Glucocorticoids have long been implicated in the etiology of primary open-angle glaucoma (POAG) and cataract. Cortisol metabolites have biologic activity and may affect aqueous humor dynamics. This study was done to determine whether these metabolites are found in human aqueous humor and can be produced by ocular tissues. Radioimmunoassays (RIA) were developed for 5α-dihydrocortisol (5α-DHF) and 5β-dihydrocortisol (5β-DHF). These assays, as well as a cortisol RIA, were used to quantify these three steroids in 20 surgically derived aqueous humor specimens from patients with and without POAG. The mean concentrations of cortisol and 5α-DHF were 2.5 and 1.3 ng/ml, respectively. In the small group studied, there was no statistically significant difference between the aqueous humor steroid levels in patients with and without POAG. The amount of 5β-DHF was at the lower limits of detection of the assay system and could not be unequivocally shown. Human lenses metabolized cortisol in vitro to 5α-DHF and 3α,5α-tetrahydrocortisol (3α,5α-THF). There was no 5β-DHF or cortisone formed. The 5α-DHF and 3α,5α-THF were identified by their positions on thin-layer chromatography, their retention times on high-performance liquid chromatography, and recrystallization with authentic standards to constant specific activity. The data suggest that the lens is the source of 5α-DHF in aqueous humor.

Materials and Methods

Steroids

All nonlabeled steroids were purchased commercially (Steraloids, Wilton, NH) and were >90% pure when analyzed by thin-layer chromatography, as described previously. The labeled steroid 1,2 3H-cortisol (55 C/mmol) was obtained commercially (New England Nuclear, Boston, MA), and 3H-5α-DHF and 3H-5β-DHF were prepared from this 3H-cortisol by Dr. J. Winter and Dr. V.D. Bockkenheuser (St. Luke’s Medical Center, New York, NY). Briefly, 3H-labeled cortisol was metabolized by appropriate strains of Clostridium paraputrificum. The metabolites were separated by celite liquid chromatography. The purity of the radioactive steroids was greater than 95% as determined by high-performance liquid chromatography (HPLC) analysis, as described below.
Aqueous Humor Specimens

Aqueous humor was obtained during cataract extraction from otherwise normal eyes and during surgical trabeculectomy of patients who had POAG. Samples were obtained from 8 patients with POAG (average age 69, 80% white, 14% male) and 12 non-POAG patients (average age 71, 80% white, 58% male). The diagnosis of POAG was based on the presence of chronically elevated IOP (>21 mm Hg), typical visual-field defects, characteristic optic nerve head changes, and gonioscopically open angles. Paracentesis was performed before the anterior chamber was entered and samples that appeared to be contaminated with serum were discarded. During these procedures, 50–150 μl of aqueous humor was recovered from the anterior chamber. The volume of each sample was determined, and the samples were then stored at −80°C until assayed.

Radioimmunoassays of Aqueous Humor Specimens

Extraction and HPLC Separation of Cortisol and Its Metabolites

Samples were defrosted and diluted with about 1 ml phosphate-buffered saline (PBS) and extracted twice with 2 ml ethyl acetate. The combined organic layers were evaporated to dryness under nitrogen, redisolved in 0.25 ml of 38% methanol, fractionated on a Zorbax C8 (DuPont Chromatography Products, Wilmington, DE) reversed phase column (25 cm × 4.6 mm), and eluted with methanol/water (38:62) at 55°C. An HPLC apparatus with ultraviolet (UV) (Shimadzu SPD-6AV, Kyoto, Japan) and radioactivity (Radiomatic Instruments, Tampa, FL) detectors was used for these procedures. The standards and their retention times in minutes with the UV detector were: cortisol, 14.8; 3β,5α-tetrahydrocortisol (3β,5α-THF) 14.9; 3β,5β-THF 15.7; cortisol, 17.8; 5α-DHF, 23.8; 5β-DHF, 32.0; 3α,5α-THF, 34.8 and 3α,5β-THF, 37.8. The fractions corresponding to cortisol (16.5–22 min), 5α-DHF (23–28 min), and 5β-DHF (31.5–37.7 min) were collected. Although the 5β-DHF fraction contains 3α,5α-THF and 3α,5β-THF, none of the tetrahydrocortisols interfered with the 5α-DHF radioimmunoassay, as described below. The HPLC fractions were divided into thirds and evaporated under vacuum in an Evapotec Vortex evaporator (A. Haake Buchler, Lenexa, KS). These tubes were used for their respective radioimmunoassays. Control specimens in which labeled cortisol, 5α-DHF, or 5β-DHF was added to aqueous humor and then extracted and separated, indicated yields of >90% and complete separation of each of these steroids.

Construction of Radioimmunoassays

Antibodies to 5β-DHF were raised in rabbits with the 21-hemisuccinate or 20-oxime derivative of the steroid conjugated to bovine serum albumin. The antisera obtained with each of these derivatives were of similar titer and when used in a radioimmunoassay, (see below) each resulted in assays of similar specificity and sensitivity. Antibodies to 5α-DHF were obtained in a similar manner. Antibodies to cortisol were purchased commercially (Radioassays Systems Laboratories, Carson, CA).

Tritium-labeled steroid (1000–4000 cpm) and appropriate nonlabeled steroids were dispensed in assay tubes in 0.2 ml of RIA buffer (0.15 M NaCl, 0.1 M Na phosphate, 0.1% Gelatin, 0.05% Na Azide, and 0.005 M EDTA, pH 7.0). The tubes were vortexed and cooled to 0°C. Then, 50–100 μl diluted antibody was added. The tubes were incubated overnight at 0°C. For the 5α-DHF and 5β-DHF assays, 0.3 ml of a precipitant of immune complexes (Tachi sorb, Calbiochemicals, LaJolla, CA) was added, and the incubation continued for 30 min at room temperature. Cold RIA buffer (3 ml) was added, the tubes were vortexed, and the bound (precipitated) labeled steroid was separated from the free steroid by centrifugation for 15 min at 2000 × g at 0°C. The supernatant was aspirated and discarded, and the pellet was dissolved in 10 ml of ACS liquid scintillation fluid (Amersham, Arlington Heights, IL) and then counted. For the cortisol, 0.3 ml of RIA, dextran-coated charcoal (Radioassays Systems Laboratories) was added to each tube, the samples were vortexed, and the free steroid (charcoal-bound) was separated by centrifugation. The bound steroid was decanted from the charcoal pellets into scintillation vials, and the radioactivity was determined, as described above. Nonimmune rabbit serum was used as a control specimen for nonspecific binding.

Metabolism of 3H-cortisol by Human Lenses in vitro

Eyes were obtained from the eyebank and used within 1–3 days after death. The eyes were rinsed in sterile Dulbecco's PBS. The anterior segment was removed, the lens zonules were cut, and the intact lens was then transferred to a sterile, 50-ml, plastic conical tube containing 5 ml Hank's balanced saline solution (HBSS). The medium was carefully removed, and HBSS containing 10–9 M 3H-cortisol was added. In some experiments, the medium contained 2% dialyzed fetal bovine serum, penicillin, streptomycin, and gentamicin. In other experiments, it contained 20 mM Hepes buffer, pH 7.4. There was no difference relative to the media supplements. The lenses were incubated in 1–2 ml of media in tightly sealed tubes at
37°C for 1–5 days. Control tubes without lenses were incubated in parallel. At the indicated times, the media were removed and the lenses were washed twice with PBS. The media and the washes were combined, extracted twice with two volumes of ethyl acetate, and the ethyl acetate fractions were combined and evaporated to dryness. The extracted material was redissolved and assayed by two-dimensional TLC, as described previously, or by HPLC, as described below. For positive identification, the TLC-separated metabolites were recrystallized to constant specific activity with authentic standards, as described previously.

Results

The antibodies raised to the 5α-DHF and 5β-DHF were suitable for use in radioimmunoassays of these steroids. Figure 1 shows typical radioimmunoassay working curves for cortisol, 5α-DHF, and 5β-DHF. The sensitivity of these assays was about 10 pg of steroid per tube, with a working range of approximately 20–500 pg. The antibodies for the dihydrocortisols showed cross-reactivity (about 10%) with cortisol, so separation of the individual steroids before radioimmunoassay was required. The 5β-DHF antibody did not cross-react with any of the THF isomers, even in large amounts (1000-fold). Human aqueous humor was extracted and separated on HPLC, as described above. The appropriate fractions from the HPLC column were assayed with the RIAs. Figure 2 summarizes the results of these assays. The mean concentration of cortisol was 2.5 ng/ml in non-POAG aqueous humor and 2.4 ng/ml in POAG aqueous humor. These average values were about 1% of that seen in normal serum. The aqueous humor levels of 5α-DHF were 1.5 ng/ml in patients without POAG and 1.1 ng/ml in patients with POAG (about 50% of the cortisol levels). The differences in aqueous humor steroid levels between patients with and without POAG were not statistically significant in this sample population. The amount of 5β-DHF found in the aqueous humor was approximately 0.3 ng/ml. Due to limits of detection of the assay system (approximately 10–20 pg of steroid per tube) and the small volume of aqueous humor specimens (0.05–0.15 ml), we cannot conclude that 5β-DHF is seen.

Table 1 shows the results of TLC analysis of the cortisol metabolites formed from nine lenses of six patients. All of the lenses metabolized the cortisol to an appreciable extent (13–90%). By contrast, similar incubations of iris-ciliary body showed barely detectable metabolism of cortisol (1–2% per 24 hr, data not shown). The lenses metabolized cortisol exclusively to the dihydrocortisols and tetrahydrocortisols, with no cortisone formed. The TLC system that was used did...
Fig. 2. Concentrations of cortisol, 5α-DHF, and 5β-DHF in aqueous humor from POAG and non-POAG patients. The mean values are indicated by horizontal bars.

not separate the different isomers of the 5-dihydrocortisols or the 3,5-tetrahydrocortisols. The shorter incubations (patients 1, 2, and 3) showed lower metabolism (13–27%) with the dihydrocortisols being the major product. The longer incubations (patients 4, 5, and 6) showed a greater amount of metabolism (34–90%), with the tetrahydrocortisols being the major product. This finding suggests that the 5α-DHF is an intermediary in the reduction of cortisol to 3α,5α-THF.

Figure 3 shows a typical HPLC radiochromatogram of the extracted metabolites of a human lens incubated with 3H-cortisol. The positions of authentic standards of cortisol, 5α-DHF, 5β-DHF, and 3α,5α-THF are shown. The retention times are slightly greater with the radioactivity detector than those indicated in the text with the ultraviolet detector due to the configuration of the apparatus.

Table 1. Metabolism of 3H-Cortisol by human lenses in vitro

<table>
<thead>
<tr>
<th>Patient</th>
<th>Lens</th>
<th>Time of incubation</th>
<th>% of Metabolite formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cortisone</td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>16 hr</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>16 hr</td>
<td>&lt;1</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>16 hr</td>
<td>&lt;1</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td>16 hr</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>20–24 hr</td>
<td>6.4</td>
</tr>
<tr>
<td>5</td>
<td>A</td>
<td>2 days</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>2 days</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>A</td>
<td>5 days</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Discussion

This study shows the presence of cortisol and 5α-DHF in human aqueous humor; 5β-DHF was not conclusively shown. Cortisol levels were approximately 2.4 ng/ml (range: 0.5–7.0 ng/ml), and 5α-DHF was found at levels that were about 50% of those of cortisol. A previous study reported aqueous humor values of cortisol from 14.1–19.8 ng/ml in patients who had POAG or cataract.22 The reason for these differences is unknown. Although there was no significant difference in the levels of cortisol or 5α-DHF in POAG vs non-POAG-derived aqueous humor, a
larger series of cases is required to determine whether there are any significant disease-related differences.

The metabolism of cortisol by human lens was limited to 5α-reduced products, such as 5α-DHF and 3α,5α-tetrahydrocortisol. These products were identified by their positions on TLC, their retention times on HPLC, and by crystallization with authentic nonlabeled standards to constant specific activity. No cortisone- or 5β-reduced metabolites were found, indicating the absence of cortisol 11-dehydrogenase and 5β-reductase activities. One abstract reported the metabolism of cortisol by human lenses to substances identified by their retention times on HPLC as 5α-DHF and 5α-THF (configuration at the third position not identified)23.

The A-ring-reduced metabolites of cortisol have a variety of biologic activities. The metabolite 5α-DHF has weak mineralocorticoid activity and may potentiate the activity of low concentrations of aldosterone.7,8 Dihydrocortisol (isomer unidentified) inhibits angiogenesis in the presence of heparin or a heparin fragment in the chick embryo chorioallantoic membrane.24 Cells cultured from the trabecular meshwork of patients with POAG metabolized cortisol predominantly to 5β-DHF.21-25 Topical application (ocular) of 5α-DHF and 5α-THF (configuration at the third position not identified).23

Table 2. Identification of cortisol metabolites produced by human lens explants by sequential recrystallization with authentic nonlabeled steroids to constant specific activity*.

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Specific activity (cpm/mg)</th>
<th>3α, 5α-tetrahydrocortisol</th>
<th>5α-dihydrocortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol/water</td>
<td>(3rd) 928 ± 38</td>
<td>(2nd) 1048 ± 44</td>
<td>(3rd) 1261 ± 170</td>
</tr>
<tr>
<td>Ethylacetate/cyclohexane</td>
<td>(2nd) 924 ± 30 (initial)</td>
<td>(2nd) 1256 ± 105</td>
<td></td>
</tr>
</tbody>
</table>

* After incubation, the 3H-metabolites were extracted and separated by TLC as described in Materials and Methods. 
† Mean ± standard deviation of quadruplicates at each crystallization.

It is unknown whether 3α,5α-THF, a major metabolite of cortisol in human lens in vitro, is seen in aqueous humor or has any effect on IOP.

Although the serum may contribute to the 5α-DHF in the aqueous humor, the relatively large amount of this metabolite, and the high activity of cortisol 5α-reductase in the lens suggest that the lens may be a significant source of the 5α-DHF in this fluid. Whereas 5β-DHF has been implicated in the pathophysiology of POAG, the physiologic function of 5α-metabolites is undetermined. The absence of detectable levels of 5β-DHF in the aqueous humor focuses attention on the trabecular meshwork, which is downstream from the source of this fluid, for studying the relationship of 5β-DHF to POAG.

Key words: cortisol metabolism, lens, aqueous humor, 5α-dihydrocortisol, 3α, 5α-tetrahydrocortisol

References


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