucocorticoid, in humans. In addition, a preliminary experiment suggested that THF was also an effective ocular hypotensive agent in patients with primary open angle glaucoma.33

The current study was conducted to determine the IOP-lowering mechanism of action of THF. Tetrahydrocortisol was tested in rats for its ability to inhibit DEX-induced systemic hypertension and loss in body weight. Tetrahydrocortisol also was evaluated for its ability to interact with the classical human glucocorticoid receptor in an in vitro ligand-binding assay. In addition, because it has been suggested that one mechanism for DEX-induced ocular hypertension is caused by the effects of DEX on the TM cell cytoskeleton, we also evaluated the effects of THF on DEX-induced cytoskeletal changes in cultured human trabecular meshwork cells.

MATERIALS AND METHODS

Effect of Steroids on Body Weight and Blood Pressure

All animal experimentation was conducted in strict compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Four groups (n = 5 per group) of normotensive Sprague-Dawley rats, each weighing 200 to 300 g, were treated daily for 14 days with subcutaneous injections at 0.1 ml/100 g body weight of sesame oil (control), dexamethasone (0.1% in sesame oil), THF (1% in sesame oil), or DEX + THF (0.1% and 1%, respectively, in sesame oil). The rats received water and laboratory chow ad libitum and were housed under a 12-hour light–12-hour dark cycle. Body weight and blood pressure were measured three times per week. Indirect blood pressure (tail cuff) was determined using a Narco (Austin, TX) Bio-Systems Physiograph.

Human Glucocorticoid Receptor Ligand-Binding Assay

The human lymphoblast cell line IM9 (ATCC, Bethesda, MD) was used as a source of the soluble glucocorticoid receptor (GR).34 The cells were grown to densities of 1 to 10 × 10⁶ cells per milliliter in RPMI 1640 media (Gibco, Grand Island, NY) containing 10% fetal bovine serum (HyClone, Logan, UT), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM L-glutamine (Gibco) at 37° and 7% CO₂ in a humidified incubator. The IM9 cells were harvested from the media by centrifugation for 10 minutes at 1500 g. Cells were washed in 12 volumes of Dulbecco’s phosphate-buffered saline (PBS; Gibco) and repelleted. Washed cells were resuspended in five to six volumes (per volume of packed cells) of homogenization buffer (10 mM TRIS, 10 mM sodium molybdate, 1 mM EDTA, pH 7.4, 20 mM 2-mercaptoethanol, and 10% glycerol), and the cells were broken by nitrogen cavitation using 2 × 15 minutes at 600 to 750 psi nitrogen in the N₂ cavitor (Parr Instrument, Moline, IL) at 0°C. Cell disruption was confirmed by Hoffman contrast microscopy using a Nikon (Garden City, NY) Diaphot. The broken cell preparation was then centrifuged at 27,000 g for 15 minutes, and the resultant supernatant was centrifuged at 103,000 g for 60 minutes at 4°C. The amount of protein in the supernatant fraction was determined using a BCA assay kit (Pierce Chemical, Rockford, IL) with a bovine serum albumin standard. Aliquots of the supernatant fraction were snap frozen in a dry ice–acetone bath and stored at −70°C. Competitive binding assays were done in duplicate in homogenization buffer (total volume of 200 μl) by mixing 1 mg of IM9 cytosol, 0.05 μCi (3 nM) of 3H-dexamethasone (Amersham, Arlington, Heights, IL), and unlabeled competitor steroids (10⁻⁵ to 10⁻¹¹ M) consisting of dexamethasone, prednisolone, cortisol, triamcinolone acetonide, progesterone, cortesolone (Sigma Chemical, St. Louis, MO), and tetrahydrocortisol (THF; 5β-pregnan-3α, 11β, 17α, 21-tetrol-20-one) (Steraloids, Wilton, NH). After incubation at 0°C for 16 to 18 hours, the assay was stopped by the addition of 100 μl of a charcoal–dextran mixture (2% activated charcoal, 0.5% dextran in 10 mM Tris, 1 mM EDTA, pH 7.4). The assay mixture was further incubated at 0°C for 10 minutes before being centrifuged for 5 minutes at 8200 g. A 100-μl sample of the supernatant (protein-bound steroid fraction) was assayed for radioactivity by liquid scintillation spectrometry, and the IC⁵₀ values were determined graphically.

Evaluation of Effects of Steroids on Trabecular Meshwork Cell Cytoskeleton

Human TM cells were cultured and characterized as described previously.20,24,25 Briefly, TM cells were grown from explants dissected from human donor eyes (obtained from a variety of regional eye banks) placed in Ham’s F10 media containing 10% fetal bovine serum (HyClone), penicillin, streptomycin, and 2 mM L-glutamine (Gibco). The TM cells were propagated by serial passage using Cytoex 5 (Sigma) microcarrier beads. Human TM cells were grown to confluence on glass coverslips for light microscopic analysis or on Formvar-coated nickel grids for wholenum transmission electron microscopy (TEM) analysis. The
TM cells were treated with dexamethasone (10^{-7} M) and/or THF (10^{-6}, 10^{-7}, or 10^{-8} M) for 0 to 14 days. Stock solutions of the steroids were prepared by dissolving DEX (10^{-4} M) and THF (10^{-3} to 10^{-5} M) in absolute ethanol. Stock solutions were diluted in the media (1 μl stock solution per milliliter of media) immediately before use. Control cells received equivalent volumes of absolute ethanol (0.1% final concentration). Trabecular meshwork cell microfilaments were examined by epifluorescence after fixing the cells in 1% glutaraldehyde (Sigma), 0.5% Triton X-100 (Sigma), 50 mM phosphate buffer (pH 7.2) and staining with rhodamine-phalloidin (Molecular Probes, Eugene, OR) as previously described. The percentage of TM cells with cross-linked actin networks (CLANs) was determined by examining approximately 200 cells on each of two coverslips per experimental condition. Each experiment was performed two or more times. The definition and characterization of CLANs have been described in detail. Trabecular meshwork cell microtubules were visualized by fixing the cells in methanol at -20°C for 10 minutes, rinsing with PBS and incubating with a 1:25 dilution of an anti-tubulin primary antibody (Boehringer Mannheim, Indianapolis, IN) in PBS per 1% bovine serum albumin (Sigma) for 1 hour. Cells were then rinsed with PBS, incubated with a 1:20 dilution of fluorescein isothiocyanate-labeled rabbit anti-mouse secondary antibody (Boehringer Mannheim), and examined by fluorescent microscopy using a Nikon Optiphot Photomicroscope (Nikon).

Trabecular meshwork cells grown on Formvar-coated (Electron Microscopy Supplies, Fort Washington, PA) nickel grids were prepared for wholemount transmission electron microscopy as described to examine the effects of steroids on microfilament and microtubule organization. Cells were fixed for 30 minutes in 0.25% glutaraldehyde and blocked for 20 minutes in 4% nonfat dry milk. They were then incubated overnight with monoclonal anti-actin or anti-tubulin primary antibodies (Amersham) using 1:100 dilutions. After rinsing five times for 2 minutes each in Tris-buffered saline, the cells were incubated for 2 hours with 1:5 dilutions of immunogold-conjugated goat anti-mouse secondary antibodies (Amersham). Cells subsequently were fixed for 30 minutes in buffered 1% glutaraldehyde, rinsed, osmicated (2% osmium) for 2 minutes, dehydrated, critical point dried, and examined in a Zeiss (Thornwood, NY) CEM-902 transmission electron microscope.

RESULTS

Effect of Dexamethasone and Tetrahydrocortisol on Body Weight and Blood Pressure

Results of daily steroid treatment for 2 weeks on rat body weight are shown in Figure 1A. Vehicle (sesame oil)-treated as well as THF-treated rats continued to gain weight during the 2 weeks of treatment, with an average weight gain of 26 to 29 g per week. In contrast,
The DEX-treated group progressively lost weight after the first 2 days of treatment, with an average loss of approximately 46 g per week. The weight loss of the group treated with the combination of THF plus DEX was identical to the group treated with DEX alone. Therefore, THF treatment alone did not have any adverse effect on body weight, and THF did not block the catabolic effect of DEX. Results of 2 weeks of steroid treatment on rat blood pressure are shown in Figure 1B. Blood pressures of control and THF-treated groups were not significantly changed during the 2 weeks of treatment. In contrast, DEX treatment caused a progressive and significant increase in systolic blood pressure, with a pressure elevation of 8 to 9 mm Hg after 10 to 12 days of DEX administration. The group treated with the combination of THF plus DEX had progressive and significant rises in systolic blood pressure that were identical to the group treated with DEX alone. Therefore, THF did not appear to block the DEX-induced systemic hypertension.

**Glucocorticoid Receptor Binding**

Glucocorticoid receptor agonists bound to the soluble receptor with affinities that closely matched their anti-inflammatory potencies (Fig. 2A): triamcinolone acetonide ≈ dexamethasone > prednisolone > cortisol, with $IC_{50}$s of $1.1 \times 10^{-8}$ M, $1.2 \times 10^{-8}$ M, $2.2 \times 10^{-8}$ M, and $5.6 \times 10^{-8}$ M, respectively. The glucocorticoid antagonists progesterone and cortexolone also bound to the glucocorticoid receptor and displaced radiolabeled DEX (Fig. 2B) with $IC_{50}$s of $1.9 \times 10^{-7}$ M and $5.8 \times 10^{-7}$ M, respectively. In contrast, THF was unable to bind competitively to the glucocorticoid receptor and to displace DEX even at high concentrations ($10^{-6}$ M). In addition, there was no binding of radiolabeled THF to the human glucocorticoid receptor in direct binding assays (data not shown).

**Effect of Dexamethasone and Tetrahydrocortisol on Trabecular Meshwork Cytoskeleton**

Dexamethasone treatment caused a time-dependent reorganization of cultured human TM cell microfilaments to form cross-linked actin networks (Fig. 3A) as seen by epifluorescent microscopy of rhodamine-phalloidin-stained cells.$^{24,25}$ Concomitant treatment of the TM cells with DEX ($10^{-7}$ M) and THF ($10^{-6}$ M) caused a progressive inhibition of DEX-induced CLAN formation (Fig. 3A). There was no difference in TM CLAN formation at days 2 and 4 between DEX and DEX + THF treatment. However, beginning at day 6, THF caused an inhibition and reversal of DEX-induced CLAN formation. In 16 different experiments using seven different TM cell lines (each cell line was examined two to three times), $10^{-6}$ M THF treatment for 14 days inhibited and reversed the DEX-induced CLAN formation almost completely (Fig. 3B). Treatment with THF alone was comparable to the untreated control cells. In dose-response studies, THF ($10^{-8}$, $10^{-7}$, and $10^{-6}$ M) was added to TM cell cultures with DEX ($10^{-7}$ M) for 14 days. Tetrahydrocortisol inhibited DEX-induced CLAN formation in a dose-dependent manner with an $IC_{50}$ of $5.7 \times 10^{-7}$ M (Fig. 3C). The THF dose-response study (Fig. 3C) was performed in a TM cell line that was a very high responder to DEX treatment (i.e., almost all the DEX-treated TM cells developed CLANs in the absence of THF). The heterogeneity in responsiveness between TM cell lines has been reported.$^{25,35}$

We examined as well the morphologic effects of DEX and THF on the TM cytoskeleton by light and electron microscopic analysis of microfilaments and microtubules. Data shown are representative of the results from hundreds of photomicrographs taken from all the TM cell lines examined. Normal cultured...
Tetrahydrocortisol Effects on the Trabecular Meshwork Cytoskeleton

FIGURE 3. Effect of dexamethasone (DEX) and tetrahydrocortisol (THF) on cross-linked actin network (CLAN) formation in cultured human trabecular meshwork cells. (A) Percent of TM cells that develop CLANs when incubated without (control) or with DEX ($10^{-7}$ M) and/or THF ($10^{-6}$ M) for 0 to 14 days (average of two independent experiments). Control (○), DEX (●), THF (○), DEX + THF (●). (B) Mean (± SEM) CLAN response ($n = 16$ assays) of seven different TM cell lines cultured with DEX ($10^{-7}$ M), THF ($10^{-6}$ M), or DEX + THF for 14 days. The response of the DEX-treated group is significantly different from the other three groups ($P < 0.001$). (C) Effect of THF dose on DEX-induced CLAN formation in cultured human TM cells. Trabecular meshwork cells were incubated with DEX ($10^{-7}$ M) in the presence or absence of $10^{-6}$, $10^{-7}$, or $10^{-8}$ M THF for 14 days ($n = 4$). The IC$_{50}$ for THF inhibition is $5.7 \times 10^{-7}$ M, and $r = 0.98$.

TM cells have abundant microfilaments that are in linear arrays and are bundled into stress fibers (Figs. 4A, 5A). Dexamethasone treatment caused the TM microfilaments to reorganize into cross-linked, geodesic, dome-like structures (Figs. 4B, 5B) composed of actin filaments but not as heavily labeled with immunogold particles compared to the untreated control TM cells. Treating the TM cells with THF alone caused an increase in microfilament bundling (Figs. 4C, 5C), and the microfilaments appeared to be arranged in a more linear fashion along the cell axis. The concomitant treatment of cultured TM cells with DEX and THF for 14 days resulted in a mixture of microfilament bundles and remnants of CLANs (Figs. 4D, 5D). Actin immunogold staining appeared to return to control levels in these cells.

In addition to the steroid-induced changes in TM cell microfilament organization, DEX and THF altered the organization of TM cell microtubules. Microtubules of untreated TM cells are organized in astral arrays extending from the microtubule organizing center at the periphery of the nucleus to the edges of the cell (Fig. 4E). Linear stretches of microtubules are decorated readily with the anti-tubulin gold complex (Fig. 5E). Treatment with THF alone does not dramatically alter TM cell microtubule organization (Fig. 5G). However, DEX-treatment caused two major changes in the TM cell microtubule structure. In many of the DEX-treated cells, the microtubule organizing center appeared to migrate to a position above the nucleus (Fig. 4F). Immuno-ultrastructural analysis revealed abundant microtubule tangles throughout the TM cell cytoplasm (Fig. 5F). The addition of THF to the DEX-treated cells appeared to normalize partially the TM microtubule organization (Fig. 5H).

DISCUSSION

The administration of glucocorticoids by a variety of routes can lead to the development of ocular hypertension and glaucoma in susceptible persons. In addition, it is possible to generate ocular hypertension in animals by ocular administration of a potent glucocorticoid. Elevated IOP associated with glucocorticoid administration is caused by increased aqueous humor outflow resistance and is associated with biochemical and ultrastructural changes in the trabecular meshwork. Accordingly, numerous investigators have studied the effects of glucocorticoids on cultured TM cells to discover the molecular mechanism(s) responsible for glaucomatous IOP elevation. We propose the following hypothesis for glucocorticoid-induced ocular hypertension. Trabecular meshwork cells contain classical GR and are, therefore, targets for glucocorticoid action. Binding of the glucocorticoid with the TM cell GR alters TM cell gene expression, leading to the differential expression of a subset of proteins. There is increased deposition of extracellular matrix molecules, decreased expression of extracellular proteinases, and a reorganization of the TM cytoskeleton. The TM nucleus and cell enlarge and various important TM cell functions are inhibited. The combination of these glucocorticoid-mediated effects on the TM leads to progressive increased resistance in aqueous humor outflow and to the development of ocular hyperten-
FIGURE 4. Effect of dexamethasone (DEX) and tetrahydrocortisol (THF) on cultured trabecular meshwork (TM) cell microfilament and microtubule cytoskeletal elements. Confluent TM cells were treated without (control) or with DEX (10^{-7} M), THF (10^{-6} M), or DEX + THF for 14 days. Actin filaments were visualized by rhodamine-phalloidin staining (A to D). Microtubules were visualized using anti-tubulin immunofluorescence (E,F). Control (A,E), DEX (B,F), THF (C), and DEX + THF (D). Microtubule organizing centers are shown by arrows. Magnification bar = 50 μm.

Given this hypothesis, it may be possible to intervene in the glucocorticoid-induced damage to the TM at various steps. For example, concomitant treatment with a potent glucocorticoid antagonist, such as RU-486, should prevent glucocorticoid-induced ocular hypertension at the TM cell by binding to the glucocorticoid receptor at the beginning of this cascade. There have been several reports of topically administered RU-486 partially blocking glucocorticoid-induced ocular hypertension in rabbits.\textsuperscript{39,40} It was suggested that THF may be a glucocorticoid antagonist because it has been reported to lower IOP in DEX-induced ocular hypertensive rabbits.\textsuperscript{42} In addition, glucocorticoid antagonists have been shown to inhibit DEX-induced changes in the cytoskeleton of cultured human TM cells.\textsuperscript{25} The administration of glucocorticoids also can cause systemic hypertension\textsuperscript{41,42} and catabolic loss in body weight.\textsuperscript{43,44} Tetrahydrocortisol was administered systemically to determine whether it could block these glucocorticoid-mediated systemic effects. Our results show that THF is not a glucocorticoid antagonist in the classical sense because it was unable to block the effects of systemically administered DEX on body weight and on systemic blood pressure, as has been previously demonstrated with a more conventional glucocorticoid antagonist such as RU-486.\textsuperscript{44} We have shown that THF is not a GR antagonist because it does not bind to the GR.

Glucocorticoids have been shown to generate many changes in cultured TM cells.\textsuperscript{18-27} One of the more dramatic changes is a major reorganization of the TM cytoskeleton, which involves the actin microfilaments\textsuperscript{24,25} as well as the microtubules.\textsuperscript{45} This glucocorticoid-mediated change in the TM cytoskeleton is associated with altered TM cell function\textsuperscript{25} and may be responsible for corticosteroid-induced ocular hypertension. Although THF is not an antagonist at the level of the glucocorticoid receptor, it was nonetheless able to inhibit and reverse DEX-induced microfilament reorganization (CLAN formation) in TM cells. There appear to be subtle effects of THF alone on TM...
cell actin microfilaments, causing increased bundling and density of the stress fibers. Topical ocular administration of THF alone did not alter the IOP of rabbits, suggesting that this effect of THF on the TM cell cytoskeleton does not cause a significant change in the outflow facility. However, the THF-mediated bundling effect on actin appears to compete with the DEX-induced formation of cross-linked actin networks and may thereby inhibit the IOP-elevating activity of DEX. Although these steroid-induced cytoskeletal effects have been shown only in cultured TM cells, it is possible that similar cytoskeletal changes occur in organ-cultured eyes in situ as well as steroid-treated eyes in vivo. We are currently testing this hypothesis by determining whether DEX-induced CLANs are generated in TM tissue.

Although the specific molecules responsible for these steroid-induced cytoskeletal changes are as yet unknown, it is possible that the IOP activity of both THF and DEX may be mediated by actin-binding or actin-associated proteins that regulate the organizational structure of actin microfilaments. Dexamethasone treatment also induced changes in the microtubule organization of TM cells. This may be the result
of a direct effect of DEX on the expression of microtubule organizing proteins, or it may indirectly be the result of the DEX-induced reorganization of the TM microfilaments or other indirect DEX effects. We have recently shown that THF and DEX can independently regulate the expression of specific proteins in cultured TM cells, and some of these proteins may be important candidates in steroidal regulation of the TM cytoskeleton.

Tetrahydrocortisol is a natural metabolite of cortisol and, as such, may be relatively free of ocular or systemic side effects. Data from the current study show that, although it is not a GR antagonist, THF does modify the cytoskeleton of cultured human trabecular meshwork cells and is capable of reversing DEX-induced changes in the cytoskeleton. Preliminary data from other studies suggest that THF may also have an IOP lowering activity in patients with primary open angle glaucoma as well as angiostatic activity. Whether these findings can be correlated directly is unknown and will require additional study.

Key Words
cytoskeleton, dexamethasone, microfilaments, tetrahydrocortisol, trabecular meshwork

References


