Key words: glutathione reductase, affinity chromatography, disc gel electrophoresis, kinetics, calf, trabecular meshwork


References

Defects in Cortisol-Metabolizing Enzymes in Primary Open-Angle Glaucoma

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Assays of cortisol-metabolizing enzymes in homogenates of human trabecular meshwork cells under optimal conditions revealed two defects in primary open-angle glaucoma (POAG): one is a marked increase in Δ4-reductase and the other is a decrease in 3-oxodioxygenase. Experiments indicated that the differences in enzyme activities seen between POAG and nonPOAG trabecular meshwork derived cell homogenates were due to altered amounts of enzymes rather than to alterations in cofactor availability, pH, or endogenous activators or inhibitors. This clearly demonstrates an enzymatic defect(s) in POAG which may be the basis for the ocular hypertension and sensitivity to exogenous glucocorticoids seen in this disorder. Invest Ophthalmol Vis Sci 26: 890–893, 1985

We reported earlier that cells cultured from trabecular meshwork specimens obtained from patients with primary open-angle glaucoma (TMPOAG cells) exhibited two major differences in cortisol metabolism when compared to similar cells from nonglaucomatous patients (TMMnonPOAG cells). One is a marked increase in Δ4-reductase activity and the other is a decrease in 3-oxodioxygenase activity leading to an accumulation of 5α (5β)-dihydrocortisol, intermediates not found with TMnonPOAG cells. In order to determine whether the differences in cortisol metabolism found in these cells were due to changes in the amount of enzymes as opposed to alterations in cofactor availability, pH, or the presence or absence of inhibitors or activators, we studied the kinetic parameters of cortisol Δ4-reductase and 3-oxodioxygenase in homogenates of these same TMPOAG and TMMnonPOAG cells where these factors can be experimentally controlled. Defining the enzymatic basis for the altered metabolism of cortisol in TMPOAG cells is important in view of the recent findings that 5β-dihydrocortisol can potentiate threshold levels of topically applied cortisol and dexamethasone in causing nuclear translocation of the cytosolic glucocorticoid receptor in rabbit iris–ciliary body tissue, an early and necessary event in steroid hormone action and potentiate threshold levels of dexamethasone in elevating intraocular pressure in young rabbits. Materials and Methods. Culture of cells and preparation of cell homogenates: Cells cultured from trabecular meshwork from two nonPOAG patients (autopsy) and two POAG patients (surgical trabeculectomy) were grown to confluence in 75-cm2 tissue culture flasks. The TMPOAG cells, used in the present
report are the same as those used previously \(^1\) for a study of cortisol metabolism in intact cells. The growth medium was removed and the cell layer was washed two times with phosphate buffered saline (PBS) at 22°C. One-half to 1 ml of buffered sucrose (0.25 M sucrose, 0.01 M Tris, 0.002 M EDTA, 0.0005 M dithiothreitol, pH 7.4) was added and the culture flasks frozen at \(-20°C\). Thirty minutes later, the flasks were defrosted and the cells were scraped from the flask with a rubber policeman. The cellular material was transferred to a glass homogenizer and disrupted with 10 strokes of a motor driven teflon pestle. The resulting homogenate contained no unbroken cells as shown by observation with a phase contrast microscope and had a protein concentration of 0.5 to 0.9 mg/ml as determined by the method of Lowry et al \(^4\) using bovine serum albumin as a standard.

**Assay procedures:** Experiments were carried out with and without cofactors and at different pH values to determine optimum conditions for the assay. All assays were run at optimum conditions except where indicated.

\(\Delta^4\)-Reductase: \(1,2,3^\text{H}-\text{cortisol} (\text{SA} 60 \text{Ci/mm})\) was added to borosilicate tubes and evaporated to dryness. One tenth milliliter of 0.2 M citrate buffer, pH 5.6, containing an NADPH-generating system \((10^{-2} \text{ M glucose-6-phosphate, } 4 \times 10^{-4} \text{ M NADP, } 20 \mu\text{g/ml glucose-6-phosphate dehydrogenase, grade I, purchased from Boehringer Mannheim})\), and 0.1 ml of cell homogenate were added. The mixture was incubated at 36°C for 90 min and then extracted with ethyl acetate and the \(3^\text{H}\)-metabolites quantitated as described previously.\(^1\)

3-Oxidoreductase: \(\text{\(3^\text{H}-\text{dihydrocortisol, isolated and puriﬁed from intact TMPOAG cells incubated with \(3^\text{H}-\text{cortisol,}\)}\) was added to borosilicate tubes and evaporated to dryness. Two hundredths milliliter of 0.2 M Tris buffer, pH 8.0, containing an NADPH-generating system as described above, and 0.02 ml cell homogenate were added. The mixture was incubated at 36°C for 90 min, extracted with ethyl acetate, and the labeled metabolites quantitated as previously described.\(^1\)

This research was reviewed and approved by the Institutional Review Board of New York Medical College.

**Results.** \(\Delta^4\)-Reductase activity: Figure 1 shows the activity of \(\Delta^4\)-reductase at varying pH values in homogenates prepared from TMPOAG cells. As can be seen, the activity of the enzyme is highest at pH 5.6 in citrate buffer. Under these conditions the amount of product formed was linear with time (through 90 min) and amount of homogenate. The reaction did not proceed in the absence of an NADPH-generating system, when incubated at 0°C or after boiling the homogenate.

Figure 2 indicates the reaction velocity as a function of substrate concentration with one of the TMPOAG cell lines. A Lineweaver–Burke plot is shown as an insert to the figure. This experiment was carried out...
three times with cells derived from TMPOAG cell lines from two different patients. All of these experiments gave similar results. The apparent $K_m$ for cortisol averaged $3 \times 10^{-7}$ M (range $1-4 \times 10^{-7}$ M) and the $V_{max}$ averaged 35 pmol dihydrocortisol formed/hr/mg cell protein (range 20-50). By contrast, homogenates prepared from TMnonPOAG cells derived from two different patients had no detectible A4-reductase activity. Assaying at other pH values gave similar results. A4-reductase activity as low as 5% of that seen in homogenates of TMPOAG cells would have been detected under these conditions.

When homogenates from TMPOAG cells and TMnonPOAG cells were mixed in a 1:1 ratio the resulting A4-reductase activity was one-half of the value seen in homogenates from TMPOAG cells. Thus, the increased levels of A4-reductase in homogenates of TMPOAG cells probably represents increased amount of enzyme rather than the presence of an activator or unknown cofactor or the absence of an inhibitor.

3-Oxidoreductase: Figure 1 shows the activity of 3-oxidoreductase as a function of pH in homogenates from TMPOAG cells. As can be seen, the activity of the enzyme is optimal at pH 8.0 in Tris buffer. Under these conditions the amount of product formed was linear with time (through 90 min) and amount of homogenate. The reaction did not proceed in the absence of an NADPH-generating system, when incubated at 0°C or after boiling the homogenate.

Figure 3 shows the enzyme activity at various concentrations of the substrate, dihydrocortisol ($10^{-8}$ to $5 \times 10^{-6}$ M). In this concentration range, the enzyme was not saturated with the substrate, indicating a high $K_m$. Similar results were obtained with homogenates from TMnonPOAG cells derived from a second patient. In other mammalian tissues, this enzyme has also been reported to have a high $K_m$ for substrate.5

Homogenates prepared from TMPOAG cells from two different patients had no detectible 3-oxidoreductase activity. This activity would have been detected if present at levels of 5% or greater than that seen in homogenates of the TMnonPOAG cells. Assaying at other pH values failed to demonstrate 3-oxidoreductase activity in these TMPOAG cell homogenates. Mixing the homogenates prepared from TMnonPOAG and TMPOAG cells in a 1:1 ratio gave an enzyme activity which was one-half that found in the TMnonPOAG Cell homogenates.

Discussion. The present study using cell homogenates demonstrates two enzyme defects in trabecular meshwork cells derived from patients with POAG as compared to similar cells from nonPOAG patients. One is a marked increase in A4-reductase and the other is a decrease in 3-oxidoreductase. Experiments indicated that the differences in cortisol metabolism seen in intact TMPOAG cells are probably due to differences in amounts of enzymes rather than to alterations in cofactor availability, pH, or endogenous activators or inhibitors. Whereas intact TMPOAG cells exhibited a four- or five-fold reduction in 3-oxidoreductase activity,1 homogenates of these cells showed a greater than 20-fold decrease in enzyme activity. This suggests a greater lability of the 3-oxidoreductase enzyme in TMPOAG cells.

Thus, in POAG there are alterations in two enzymes involved in cortisol metabolism. Whether this is the result of a primary defect in one of the enzymes with subsequent alterations in the other remains to be determined. In any event, the net effect is an accumulation of dihydrocortisol. 5α-dihydrocortisol has been shown to have weak mineralocorticoid activity and may potentiate the activity of low concentrations of aldosterone.6 Since 5β-dihydrocortisol has been shown to potentiate the ocular effects of glucocorticoids in the rabbit (nuclear translocation of the cytosolic glucocorticoid receptor and elevation of intraocular pressure) a similar potentiation in man may account for the ocular hypertension of POAG patients and their sensitivity to exogenous glucocorticoids.

Key words: cortisol A4-reductase, 3-oxidoreductase, primary open angle glaucoma

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References

The Production and Mechanism of Ghost Cell Glaucoma in the Cat and Primate
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Fresh human ghost blood cells (GBCs) have been shown to cause increased resistance to outflow in enucleated human eyes. In addition, glutaraldehyde-fixed GBCs can cause glaucoma in the rabbit and primate in vivo. The present study shows for the first time that fresh autologous GBCs can cause an acute in vivo rise of intraocular pressure when injected into the anterior chambers of the cat and primate. This rise was of greater magnitude and longer duration than that caused by the injection of a greater number of pliable, fresh red blood cells. It has been theorized that ghost cell glaucoma (GCG) is due to cellular obstruction of the intertrabecular spaces by the nonpliable GBCs. Histologic results from the present study confirm this belief. No evidence of significant trabecular meshwork degeneration or significant GBC phagocytosis was seen. Invest Ophthalmol Vis Sci 26:893–897, 1985

Ghost cell glaucoma is a recently described glaucoma that occurs as a consequence of vitreous hemorrhage. Following vitreous hemorrhage, the red blood cells (RBCs) degenerate into GBCs and pass slowly into the anterior chamber through a disruption in the anterior hyaloid face. The cells then flow into the outflow pathways, causing an increase in intraocular pressure (IOP). When the glaucoma was initially described, it was felt that the reduced pliability of the cells made them effective obstructors of the intertrabecular spaces. This theory was supported by perfusion studies in enucleated human eyes.1,2

Grierson and Lee,3 however, have shown that endothelial cells can phagocytose GBCs in traumatized eyes, leading to speculation that this process may somehow contribute to the rise in IOP. Furthermore, Quigley,4 using glutaraldehyde-fixed GBCs injected into the anterior chambers of rabbits and monkeys, suggested that endothelial swelling might be important in the pathophysiology of GCG. The purpose of our study was to determine whether GCG is due to primary obstruction of the intertrabecular spaces, as was originally believed, or whether it is due to a secondary effect. A second purpose of this study was to show for the first time that fresh, autologous GBCs could cause an acute, in vivo rise in IOP, and to compare the rise with that caused by fresh RBCs.

Materials and Methods. Ghost blood cells were prepared by incubating washed RBCs in a 37°C water bath for 10 days.3 The tubes were then placed in a centrifuge at 1000 000 × g for 1 hr until the GBCs separated from the undegenerated RBCs and formed a khaki-colored layer. This layer was then removed, and GBC formation was verified by phase microscopy. The concentration of GBCs was determined using a manual hemocytometer.

Adult cats and rhesus monkeys were utilized in this in vivo study. Eight cat and two monkey anterior chambers were injected with autologous GBCs. Five cat anterior chambers were injected with autologous RBCs. In addition, control experiments were conducted on two cats and one primate using injections of mock aqueous solution.

Blood cell injection was accomplished by inserting a 25-gauge needle obliquely through the peripheral cornea into the anterior chamber of anesthetized animals (xyazine and ketamine, in the cats; and ketamine, in the primates). Approximately 60% of the aqueous humor was withdrawn, and the needle was removed. A new needle was inserted through the