Original papers

Inhibition of 11β-hydroxysteroid dehydrogenase type 1 lowers intraocular pressure in patients with ocular hypertension

S. RAUZ1,2, C.M.G. CHEUNG1, P.J. WOOD3, M. COCA-PRADOS4, E.A. WALKER2, P.I. MURRAY1 and P.M. STEWART2

From the 1Academic Unit of Ophthalmology, Division of Immunity and Infection, and 2Department of Endocrinology, Division of Medical Sciences, University of Birmingham, Birmingham, UK, 3Regional Endocrine Unit, Southampton General Hospital, Southampton, UK, and 4Department of Ophthalmology and Visual Science, Yale University School of Medicine, Connecticut, USA

Received 4 November 2002 and in revised form 3 April 2003

Summary

Background: Intraocular pressure (IOP) is maintained by a balance between aqueous humour (AH) production (dependent on sodium transport across a ciliary epithelial bi-layer) and drainage (predominantly through the trabecular meshwork). In peripheral epithelial tissues, sodium and water transport is regulated by corticosteroids and the 11β-hydroxysteroid dehydrogenase (11β-HSD) isozymes (11β-HSD1 activating cortisol from cortisone, 11β-HSD2 inactivating cortisol to cortisone).

Aim: To analyse expression of 11β-HSD in the human eye and investigate its putative role in AH formation.

Design: Multipart prospective study, including a randomized controlled clinical trial.

Methods: The expression of 11β-HSD1 in normal human anterior segments was evaluated by in situ hybridization (ISH). RT-PCR for 11β-HSDs, glucocorticoid and mineralocorticoid receptors (GR, MR) was performed on human ciliary body tissue. AH cortisol and cortisone concentrations were measured by radioimmunoassay on specimens taken from patients with primary open-angle glaucoma (POAG) and age-matched controls. Randomized, placebo-controlled studies of healthy volunteers and patients with ocular hypertension (OHT, raised IOP but no optic neuropathy) assessed the effect of oral carbenoxolone (CBX, an inhibitor of 11β-HSD) on IOP.

Results: ISH defined expression of 11β-HSD1 in the ciliary epithelium, while RT-PCR analysis of ciliary body tissue confirmed expression of 11β-HSD1, with additional GR and MR, but not 11β-HSD2 expression. In both POAG patients and controls, AH concentrations of cortisol exceeded those of cortisone. The CBX-treated healthy volunteers who demonstrated the largest change in urinary cortisol metabolites, indicative of 11β-HSD1 inhibition, had the greatest fall in IOP. Patients with OHT showed an overall reduction of IOP by 10% following CBX administration, compared to baseline (p < 0.0001).

Discussion: CBX lowers IOP in patients with ocular hypertension. Our data suggest that this is mediated through inhibition of 11β-HSD1 in the ciliary epithelium. Selective and topical inhibitors of 11β-HSD1 could provide a novel treatment for patients with glaucoma.

Address correspondence to Professor Paul M. Stewart, Endocrinology, Division of Medical Sciences, University of Birmingham, Queen Elizabeth Hospital, Edgbaston, Birmingham B15 2TH. e-mail: p.m.stewart@bham.ac.uk © Association of Physicians 2003; all rights reserved.
Introduction

The sodium-transporting human ocular ciliary epithelium is a complex bi-layer of non-pigmented (NPE) and pigmented (PE) polarized, neuroepithelial cells. The inner NPE layer lies in direct contact with the aqueous humour (AH) in the posterior chamber, while the outer PE layer is adjacent to the highly vascularized connective tissue stroma of the ciliary body. The ciliary epithelial bi-layer is primarily involved in AH formation, a clear fluid of high optical quality that provides nutrition to the transparent and avascular structures of the eye. Production of AH, together with drainage, is also fundamental to maintaining a normal intraocular pressure (IOP). AH is secreted into the posterior chamber of the eye flowing from the ciliary epithelium, between the iris and the lens, through the pupillary aperture, entering the anterior chamber, and finally flowing radially to the periphery, where it exits predominantly via the canal of Schlemm in the trabecular meshwork (TM), and to a lesser extent through uveoscleral outflow routes.2,3

In the kidney and colon, epithelial sodium transport is regulated by corticosteroids through the induction of both epithelial sodium channels (ENaC) adjacent to the lumen, and the basolateral Na\(^+\)K\(^+\)ATPase pump adjacent to the stroma.4–9 Corticosteroid action is dependent upon the expression of corticosteroid hormone receptors—mineralocorticoid (MR) and glucocorticoid (GR)—and the pre-receptor metabolism of cortisol by 11\(\beta\)-hydroxysteroid dehydrogenase type 2 (inactivating cortisol to cortisone).10–14 Studies have localized the MR and GR, and perhaps surprisingly, 11\(\beta\)-HSD1 and not 11\(\beta\)-HSD2, to the human NPE.15–18 11\(\beta\)-HSD1 catalyses the opposite reaction to 11\(\beta\)-HSD2, i.e. the generation of cortisol from cortisone,19–21 and the documentation of a high cortisol/cortisone ratio in aqueous humour of 14:1 is consistent with this local cortisol-generating system.15 Furthermore, in a pilot uncontrolled study, IOP fell by 17.5% following the administration of oral carbenoxolone (CBX), an inhibitor of 11\(\beta\)-HSD1, suggesting that 11\(\beta\)-HSD1 activity may partly regulate sodium transport across the NPE-PE bi-layer, and consequently aqueous humour secretion.15

In this study, we further define expression of the 11\(\beta\)-HSD isozymes in the human ciliary epithelium and investigate in vivo the role of 11\(\beta\)-HSD1 in regulating intraocular pressure.

Methods

Study A: In situ hybridization

Using RNase-free conditions, in situ hybridization (ISH) was carried out on 5 \(\mu\)m, formalin-fixed, paraffin-embedded human ocular sections obtained from the Academic Unit of Ophthalmology, University of Birmingham, UK, as previously described.22,23 Eyes were acquired at surgical enucleation; in all cases the underlying diagnosis was choroidal malignant melanoma, and only adjacent anatomically normal anterior segment structures were studied. Sections were pre-heated for 4 h at 60°C, then dewaxed, rehydrated and permeabilized with 20 \(\mu\)g/ml of RNase-free proteinase K in 50 mM Tris-HCl, at 37°C for 20 min. Sections were re-fixed at 4°C with 4% paraformaldehyde in phosphate-buffered saline (PBS), and hybridized overnight with in-house-generated 11\(\beta\)-HSD1 antisense DIG-labelled cRNA probes (80 ng/100 \(\mu\)l).23 The sections were washed to a final stringency of [0.05 \(\times\)]SSC:50% (v/v) deionized formamide at 50°C for 60 min. Hybridized DIG-labelled probes were detected following either: (i) incubation with anti-DIG alkaline phophatase Fab fragments, then visualization using 4-nitroblue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) chromagen precipitation; or (ii) overnight incubation with a 1:6 dilution of anti-DIG-fluorescein Fab fragments (Roche Molecular Biochemicals), mounting in vectashield mounting medium containing 4‘6-diamidino-2-phenylindole (DAPI) (Vector laboratories, UK), and then visualization with 494 nm (fluorescein) and 360 nm (DAPI) wavelength excitation filters, emitting 523 nm (yellow) and 460 nm (blue) fluorescence, respectively. Control experiments used no probe, sense DIG-labelled cRNA probes, and antisense DIG-labelled cRNA probes incubated in the presence of a 60-fold excess of unlabelled antisense cRNA probe.

Study B: RT-PCR

RNA was prepared from human ciliary body tissue 24–48 h after surgical enucleation using a single-step extraction method (RNAzol Bl RNA isolation kit, AMG Biotechnology) according to the manufacturer’s protocol. Enucleated eyes had a normal anatomical structure, but the tissue was considered to be of inadequate quality for transplantation purposes. Donors had given consent for the tissue to be used for research if transplantation could not be performed. After conducting the reverse-transcriptase reaction using a Promega
reverse transcription system, a 5 μl aliquot was taken for subsequent PCR reactions using primer pairs for 11β-HSD1, 11β-HSD2, GR and MR, generating transcript sizes of 571 bp, 477 bp, 693 bp and 450 bp, respectively, as previously described.15,24,25 Human hepatocyte cDNA was used as a positive control for 11β-HSD1 and GR, whilst human placental cDNA was used for 11β-HSD2 and MR. The negative control specimen substituted nuclease-free water for the cDNA template. 18S ribosomal RT-PCR was carried out to confirm the presence and integrity of RNA in all samples. In each case, PCR products were sequenced to confirm identity.

**Study C: Aqueous humour analysis**

Forty control patients (mean ± SEM age 70 ± 12 years, 13 males) and 38 with primary open-angle glaucoma (POAG) (mean ± SEM age 77 ± 9 years, 20 males, duration of diagnosis 4.2 ± 1.1 years) were identified from patients undergoing routine phacoemulsification for cataracts under local anaesthesia at the Birmingham and Midland Eye Centre, UK. All POAG patients were normotensive at the time of surgery: 31 were being treated medically with topical anti-glaucoma therapy, and nine had a previous history of glaucoma filtration surgery. Of the patients who were being treated medically, 11 were on monotherapy (β-blockers, topical prostaglandin analogues or sympathomimetics), 20 patients were being treated with more than two drugs or combined drug therapy (topical carbonic anhydrase inhibitors plus β-blockers, topical prostaglandin analogues plus β-blockers), and two had additional glaucoma filtration surgery. Patients with diabetes, uveitis or underlying endocrine disease, and those on topical or systemic corticosteroids, were excluded. In each case, 25–75 μl AH was collected at the start of the surgical procedure and stored at −70°C until further analysis. Both cortisol and cortisone concentrations determined by an adaptation of published radioimmunoassay methods for measuring cortisol and cortisone in saliva.26 In cases where there was insufficient sample (five controls, nine POAG), only one of the assays was performed. Both cortisol and cortisone assays have been validated in terms of recovery (93–113%), parallelism precision (CVs < 12% over the working range) and detection limits (0.5–1.0 nmol/l).

**Study D: Clinical study: healthy volunteers**

A randomized, double-masked, placebo-controlled trial was conducted to evaluate the effect of an inhibitor of 11β-HSD1 on IOP in normal volunteers. Fourteen healthy, male volunteers, on no systemic or topical medications, and with no family history of glaucoma were recruited to receive either carbadoxolone (CBX) 100 mg three times daily (age 31.1 ± 12.3 years) or placebo (age 29.1 ± 8.8 years) for 7 days. Pure carbenoxolone sodium was supplied by Sanofi Winthrop. Both CBX and placebo were prepared in identical capsules, the placebo consisting predominantly of a lactose-based inert substance, not known to affect IOP. Randomization used a block, computerized method so that the investigator and volunteers were masked from the process.

Baseline IOP readings were measured on two consecutive days using the same Goldmann applanation tonometer at 07:00, 14:00, and 19:00 h, by a single observer. Systolic and diastolic blood pressures (recorded with an Omron HEM-705CP automated digital blood pressure monitor) were measured at each time point. Urine was collected for cortisol (tetrahydrocortisol (THF), allo-THF, urinary free cortisol (UFF)) and cortisone (tetrahydrocortisone (THE), urinary free cortisone (UFE)) metabolites to evaluate 11β-HSD1 (THF + alloTHF/THE) and 11β-HSD2 (UFF/UFE) activities.27,28 IOP measurements and blood pressure recordings were repeated at each time point on the first, third and seventh day of treatment. A further 24-h urine collection was performed on the last day of treatment. Serum electrolytes were measured throughout the study.

**Study E: Clinical study of ocular hypertensive patients**

Based on the results of steroid analytes from the above study, a second randomized, double-masked, placebo-controlled trial was conducted, this time with crossover, assessing the effects of oral CBX (100 mg three times a day for 4 days) or placebo, on IOP in patients with ocular hypertension (OHT). OHT patients have raised IOP but do not have optic nerve damage, although they may have other risk factors for POAG. Sample size calculations aiming for a power of 0.9, using data from the previous study, indicated that 20 patients would be required to demonstrate a statistically significant drop in IOP following ingestion of CBX at a confidence level of 99%. A 20% fall in IOP was considered to be clinically significant when performing the calculations. Patients with raised IOP who were not on ocular-hypotensive medications were identified from general and glaucoma clinics. Patients with systemic hypertension, underlying endocrine...
Ethics and statistical methods

The collection of human tissues for in vitro investigation, and the clinical studies were approved by the local ethics committee. The clinical studies followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all volunteers. Statistical analysis was performed using the software packages Minitab 13.1 for Windows and SPSS 10 for Windows. The two-sample paired t-test and analysis of variance were used to analyse AH cortisol and cortisone concentrations from POAG patients and controls (Study C), and for the analysis of urinary corticosteroid metabolite profiles during pre- and post-CBX/placebo (Study D). Regression analysis and Spearman Rank correlation were used to analyse changes in IOP (baseline versus last day of treatment) and corresponding urinary corticosteroid metabolite profiles (Study D). The general linear model for analysis of variance was used to analyse IOP with pairwise comparisons of the various stages of the clinical studies (Studies D and E).

Results

Study A: In situ hybridization

11β-HSD1 isozyme mRNA was demonstrated in the NPE (Figure 1A), while control analyses revealed minimal or no NBT/BCIP chromagen precipitation (Figure 1 B–D). Indirect fluorescent-ISH confirmed expression in the NPE and some evidence of 11β-HSD1 expression in the PE, although visualization of full expression was compromised by the pigment (Figure 1E). Sections incubated with DIG-labelled sense cRNA revealed only minimal fluorescence (Figure 1F).

Study B: RT-PCR

11β-HSD type 1, MR and GR, but not 11β-HSD2 mRNA expression, was identified in human ciliary body tissue (Figure 2). Integrity of the RNA samples was confirmed by the presence of 18S ribosomal RNA (18S).

Study C: Aqueous humour analysis

In every patient, POAG or control, AH concentrations of cortisol exceeded those of cortisone (POAG cortisol 5.5 ± 0.47 nmol/l, cortisone 2.8 ± 0.35 nmol/l; controls cortisol 4.8 ± 0.38 nmol/l and cortisone 1.9 ± 0.13 nmol/l, means ± SEM) (Figure 3). There was no statistical difference between the POAG and control groups.

Study D: Clinical study: healthy volunteers

The urinary THF/alloTHF/THE ratio decreased significantly (mean ± SD 1.09 ± 0.25 vs. 0.78 ± 0.58, p < 0.05) following CBX administration compared to baseline, compatible with inhibition of 11β-HSD1 activity (Figure 4A). Despite this, the UFF/UFE ratio increased significantly (0.81 ± 0.30 vs. 1.62 ± 0.58, p < 0.01), indicating concomitant inhibition of 11β-HSD2 (Figure 4B). These changes were not observed in the placebo-treated group.

In normal volunteers, there was no significant difference in IOP between the CBX and placebo groups. A positive trend was seen when comparing the change in IOP and change in urinary THF/alloTHF/THE ratio (indicative of 11β-HSD1 inhibition) in the CBX-treated group, although not significant, (b (size of effect) = +3.5, r² = 27.6%, p = 0.226), a trend not seen with the UFF/UFE ratio (b = −0.004, r² = 0.0%, p = 0.998), or in the placebo-treated group (b = −3.82, r² = 11.3%, p = 0.897, and b = −0.68, r² = 0.0%, p = 0.730, respectively). Furthermore, on examining changes in urinary metabolites and IOP in each of the subjects, it was clear that those who demonstrated the largest change in steroid metabolites consistent with 11β-HSD type 1 inhibition, also had a fall in IOP (data not shown). There was no change in blood pressure or serum electrolytes.

Study E: Clinical study of ocular hypertensive patients

There was an overall significant reduction of IOP by 10% following the ingestion of CBX (p < 0.0001) (Figure 5). Compared to baseline values, the reduction was statistically significant when patients were treated with CBX, but not following placebo, or during the intermediate washout stage. Blood pressure remained within normal limits throughout the course of the study.
Discussion

Glaucoma is a leading cause of irreversible visual loss responsible for 13.5% of global blindness (5.1 million people), of which POAG is the most prevalent. It is characterized by an optic neuropathy with corresponding visual field loss, associated with a range of risk factors including elevated IOP—the only treatable ocular risk factor. In turn, IOP is regulated by a fine balance between production through the ciliary epithelium and drainage via the trabecular meshwork, canal of Schlemm, and uveoscleral outflow routes. Corticosteroids may regulate this process, as approximately one-third of the normal population develop a moderate increase in IOP after topical corticosteroid use, whereas almost all patients with POAG or normal tension glaucoma develop raised IOP after topical therapy. Raised IOP is also a recognized feature of endogenous Cushing’s syndrome.

In tissues such as the kidney, colon and salivary gland, the control of epithelial sodium transport is mediated by mineralocorticoid and glucocorticoid effector mechanisms through the transcriptional activation of novel, recently characterized, target genes, including serum and glucocorticoid regulated kinase isoform 1 (SGK1), which is involved in activation of pre-existing ENaCs. At a pre-receptor level, 11β-HSD2 protects the MR from cortisol by inactivating it to cortisone, thereby enabling aldosterone to interact with the MR. When 11β-HSD2 activity is compromised, as seen
in the inherited form of hypertension, the syndrome of Apparent Mineralocorticoid Excess, or following liquorice or carbenoxolone ingestion, cortisol gains access to the MR to act as a potent mineralocorticoid. The opposite enzyme, 11β-HSD1, is not present to any extent in human kidney, but has been shown to modulate cortisol exposure to the GR at other sites, notably liver, adipose tissue, and bone. Manipulation of 11β-HSD1 expression has been achieved in vitro and in vivo by using carbenoxolone, which inhibits 11β-HSD1 in addition to 11β-HSD2. In this study, systemic inhibition of 11β-HSDs resulted in a fall in intraocular pressure in patients with ocular hypertension, an effect that we suggest is mediated through 11β-HSD1 in the ciliary epithelium. Although earlier studies by Southren et al. provided evidence of cortisol metabolism within both human and rabbit ocular tissues, these data mainly refer to the cortisol A-ring metabolism (5α/β-reductase, tetrahydrocortisol) and not C-ring metabolism involved in the interconversion of cortisol and cortisone by 11β-HSD.

The data presented here support our earlier work showing only expression of 11β-HSD1 and not 11β-HSD2 in human ciliary epithelial cells. This has now been confirmed at both the mRNA and protein level, and is somewhat surprising in view of the established role of the ciliary epithelium to active sodium transport. Nevertheless, the corticosteroid regulation of SGK1 via the GR and MR, and expression of ENaC within the human ocular ciliary

Figure 2. RT-PCR analysis of ciliary body (CB) tissue from a recently enucleated human eye. GR, MR and 11β-HSD1 mRNAs were expressed, but 11β-HSD2 mRNA was not detected. Integrity of the RNA samples was confirmed by the presence of 18S ribosomal RNA (18S). (+ve, human hepatocyte cDNA for GR and 11β-HSD1, and human placental cDNA for MR and 11β-HSD2; -ve, no DNA template).

Figure 3. Cortisol and cortisone levels in aqueous humour from 40 controls (□), and 38 patients (■) with primary open angle glaucoma. (A) all patients, (B) males, (C) females. Results are shown as means ± SEM. Although cortisol levels exceed those of cortisone in each case, there was no statistical significance between glaucoma and controls.

epithelium indicate that this mechanism may be an integral feature of the sodium transport signalling cascade. Analysis of AH confirmed cortisol levels in excess of cortisone in every patient; when contrasted with an excess of urinary cortisone to cortisol (reflecting 11β-HSD2 in the kidney), these data are consistent with the predominant expression of 11β-HSD1 in ocular tissues. Although drainage of AH has been thought to be the rate-limiting step in the regulation of IOP, our earlier analysis of 11β-HSD isozyme expression in several trabecular meshwork samples failed to identify the expression of either isozyme. It would be difficult, therefore, to surmise how CBX could modulate IOP through an effect upon the trabecular meshwork per se. CBX
inhibits both 11β-HSD isozymes as demonstrated in this study, but based on existing knowledge we hypothesize that it modulates IOP by inhibiting the 11β-HSD1-mediated generation of cortisol in the ciliary epithelium, causing a reduction in corticosteroid-induced sodium transport and a lowering of IOP (Figure 6).

There were no differences in AH cortisol/cortisone ratios between POAG and controls, and subgroup analysis of males and females, with and without glaucoma, also failed to demonstrate a difference. It seems unlikely that increased expression of 11β-HSD1 within the NPE is a primary pathogenic mechanism in POAG. However, all glaucoma patient volunteers were normotensive, and many were being treated with aqueous suppressing topical agents that may confound our findings. Rozsival et al. reported an evaluation of aqueous humour cortisol levels in glaucoma (n = 35) and cataract (n = 35) patients.49 Patients with a variety of glaucoma diagnoses were identified, and aqueous humour matched with venous blood samples were taken for cortisol quantification by RIA, but cortisone levels were not measured. In every case, including the controls, aqueous humour cortisol exceeded that of plasma, the highest plasma and aqueous cortisol levels being found in patients with POAG (n = 19). The authors concluded that

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**Figure 4.** Changes in urinary corticosteroid metabolites after the administration of CBX (n = 7) or placebo (n = 7) confirming systemic inhibition of 11β-HSD1 (A) and 11β-HSD2 (B). Results are shown as means ± SD. ( ), stage I—baseline; ■, stage II—treatment: CBX or placebo as indicated; *p < 0.05; **p < 0.001.

**Figure 5.** Change in IOP in patients with ocular hypertension after oral carbenoxolone (CBX) or placebo. The reduction was highly significant when patients (n = 20) were treated with CBX, but not when patients were taking placebo or during the intermediate washout stage. (A) Right eye; (B) left eye. (IOP in mmHg; boxes, 99% CI; +, mean; whiskers, range.)
intraocular cortisol homeostasis was disturbed in patients with POAG, and proposed an increased release of cortisol from ciliary body tissues into the aqueous humour, consistent with our demonstration of 11β-HSD1 at this site, or a defect in reabsorption resulting from detrimental effects of cortisol on trabecular meshwork morphology and physiology.

Whereas IOP fell following CBX in patients with ocular hypertension, our placebo-controlled trial in healthy volunteers was less conclusive. Nevertheless, individuals who demonstrated the largest fall in their IOP, also had the greatest reduction in their urinary corticosteroid metabolites consistent with systemic 11β-HSD1 inhibition, suggesting that interindividual variability in 11β-HSD1 activity within the NPE may be a susceptibility factor. Although the prime aim of our study was the evaluation of a 11β-HSD1 inhibitor in the formation of aqueous humour, and not as a novel therapy for glaucoma, it is interesting to note that in the pioneering study evaluating systemic β-adrenergic blockers (propranolol) as an ocular hypotensive agent, reduction in IOP was only seen following an initial parenteral bolus of propranolol, and was not sustained when subjects were subsequently maintained on oral treatment. This suggested either that an inadequate oral maintenance dose of propranolol was used, or that local application would offer better ocular penetration and efficacy. This could account for the failure of CBX to show a reduction in IOP and further studies are now indicated with more selective 11β-HSD1 inhibitors applied topically.

In summary, we have localized 11β-HSD1 to the NPE, and have provided further evidence of a local cortisol-generating system within the eye. Inhibition of 11β-HSD1 after administration of systemic CBX resulted in a reduction of IOP, presumably through inhibition of the sodium transporting capacity of the NPE. These data link 11β-HSD1 to AH formation, and selective inhibition of 11β-HSD1 activity, perhaps through topical administration, could provide a potential therapeutic target for lowering IOP in the treatment of glaucoma.

Acknowledgements
This work was supported by grants from the Medical Research Council, UK, and Research in Eye Disease Trust, UK. The data were presented in part at the
XXIVth International Congress of Ophthalmology, Sydney, Australia April 2002, and Association for Research in Vision and Ophthalmology annual meeting, Fort Lauderdale, Florida, May 2002. The authors have no proprietary interest in the products described in this article, although the work forms the basis of a International Patent Application (No. 9914648.2), ‘Glaucma Treatment’.

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