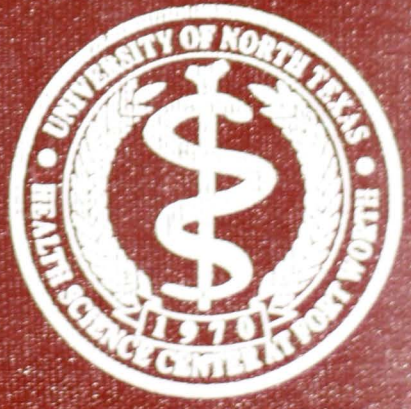

UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

UNTHSC - FW
MC3N5Q

LEWIS LIBRARY
UNT Health Science Center
3500 Camp Bowie Blvd.
Ft. Worth, Texas 76107-2699

Zhang, Xinyu, Mechanisms of glucocorticoid-induced ocular hypertension. Doctor of Philosophy (Pharmacology & Neuroscience). August 2003; 163p; 4 tables; 24 figures; 102 titles.

Glucocorticoids, frequently used anti-inflammatory and immunosuppressive agents, are associated with ocular hypertension and glaucoma. Endothelin-1 (ET-1) is also implicated in glaucoma pathology and optic neuropathy as its concentration is elevated in glaucoma patients and in animal models of glaucoma, and chronic administration of ET-1 produces damage to the optic nerve head in rats. Glucocorticoids have been reported to regulate the expression of ET-1 gene and ET receptors in the cardiovascular system. However in the eye, the interactions between glucocorticoids and endothelin have not yet been investigated, particularly as both glucocorticoids and ET-1 have been implicated in the regulation of intraocular pressure and contribute to glaucoma pathology. Therefore, the purpose of the investigations described herein was to determine the novel mechanisms that may be involved in the regulation of intraocular pressure by glucocorticoids through interactions with ET-1 and ET receptors in NPE cells, a source of ET-1, and in TM cells where both glucocorticoids and ET-1 effect aqueous humor outflow. The hypothesis was that ET-1 exacerbates the actions of glucocorticoids on TM cells and contributes to increased outflow resistance.

Furthermore, individual sensitivities to glucocorticoids differ considerably. About one in every three people in the general population is considered potential steroid responders while almost all primary open angle glaucoma (POAG) patients are steroid responders and develop ocular hypertension after ocular administration of glucocorticoids. The molecular mechanisms underlying the higher glucocorticoid

responsiveness among POAG patients remain unknown. The glucocorticoid receptor beta isoform (hGRβ) has become a candidate for glucocorticoid resistance in some diseases, especially in asthma, based on the reports of its negative activity. The purpose of this segment of the investigations was to test the hypothesis that glucocorticoid responsiveness was regulated by the expression of hGRβ in TM cells.

We demonstrated that dexamethaosone (Dex), a synthetic glucocorticoid, increased ET-1 synthesis and release from human non-pigmented ciliary epithelial (HNPE) cells. Dex also suppressed ET_B receptor protein expression and attenuated ET-1 mediated increase in nitric oxide (NO) while Dex had no effect on ET_A receptor expression and ET_A receptor mediated intracellular Ca^{2+} mobilization in TM cells. The increase in the release of ET-1 from HNPE cells with a concomitant decrease of ET_B receptor protein expression and ET_B receptor mediated NO release by Dex in TM could result in an increase in the contraction and decrease in relaxation of trabecular meshwork thus reducing the intratrabecular space. Such actions by ET-1 may exacerbate Dex effects on the outflow pathway leading to increased outflow resistance and consequently elevated intraocular pressure that typically is associated with glucocorticoids.

We have also found a significant difference in hGRβ levels among normal versus glaucomatous TM cell lines, with the POAG TM cell lines having lower hGRβ receptor expression. This is coincidence with the fact that in the normal population, there is a low rate of glucocorticoid responders as compared to almost all POAG patients considered as glucocorticoid responders. Overexpression of hGRβ in TM cells, produced by transfecting a hGRβ expression construct, inhibited Dex-induced expression of myocilin,

a glaucomatous gene, supporting the contention that hGRβ acts as a negative regulator of glucocorticoid activity. In addition, we studied the machinery of cytoplasm to nuclear transport of hGRβ. We identified that a chaperon protein, hsp90, is a requirement for the nuclear translocation of hGRβ.

In conclusion, we have described a novel-signaling pathway for glucocorticoids through the regulation of ET-1 and ET receptors in the anterior segment which have consequences on aqueous humor outflow. We have also demonstrated a possible molecular mechanism by which glucocorticoid responsiveness in POAG patients is achieved as a result of the low level of nuclear hGRβ receptor isoform expression. Furthermore, we have, for the first time, identified hap90 as a chaperon protein for the translocation of hGRβ from the cytoplasm to the nucleus.

MECHANISMS OF GLUCOCORTICOID – INDUCED OCULAR HYPERTENSION

Xinyu Zhang, M.S.

APPROVED:

Major Professor

Committee Member

Committee Member

Committee Member

University Member

Chair, Department of Pharmacology & Neuroscience

Dean, Graduate School of Biomedical Sciences

MECHANISMS OF GLUCOCORTICOID – INDUCED OCULAR HYPERTENSION

DISSERTATION

Presented to the Graduate Council of the

University of North Texas Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Xinyu Zhang, B.S. M.S.

Fort Worth, Texas

August 12, 2003

ACKNOWLEDGEMENTS

I express my deep gratitude to my mentor, Dr. Thomas Yorio, for his excellent guidance, expertise and support throughout my graduate education. His insight, interest, warmth and humor have contributed immeasurably to both the academic and personal aspects of my scientific education. I will carry his unfailing guidance, support and affection with me long after my graduation. I hope that I can someday repay a fraction of my debt to him by imparting to future scientists some of his spark and inspiration.

I also gratefully acknowledge Dr. Abbot F. Clark for making significant contribution to shape my research project. He has been an invaluable teacher for his expertise in the research field of steroids and glaucoma. The work presented in this dissertation would not have been possible without his intellectual and material assistance.

I am indebted to my committee members, including Dr. Glenn Dillon, Dr. Neeraj Agarwal, and Dr. Robert Wordinger, for their generosity in their time and suggestions.

I would also like to express gratitude to my lab-fellows Dr. Ganesh Prasanna, Dr. Raghu Krishnamoorthy and Santosh Narayan, for their valuable discussion, especially to Dr. Raghu Krishnamoorthy for always generating an enjoyable working environment.

I must, of course, thank my entire family: my parents, Mr. Rongshun Zhang and Mrs. Airong Min, for instilling the values of compassion, hard work and persistence; my brothers and sister, Xinnong Zhang, Xinsheng Zhang, and Xinwei Zhang, for their moral support; specially, my husband, Xiaoren Lin for his love and support throughout my graduate study and my lovely daughter, Yiman Lin, who is the joy of my life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Glaucoma

Glaucoma is the second leading cause of blindness in the world with a prevalence of approximate 66 million people worldwide.[1] Elevated intraocular pressure (IOP) remains one of the most important risk factors for the development of glaucomatous optic neuropathy.[2, 3] While no cure exists for glaucoma, surgery and medial therapy to reduce the elevated IOP has been used to delay the progression of damage to the optic nerve. Of the many forms of glaucoma, primary open-angle glaucoma (POAG) is the most prevalent.[4] POAG is associated with elevated IOP, which is due to impaired aqueous humor outflow and is correlated with morphologic and biochemical changes in the trabecular meshwork (TM),[5] a main resistance in the aqueous humor outflow pathway. Although the actual mechanism responsible for optic nerve damage in POAG is unclear, POAG has a complex or multifactorial etiology and genetic factors have long been implicated in its pathophysiology. By application of molecular genetic techniques, the chromosomal locations of six genes that can independently cause POAG has been mapped (GLC1A, GLC1B, GLC1C, GLC1D, GLC1E and GLC1F), including a glaucoma gene, myocilin (GLC1A), located in chromosome 1.[6] Myocilin was identified and detected in high amounts in the trabecular meshwork. Although the function of

myocilin remains unknown, mutations in the myocilin gene were shown to be associated with both juvenile and adult-onset POAG.[6] In addition to localizing and identifying genes for specific types of glaucoma, researchers have begun to characterize the gene products and investigate the molecular mechanisms involved in glaucoma.

Glucocorticoids and Glaucoma

Glucocorticoids, important steroid hormones for treating a variety of ocular diseases and conditions, has gained notoriety due to its tendency to induce multiple side effects, including the development of ocular hypertention and glaucoma. Glucocorticoids appear to be associated with POAG. It has been reported that POAG patients have elevated levels of the endogeneous glucocorticoid, cortisol, in their blood[7, 8, 9, 10, 11] and aqueous humor[8] compared to non-glaucomatous subjects. The administration of glucocorticoids can cause ocular hypertention which is dependent on the potency, the dose and the duration of treatment of glucocorticoids.[12] Topical ocular administration of a potent glucocorticoid for 4-6 weeks can produce an elevated IOP in approximately 35% of the general population and 20% of responders, classified as high steroid responders, are more likely to eventually develop POAG.[13, 14] In contrast to the normal population, almost all POAG patients are considered steroid responders.[13, 15] Moreover glucocorticoid-induced glaucoma is similar in many ways to POAG including increasing outflow resistance and an increase in IOP.[12] Since the trabecular meshwork is located in the aqueous humor outflow pathway and plays a critique role in aqueous humor outflow, the effects of glucocorticoids on the trabecular meshwork have been studied for many years in either cell culture, whole explant organ culture system, perfusion human eye, or *in vivo*.

Glucocorticoids change the morphology and activity of trabecular meshwork, including reorganization of the cytoskeleton to form ordered cross-linked actin networks (CLANs),[16] altered expression of extracellular matrix glycoprotein fibronectin[17] and laminin,[18] changes in extracellular metalloproteinase activity,[19, 20] altered phagocytosis or migratory activities,[16, 21] increase in cell adhesion molecules,[22] and enhanced expression of myocilin, a glaucoma gene (*MYOC*).[23, 24, 25] Overall glucocorticoids increase extracellular material deposits in trabecular meshwork, and subsequently increase outflow resistance and increase IOP.

Human Glucocorticoid Receptor Isoforms GRα and GRβ

Glucocorticoids action on target tissue to elicit specific biological responses is dependent on the presence of glucocorticoid receptors.[26] The glucocorticoid receptor (hGR) has been detected in many ocular tissues, including trabecular meshwork.[27] The cloning of the full length human glucocorticoid receptor[28] predicted the existence of two receptor isoforms termed hGRα and hGRβ, which differ only at the carboxyl terminus. Such findings are expected if the two hGR isoforms were formed via alternative splicing. Encio and Detera-Wadleigh[29] ascertained the genomic structure of human GR and confirmed that hGRα and hGRβ originate from the same gene by alternative splicing. Based on these observations, it is clear that the human GR gene contains 10 exons, and alternative splicing of the hGR gene generates hGRα with exons of 1-8 and 9α which translates into a 777 amino acid protein, whereas hGRβ with exons of 1-8 and 9β translates into a 742 amino acid protein. Hence hGRα and hGRβ are identical through

amino acid 727 but diverge subsequently with hGRα having an additional 50 amino acids and hGRβ an additional, nonhomologous 15 amino acids (see **Figure 1**).[28, 29] Besides alternative splicing of human hGR, the hGR gene is subjected to alternative translation initiation from a downstream in-frame ATG codon, a potential alternative translation initiation site, which produces two forms of hGR, named hGR-A and hGR-B.[30] Because hGRα is predominantly expressed and is shown to be most abundant with ligand binding studies,[28] it has been the primary focus of subsequent research. As a result, hGRα expression, biochemical properties, and physiological function have been well characterized. hGRα belongs to the superfamily of steroid/thyroid/retinoic acid receptor proteins that function as ligand-dependent transcription Factors.[26] It is comprised of a unique amino-terminal region including a transactivation domain, a central DNA-binding domain, crucial for specific interaction of the receptor with DNA sequences containing glucocorticoid response elements (GRE) and a carboxyl terminus. The carboxyl terminus has the hormone-binding domain as well as sequences important for interaction with heat shock protein 90 (hsp90),[31] nuclear translocation,[32] receptor dimerization,[33] and transactivation.[34] The ability of both natural and synthetic glucocorticoids to elicit cellular responses is through hGRα. Upon hormone binding, hGRα regulates positively or negatively transcription. hGRα predominantly exists in the ctytoplasm and undergoes ligand-dependent translocation to the nucleus,[32, 35, 36] where it binds to glucocorticoid response elements in the promoter regions of target genes and regulates gene expression. hGRα in the nucleus can also physically interact with other transcription factors, such as AP-1[37] and NF-κB,[38] modulate gene expression apart from DNA binding. Moreover, the

ALTERNATIVE SPLICING GENERATES GRα AND GRβ ISOFORMS

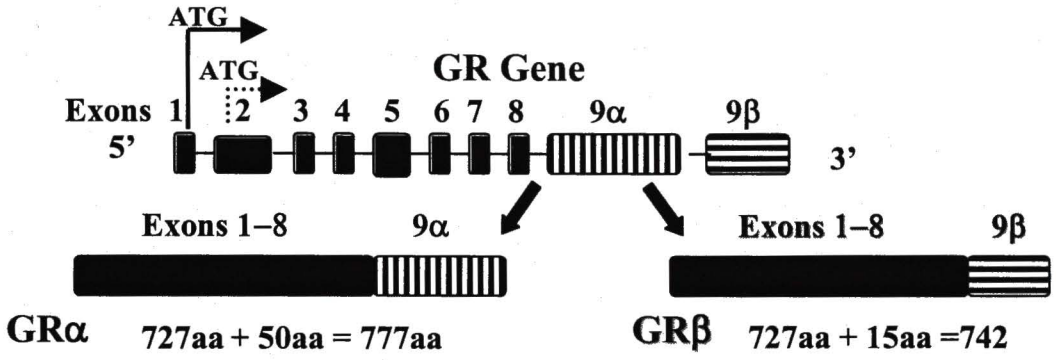


Figure 1. GRα and GRβ originate from the same GR gene which contains 10 exons. Alternative splicing of GR gene generated GRα with exons of 1-8 and 9α which translated into 777 amino acid protein, where GRβ with exons 1-8 and 9β translated into 742 amino acid protein. Hence GRα and GRβ are identical through amino acid 727, but then diverge with GRα having an additional 50 amino acid and GRβ having an additional non-homologous 15 amino acids. It is these different amino acids in the carboxyl terminus which allow GRα but not GRβ to bind to hormones and regulate gene expression. Besides the original initiation site, there is an internal ATG codon, which is a potential alternative translation initiation site for producing two isoforms of GR, named GR-A and GR-B.

actions of hGRα are known to vary among different cells due to variations in the concentration of many cellular modulatory factors, such as coactivators like SRC-1 or TIF2, corepressors like NcoR or SMRT, and heat shock protein HSP90 etc. In contrast to hGRα, the unique end of carboxyl terminal of hGRβ influences several key biochemical properties that distinguish it from hGRα. It does not bind to glucocorticoids and lacks transcriptional activity by itself.[28], [39] Currently the physiological significance of hGRβ is not clear.

The Dominant Negative Activity of the hGRβ Isoform

At the time Hamberger et al[28] discovered hGRβ, they found the hGRβ protein did not bind to glucocorticoid analogues, and immunoblot analysis using polyclonal antibody directed against the human glucocortiocid receptor only recognized hGRα in most human and mouse cell lines. They concluded that the predominant physiological form of the glucocorticoid receptor is the hGRα. Little was known about hGRβ until Bamberger et al[40] found that mRNA of hGRβ were expressed throughout the human body, and overexpression of hGRβ in transfected COS cells disrupted the enhancing effects of hGRα on gene transcription. Oakley et al[39] also observed that mRNA of hGRβ is expressed in various human adult and fetal tissues. Their group found in transfected cell lines that hGRβ did not bind glucocorticoids and antiglucocorticoids, was transcriptionally inactive on the glucocorticoid responsive element, and hGRβ repressed the transcriptional activity of hGRα on the glucocorticoid responsive promoter.[39, 41]

Although the concept of hGRβ as a dominant negative inhibitor of hGRα activity remains controversial,[42] there are increased reports that glucocorticoid insensitvity is associated with increased expression of hGRβ, with the most extensive study in chronic asthma. Several investigators have found that peripheral blood mononuclear cells (PBMC) from patients with glucocorticoid-insensitive asthma have a decrease ability of their hGR to bind its glucocorticoid response elements.[43, 44] Hamid et al[45] reported that airway cells PBMC from patients with glucocorticoid resistant asthma express significantly higher levels of hGRβ than the same cells from patients with glucocorticoid-sensitive asthma, and GRβ expression in glucocorticoid-insensitive asthma was particularly high in airway T cells. The inflammatory cells from patients with glucocorticoid-sensitive asthma had much higher ratio of hGRα/hGRβ than from glucocorticoid-resistant asthma patients.[46] Increased expression of hGRβ at night in PBMC from patients with nocturnal asthma was associated with decreased steroid responsiveness in nocturnal asthma.[47] Overall the amount of hGRβ was found to correlate with glucocorticoid resistance and therapy efficacy in asthma.[48] Besides research in asthma diseases, it has also been observed that hGRβ was expressed much higher in peripheral lymphocytes from patients with steroid-resistant unlcerative colitis (UC) comparing with the steroid-sensitive UC patients.[49] High constitutive expression of hGRβ by human neutrophils prevented these cells from experiencing glucocorticoid-induced cell death.[50] Interestingly, mice are known to be deficient in the expression of hGRβ,[51] when virally trnasduced hGRβ cDNA into mouse hybridoma cells, the stable expression

of hGRβ results in glucocorticoid insensitivity of these cells.[52] Although the molecular mechanism underlying negative activity of hGRβ is not known yet, formation of transcriptional impaired hGRα-hGRβ heterodimers may be responsible for the antagonism.[41, 52]

Glucocorticoid Receptor nuclear translocation

Glucocorticoid receptors, like other steroid receptors, are capable of shuttling between nuclear and cytoplasmic compartments.[53, 54] hGRα undergoes hormone-dependent nuclear import in most cells,[35] and has served as a useful paradigm for cytoplasmic anchoring.[55] In the absence of glucocorticoids, GRα resides predominantly in the cytoplasm of cells as an oligomeric complex containing one molecule of receptor, and two molecules of heat shock protein 90 (hsp90) and often one molecule of hsp70, which directly bind to the receptor,[55, 56] and one molecule of immunophilin either FKBP52, FKBP51, Cyp40, or PP5, which have tetratricopeptide repeat (TPR) domains interacting with hsp90.[54, 57] Hsp90 and hsp70 are both required for glucocorticoid receptor complex assembly and for proper folding to the steroid-binding conformation.[58] It is generally accepted that once hormone binds to hGRα, receptors undergo conformational change and dissociate from Hsp90, Hsp70 and the TPR proteins, followed by nuclear translocation and binding to GRE to regulate gene transcription.[59, 60, 54] However it is also found that the complex of hGRα and hsp90 is dynamic in the sense that assembly and disassembly occurs constantly,[56] and hGRα and hsp90 can move together from the

cytoplasm to the nucleus.[61] A couple of observations suggested that the dynamic assembly and disassembly of GR-hsp90 heterocomplexes is required for receptor movement.[62, 63, 64] hGRα has been found associated with microtubules,[65] and binding of GR-hsp90 to immunophilins is involved in both dynein interaction and hGR nuclear movement.[66, 67, 68] The machinery of hGRα transportation to the nucleus has been suggested as a dynamic interaction of GR-hsp90-FKBP52 heterocomplexes and requiring the retrograde motor protein, dynein, to move it along microtubular tracks.[68] Recently, Davies et al[69] found that Dex binding switches GR-hsp90-FKBP51 to GR-hsp90-FKBP52 and recruits dynein, then GR-hsp90-FKBP52-dynein complex shuttles to the nucleus, and release GR into the nucleus (see **Figure 2**). While nucleocytoplasmic translocation of hGRα is becoming more and more clear, still nothing is known about hGRβ translocation. Although Oakley et al[70] detected hGRβ predominantly in the nucleus independent on glucocorticoid treatments, other groups identified that it also exists in the cytoplasmic fraction in some human original cells.[71, 42] In addition, hGRβ can be associated with hsp90.[41, 42] It is possible that the transport of hGRβ also requires an association with hsp90. Since hGRα activation needs ligand binding to recruit adapter protein FKBP52 and motor protein dynein,[68, 69] and hGRβ does not bind to ligand,[40, 39] the transport mechanism of hGRα and hGRβ may be not exactly the same. However the stability of hGRβ interaction with hsp90 may impact upon the efficiency of receptor nuclear translocation and alter their subcellulular distribution.

MODEL OF THE INHIBITION OF hGRβ ON hGRα

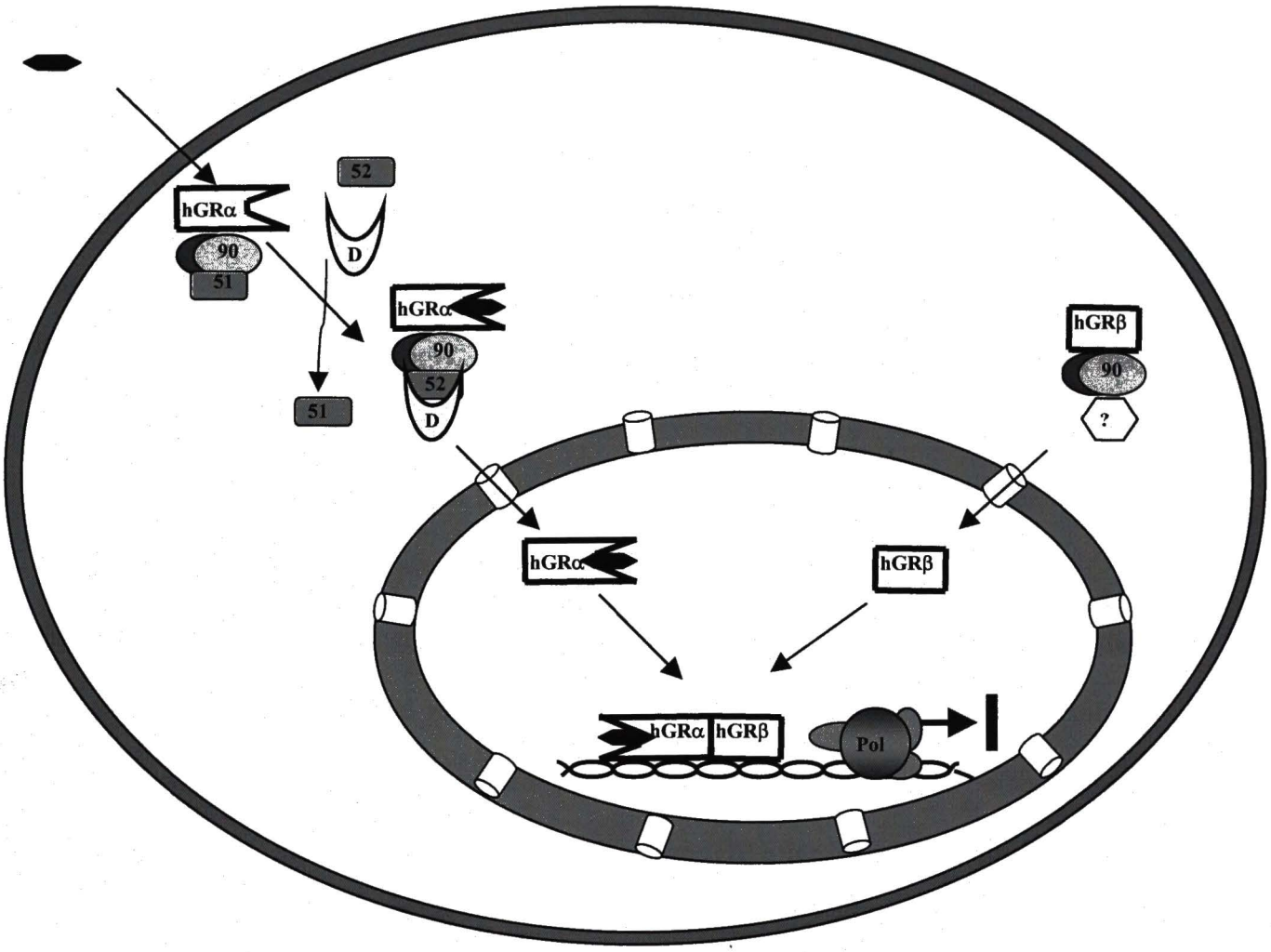


Figure 2. This model presents the translocation of hGRα and hGRβ and formation of the transcriptional inactive heterocomplex of hGRα and hGRβ in the trabecular meshowork cell. In the absence of hormone, hGRα is primarily located in the cytoplasm of the cells as an oligomeric complex containing hsp90 and immunophilin FKBP51. As the consequence of glucocorticoid binding, immunophilin FKBP52 interchanges with FBKP51 followed by concomitant recruitment of dynein, and then hGRα-hsp90-FKBP52-dynein complex associate with microtubules and translocates toward the nucleus and finally release of hGRα into the nucleus. However, hGRβ is spontaneously transported and released into the nucleus along microtubular tracks. In the nucleus, hGRβ competes with hGRα to form transcriptional inactive heterocomplex of hGRα-hGRβ and inhibits or attenuates the ability of hGRα to regulate gene expression.

Endothelin-1 and Glaucoma

The endothelin (ET) family of peptides comprises mainly three isoforms, ET-1, -2, and -3.[72] ET-1 and ET-3 are expressed in the eye, including iris, choroid, retina, optic nerve head, ciliary body, lens and corneal endothelium, and studies also show their presence in the aqueous and vitreous humors. ET-1, well known as a potent vasoactive peptide, may contribute to the etiology of glaucoma, as its concentration is elevated in the aqueous humor in POAG patients and in animal models of glaucoma.[73, 74] Moreover, chronic administration of ET-1 can produce an optic neuropathy.[75] In the anterior segment, the non-pigmented ciliary epithelial cells have been shown to be a source for ET-1, as they endogenously synthesize and release ET-1.[76, 77] ET-1 has also been shown to inhibit Na+/K+-ATPase and decrease aqueous humor formation.[78, 79] Such effects could contribute to the functional response in the decrease IOP after intravitreal administration of ET as shown in rabbits.[80, 81] In the classical model (Fig. 3), ET-1's action on the regulation of intraocular pressure is explained by paracrine effects on contractions of the ciliary muscle (CM) and trabecular meshwork (TM), with the net effect depending on which of the contractions dominate. For instance, in bovine eyes, the contraction of TM would reduce intratrabecular space and decrease aqueous humor outflow even in the presence of CM contraction. Since glucocorticoids cause changes in the morphology and activity of trabecular meshwork with concomitant increases in the deposition of extracellular matrix materials,[82] the contraction of the TM in the presence of glucocorticoids could reduce the intertrabecular space and consequently increase outflow resistance and IOP as a maladaptation.

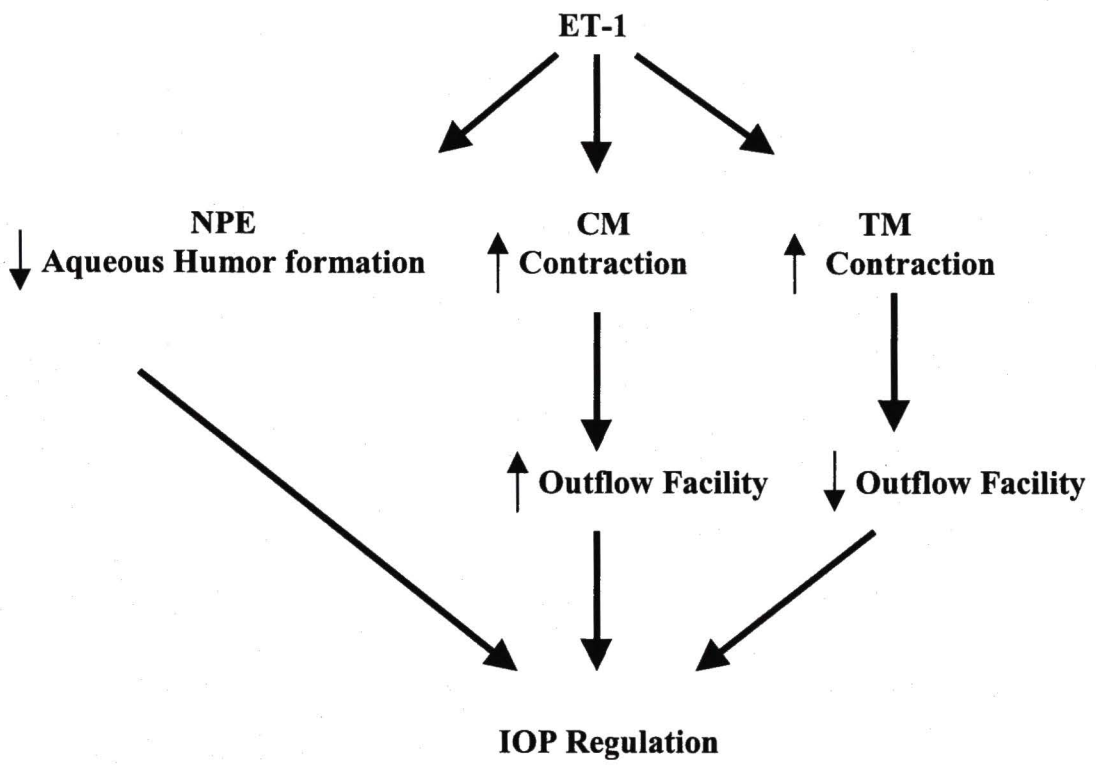


Figure 3. Several actions of endothelin-1 (ET-1) in regulating intraocular pressure are involved in the anterior chamber. Classic ET-1's action on the regulation of intraocular pressure is explained by paracrine effect on contractions of the ciliary muscle (CM) and trabecular meshwork (TM), with the net effect depending on which of the contractions dominate. For instance, in bovine eyes or glucocorticoid treated eyes, the contraction of TM would reduce intratrabecular space and decrease aqueous humor outflow even in the presence of CM contraction. In addition, ET-1 can also inhibit Na+/K+-ATPase in non-pigmented ciliary epithelium and decrease aqueous humor formation.

SPECIFIC AIMS

Glucocorticoids and endothelin-1 are both implicated in glaucoma pathology and optic neuropathy. In vascular smooth muscle cells and other cell types, glucocorticoids have been reported to increase ET-1 gene expression and regulate ET receptors.[83, 84, 85, 86] However in the eye, the interactions between glucocorticoids and endothelin have not yet been investigated. The first part of this research project intends to address the interaction of endothelin with glucocorticoids in human non-pigmented ciliary epithelial (HNPE) cells, which have been shown to be a source for ET in the anterior chamber, and additionally in human TM, a main resistance in aqueous humor outflow pathway. Such studies would describe novel signaling pathways for glucocorticoids in the anterior segment and define consequences on aqueous humor outflow and possibly glaucoma pathology. Glucocorticoids can increase IOP, however this response is not consistent within the population. Approximately 90% of primary open angle glaucoma (POAG) patients respond to glucocorticoids with an increase in IOP following topical ocular administration of glucocorticoids, whereas only 35% of general population are referred as glucocorticoid responders.[15] Little is known about the molecular basis underlying steroid responsiveness in glaucoma. It has been reported that glucocorticoid receptor β (hGRβ) may be associated with glucocorticoid resistance in other diseases, especially in asthma.[43, 44, 45, 46, 47, 48] The secondary part of this research project intends to investigate the potential role of hGRβ in regulating glucocorticoid sensitvity in glaucoma. The hypothesis to test is that glucocorticoids upregulate endothelin-1 and regulate endothelin receptor subtypes in the anterior segment which contributes to the glucocorticoid-induced

ocular hypertension and that low expression of hGRβ in glaucomatous TM leads to glucocorticoid sensitivity in glaucoma. The following specific aims will address these mechanisms.

Aim 1: Determine the regulation of glucocorticoids on the release of ET-1 and the expression of ET receptors in HNPE cells.

1.1 Quantitate the levels of mRNA expression of ET-1, ET_A and ET_B receptors in the presence and absence of dexamethasone treatment in cultured transformed human non-pigmented ciliary epithelial (HNPE) cells.

1.2 Measure the amount of ET-1 peptide released and differential expression of ET_A and ET_B receptor protein in the presence and absence of dexamethasone in HNPE cells.

Aim 2: Determine the effect of glucocorticoids on the expression of ET receptor subtypes in TM cells.

2.1 Measure the regulation of mRNA expression of ET_A and ET_B receptors by dexamethasone in normal and glaucomatous TM cell lines.

2.2 Measure changes in ET_A and ET_B receptor protein expression by dexamethasone in normal and glaucomatous TM cell lines.

Aim 3: Measure the relative expression of hGRα and hGRβ and their regulation by dexamethasone in various TM cell lines as well as determining the subcellular distribution of hGRβ in normal and glaucomatous TM cell lines.

3.1 Study the protein expression of hGRβ in at least 3 normal and 3 glaucomatous primary TM cell lines in the presence or absence of dexamethasone.

3.2 Further determine the subcellular distribution and the relative amounts of hGRβ between those 3 normal and 3 glaucomatous TM cell lines using immunofluorescence microscopy.

Aim 4: Identify the mechanism of translocation of hGRβ from the cytoplasm to the nucleus in TM cells and the inhibitory activity of hGRβ in hGRα action on glaucoma gene expression or glaucoma phenotype formation.

4.1 Determine if heat shock protein 90 (hsp90) is a chaperone for hGRβ translocation from the cytoplasm to the nucleus.

4.2 Determine if microtubules are involved in the translocation of hGRβ from the cytoplasm to the nucleus.

4.3 Evaluate the role of hGRβ in the regulation of glucocorticoid sensitivity in trabecular meshwork by determining if it inhibits the expression of a glaucoma marker gene.

EXPERIMENTAL DESIGN

Three individual experiments were designed to investigate specific aim I, II, III, and IV. These experiments are discussed in detail in chapters 2, 3, and 4. However, a brief description of the experimental rational and methodology is provided below.

Study # 1: We tested the hypotheses that glucocorticoids upregulate endothelin-1 and regulate endothelin receptor subtypes in non-pigmented ciliary epithelial cells. This study was conducted by comparing mRNA and protein levels of ET-1, ET_A, and ET_B receptors between dexamethasone treatments and vehicle control. This study allowed us to detect the novel cross-talk between endothelin-1 and glucocorticoids in the eye (*Zhang X, Krishnamoorthy RR, Prasanna G, Narayan S, Clark A,Yorio T. Dexamethasone regulates endothelin-1 and endothelin receptors in human non-pigmented ciliary epithelial (HNPE) cells. Exp Eye Res. 2003;76:261-72).*

Study # 2: We tested the hypotheses that glucocorticoids regulate endothelin receptor subtypes in trabecular meshwork cells, which contribute to the glucocorticoid-induced ocular hypertention. This study was accomplished by measuring mRNA and protein levels of ET_A and ET_B receptors in normal and glaucomatous TM cells with or

without dexamethasone treatment conditions. We also evaluated the function of ET_A and ET_B receptors by measuring the mobilization of intracellular Ca^{2+} induced by ET-1 and ET-1-stimulated nitric oxide release respectively. These experimental designs were aimed to detect the novel signaling pathways for dexamethasone in the regulation of outflow resistance besides increasing the deposition of extracellular matrix materials in the trabecular meshwork *(Zhang X, Clark AF, and Yorio T. Interactions of Endothelin-1 with Dexamethasone in Primary Cultured Human Trabecular Meshwork Cells. Invest Ophthalmol Vis Sci. In Press).*

Study # 3: We tested the hypotheses that glucocorticoid receptor β (hGRβ) is a key component in regulating glucocorticoid responsiveness and that low expression of hGRβ in glaucomatous TM cells leads to glucocorticoid sensitivity in glaucoma. We focused on comparing the expression of hGRβ in several human normal and glaucomatous TM cell lines. Such study allowed us to observe the obvious differences in relative amount of hGRβ between normal and glaucomatous TM cells. This study also investigated the transport mechanism involving in moving hGRβ from the cytoplasm to the nucleus, which may give rationale and explanations about the different nuclear amounts of hGRβ as seen in normal and glaucomatous TM cells. Finally, in this study, we directly tested if hGRβ could inhibit a glucocorticoid response by determining if overexpression of hGRβ reduced dexamethaonse induction of myocilin, a well known glaucoma gene. Data from this study could provide the most direct evidence for the potential role of hGRβ in regulating glucocorticoid sensitivity in an *in vitro* model system

(Zhang X, Clark AF, and Yorio T. Expression and Translocation of Glucocorticoid Receptor β and its Regulation of Glucocorticoid Responsiveness in Trabecular Meshwork Cells. To be sumitted).

METHODS

The methodology used in each investigation is described in detail in each of the chapters (II, III, and IV); however, it is appropriate to discuss these techniques briefly with respect to each specific aim.

Quantitative Polymerase Chain Reaction (QPCR)

QPCR was used to compare mRNA levels of ET-1, ET_A, and ET_B between Dex treatment and control in HNPE and TM cells. Total cellular RNA was isolated from cells using the Trizol B reagent (Life Technologies, Rockville, MD). cDNA was synthesized from total RNA from each sample using random primers and AMV Reverse Transcriptase (Promega, Madison, WI). QPCR amplification of each sequence was performed using SYBR Green PCR Core regents (PE Applied Biosystems, Foster City, CA) in a Cepheid Smart Cycler (Cepheid, Sunnyvale, CA). Quantification of relative RNA level was achieved using the comparative C_T method (as described in PE Biosystems User Bulletin#2: http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf.

ET-1 Radioimmunoassay

Radioimmunoassay of ET-1 was performed to measure the ET-1 released in the culture media from HNPE cells in the presence or absence of Dex (100 nM) for 24 h. A

commercially available radioimmunoassay (RIA) kit for ET-1 (Peninsula Laboratories, Belmont, CA) was used according the manufacture's protocol. The sample ET-1 concentration was obtained from a standard curve.

Western Blot Analysis for ET_A and ET_B Receptors

In order to measure the expression of ET_A and ET_B receptors in HNPE and TM cells under Dex (100 nM) or control conditions, the plasma membrane fractions were isolated. Western Blot analysis was performed using primary rabbit polyclonal ET_A or ET_B antibodies (Alomone Labs, Jerusalem, Israel) respectively, and secondary horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ). The non-specific bands in the immunoblots for ET_A and ET_B receptors were identified by pre-incubating the anti-ET_A or anti-ET_B antibodies with their respective receptor peptides (Alomone Labs, Jerusalem, Israel).

[^{125}I]ET-1 Binding Assay

In order to verify and better quantify the ET receptor density changes by Dex (100 nM) for 24 h in HNPE cells, [^{125}I]ET-1 binding to its receptors was performed. First the plasma membrane fractions were isolated and [^{125}I]ET-1 (NEN Life Science Product. Inc., Boston, MA) binding to total ET receptors (ET_A and ET_B) was measured. Non-specific binding was defined as filter-bound radioactivity measured in the presence of unlabeled ET-1 (Sigma Chemical, St Louis, MO) before addition of [^{125}I] ET-1. The binding of ET_A receptor was determined by pre-treating with sarafatoxin S6C (Peninsula

Lab Inc. Belmont, CA), an ET_B agonist, to block the binding of [^{125}I]ET-1 to ET_B receptors. ET_B receptor binding was defined as the total specific [^{125}I]ET-1 binding minus the amount of ET_A receptor binding.

Measurement of Intracellular Ca^{2+} Mobilization

In order to determine whether ET_A receptor function was altered by Dex (100 nM) treatment for 72 h, ET-1 effects on intracellular calcium were measured using the Fura-2, AM fluorescent probe technique in both normal and glaucomatous TM cells under vehicle control and Dex treatment conditions. The intracellular $[Ca^{2+}]_i$ within single cells was measured with a Nikon fluorescent microscope using Imaging Metafluor software (Universal Imaging Co., West Chester, PA). ET-1 (100 nM) was added and changes in fluorescence ratios were recorded. BQ610 (Peninsula Lab Inc. Belmont, CA), an antagonist of ET_A receptor, was used at concentration of 1 μM to determine whether ET-1 induced-Ca^{2+} mobilization was mediated through ET_A receptor.

Determination of Nitrite Accumulation in Culture Medium

In order to determine whether ET_B receptor function was altered by Dex (100 nM) treatment for 72 h in TM cells, the effect of Dex on ET-1-induced nitric oxide (NO) release was measured using a Griess colorimetric nitric oxide synthase assay kit. The concentration of Nitrite, the final oxidation product of NO in the culture medium, was calculated from a constructed nitrite standard curve. An ET_B receptor antagonist, BQ788 (Peninsula Lab Inc. Belmont, CA), was used to determine the role of ET_B receptor on NO release induced by ET-1.

Conventional or confocal Immunofluorescence Microscopy or Double Immunofluorescence Microscopy

Immunofluorescence microscopy was used to compare the relative amount of hGRβ between several normal and glaucomatous TM cell lines, and double immunofluorescence microscopy was used to detect the colocalization of hGRβ with β-tubulin or hsp90 in normal and glaucomatous TM cells. TM cells grown on sub-confluence coverslips was fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100. Polyclonal rabbit anti-hGRβ antibody (Affinity PA3-514) and Alexa Fluor 594 goat anti-rabbit IgG (red fluorescence, Molecular Probe) was used to stain hGRβ. For double imuunofluorescence, either primary rabbit anti-hGRβ antibody + mouse anti-β-tubulin antibody (Santa Cruz sc-5274), or primary anti-hGRβ antibody + mouse anti-hsp90 antibody (Santa Cruz sc-13119) and secondary Alexa Fluor 594 goat anti-rabbit IgG + Alexa Fluor 488 goat anti-mouse IgG (green fluorescence, Molecular Probe) was used to stain either hGRβ and β-tubulin or hGRβ and hsp90. DAPI was used to define nuclear regions. Immunofluorescence was viewed under a Nikon Diaphot Fluorescence Microscope or Zeiss LSM-410 Confocal Scanning Laser Microscopy System.

Western Blot Analysis for hGRβ in Cytoplasmic and Nuclear Fractions

In order to confirm the subcellular distribution of hGRβ in TM cells, cytosolic and nuclear fractions were isolated and Western Blot analysis was conducted to detect both

hGRα and hGRβ in those isolated fractions. The effects of Dex (100 nM) in the subcellular distribution of hGRα and hGRβ were also investigated.

Co-immunoprecipitation of hGRβ-hsp90 Complexes

In order to find out whether hGRβ is physically associated with hsp90, Co-immunoprecipitation of hGRβ-hsp90 complex was performed. First, we used mouse anti-hsp90 monoclonal antibody to immunoprecipitate hsp90 complexes and used anti-hGRβ antibody to run immunoblotting to detect GRβ, then we used anti-hGRβ antibody to do immunoprecipitation and used anti-hsp90 antibody to perform immunoblotting to detect hsp90.

Transfection of TM cells with hGRβ expression vectors

To test the negative activity of hGRβ, we transfected pCMX-hGRβ, a hGRβ expression vector, into TM cells and detected whether the overexpression of hGRβ inhibited the induction of myocilin by Dex (100 nM). Transfection was conducted using CalPhos™ Mammalian Transfection Kit (BD Biosciences) and Western Blotting was performed to detect the myocilin expression using polyclonal anti-myocilin antibody.

References:

1. Quigley HA. Number of people with glaucoma worldwide. *Br J Ophthalmol.* 1996;80:389-93.
2. Hart WM, Jr., Yablonski M, Kass MA,Becker B. Multivariate analysis of the risk of glaucomatous visual field loss. *Arch Ophthalmol.* 1979;97:1455-8.
3. Sommer A. Intraocular pressure and glaucoma. *Am J Ophthalmol.* 1989;107:186-8.
4. Kahn HA,Milton RC. Alternative definitions of open-angle glaucoma. Effect on prevalence and associations in the Framingham eye study. *Arch Ophthalmol.* 1980;98:2172-7.
5. Rohen JW,Jikihara S. [Morphology of the aqueous outflow system in different forms of glaucoma]. *Fortschr Ophthalmol.* 1988;85:15-24.
6. Fingert JH, Stone EM, Sheffield VC,Alward WL. Myocilin glaucoma. *Surv Ophthalmol.* 2002;47:547-61.
7. Ray S, Mehra KS, Misra S,Singh R. Plasma cortisol in glaucoma. *Ann Ophthalmol.* 1977;9:1151-4.
8. Rozsival P, Hampl R, Obenberger J, Starka L,Rehak S. Aqueous humour and plasma cortisol levels in glaucoma and cataract patients. *Curr Eye Res.* 1981;1:391-6.
9. Schwartz B, McCarty G,Rosner B. Increased plasma free cortisol in ocular hypertension and open angle glaucoma. *Arch Ophthalmol.* 1987;105:1060-5.

10. Meredig WE, Pulhorn G, Jentzen F,Hartmann F. [Plasmacortisol level in patients suffering from glaucoma]. *Albrecht Von Graefes Arch Klin Exp Ophthalmol.* 1980;213:215-8.

11. McCarty GR,Schwartz B. Increased plasma noncortisol glucocorticoid activity in open-angle glaucoma. *Invest Ophthalmol Vis Sci.* 1991;32:1600-8.

12. Clark AF, Wilson K, de Kater AW, Allingham RR,McCartney MD. Dexamethasone-induced ocular hypertension in perfusion-cultured human eyes. *Invest Ophthalmol Vis Sci.* 1995;36:478-89.

13. Armaly MF,Becker B. Intraocular pressure response to topical corticosteroids. *Fed Proc.* 1965;24:1274-8.

14. Kitazawa Y,Horie T. The prognosis of corticosteroid-responsive individuals. *Arch Ophthalmol.* 1981;99:819-23.

15. Lewis JM, Priddy T, Judd J, et al. Intraocular pressure response to topical dexamethasone as a predictor for the development of primary open-angle glaucoma. *Am J Ophthalmol.* 1988;106:607-12.

16. Clark AF, Wilson K, McCartney MD, Miggans ST, Kunkle M,Howe W. Glucocorticoid-induced formation of cross-linked actin networks in cultured human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 1994;35:281-94.

17. Steely HT, Browder SL, Julian MB, Miggans ST, Wilson KL,Clark AF. The effects of dexamethasone on fibronectin expression in cultured human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 1992;33:2242-50.

18. Dickerson JE, Jr., Steely HT, Jr., English-Wright SL,Clark AF. The effect of dexamethasone on integrin and laminin expression in cultured human trabecular meshwork cells. *Exp Eye Res*. 1998;66:731-8.

19. Samples JR, Alexander JP,Acott TS. Regulation of the levels of human trabecular matrix metalloproteinases and inhibitor by interleukin-1 and dexamethasone. *Invest Ophthalmol Vis Sci*. 1993;34:3386-95.

20. Snyder RW, Stamer WD, Kramer TR,Seftor RE. Corticosteroid treatment and trabecular meshwork proteases in cell and organ culture supernatants. *Exp Eye Res*. 1993;57:461-8.

21. Johnson D, Gottanka J, Flugel C, Hoffmann F, Futa R,Lutjen-Drecoll E. Ultrastructural changes in the trabecular meshwork of human eyes treated with corticosteroids. *Arch Ophthalmol*. 1997;115:375-83.

22. Underwood JL, Murphy CG, Chen J, et al. Glucocorticoids regulate transendothelial fluid flow resistance and formation of intercellular junctions. *Am J Physiol*. 1999;277:C330-42.

23. Polansky JR, Fauss DJ, Chen P, et al. Cellular pharmacology and molecular biology of the trabecular meshwork inducible glucocorticoid response gene product. *Ophthalmologica*. 1997;211:126-39.

24. Clark AF, Steely HT, Dickerson JE, Jr., et al. Glucocorticoid induction of the glaucoma gene MYOC in human and monkey trabecular meshwork cells and tissues. *Invest Ophthalmol Vis Sci*. 2001;42:1769-80.

25. Shepard AR, Jacobson N, Fingert JH, Stone EM, Sheffield VC,Clark AF. Delayed secondary glucocorticoid responsiveness of MYOC in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 2001;42:3173-81.

26. Evans RM. The steroid and thyroid hormone receptor superfamily. *Science.* 1988;240:889-95.

27. Stokes J, Noble J, Brett L, et al. Distribution of glucocorticoid and mineralocorticoid receptors and 11beta-hydroxysteroid dehydrogenases in human and rat ocular tissues. *Invest Ophthalmol Vis Sci.* 2000;41:1629-38.

28. Hollenberg SM, Weinberger C, Ong ES, et al. Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature.* 1985;318:635-41.

29. Encio IJ,Detera-Wadleigh SD. The genomic structure of the human glucocorticoid receptor. *J Biol Chem.* 1991;266:7182-8.

30. Yudt MR,Cidlowski JA. Molecular identification and characterization of a and b forms of the glucocorticoid receptor. *Mol Endocrinol.* 2001;15:1093-103.

31. Dalman FC, Scherrer LC, Taylor LP, Akil H,Pratt WB. Localization of the 90-kDa heat shock protein-binding site within the hormone-binding domain of the glucocorticoid receptor by peptide competition. *J Biol Chem.* 1991;266:3482-90.

32. Picard D,Yamamoto KR. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *Embo J.* 1987;6:3333-40.

33. Dahlman-Wright K, Wright A, Gustafsson JA,Carlstedt-Duke J. Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimer is mediated by a short segment of five amino acids. *J Biol Chem.* 1991;266:3107-12.

34. Hollenberg SM,Evans RM. Multiple and cooperative trans-activation domains of the human glucocorticoid receptor. *Cell.* 1988;55:899-906.

35. Wikstrom AC, Bakke O, Okret S, Bronnegard M,Gustafsson JA. Intracellular localization of the glucocorticoid receptor: evidence for cytoplasmic and nuclear localization. *Endocrinology.* 1987;120:1232-42.

36. Cidlowski JA, Bellingham DL, Powell-Oliver FE, Lubahn DB,Sar M. Novel antipeptide antibodies to the human glucocorticoid receptor: recognition of multiple receptor forms in vitro and distinct localization of cytoplasmic and nuclear receptors. *Mol Endocrinol.* 1990;4:1427-37.

37. Yang-Yen HF, Chambard JC, Sun YL, et al. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell.* 1990;62:1205-15.

38. McKay LI,Cidlowski JA. Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr Rev.* 1999;20:435-59.

39. Oakley RH, Sar M,Cidlowski JA. The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. *J Biol Chem.* 1996;271:9550-9.

40. Bamberger CM, Bamberger AM, de Castro M,Chrousos GP. Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. *J Clin Invest.* 1995;95:2435-41.

41. Oakley RH, Jewell CM, Yudt MR, Bofetiado DM,Cidlowski JA. The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanisms of action. *J Biol Chem.* 1999;274:27857-66.

42. Hecht K, Carlstedt-Duke J, Stierna P, Gustafsson J, Bronnegard M,Wikstrom AC. Evidence that the beta-isoform of the human glucocorticoid receptor does not act as a physiologically significant repressor. *J Biol Chem.* 1997;272:26659-64.

43. Adcock IM, Lane SJ, Brown CR, Peters MJ, Lee TH,Barnes PJ. Differences in binding of glucocorticoid receptor to DNA in steroid-resistant asthma. *J Immunol.* 1995;154:3500-5.

44. Leung DY, Hamid Q, Vottero A, et al. Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. *J Exp Med.* 1997;186:1567-74.

45. Hamid QA, Wenzel SE, Hauk PJ, et al. Increased glucocorticoid receptor beta in airway cells of glucocorticoid-insensitive asthma. *Am J Respir Crit Care Med.* 1999;159:1600-4.

46. Sousa AR, Lane SJ, Cidlowski JA, Staynov DZ,Lee TH. Glucocorticoid resistance in asthma is associated with elevated in vivo expression of the glucocorticoid receptor beta-isoform. *J Allergy Clin Immunol.* 2000;105:943-50.

47. Kraft M, Hamid Q, Chrousos GP, Martin RJ,Leung DY. Decreased steroid responsiveness at night in nocturnal asthma. Is the macrophage responsible? *Am J Respir Crit Care Med.* 2001;163:1219-25.

48. Leung DY,Chrousos GP. Is there a role for glucocorticoid receptor beta in glucocorticoid-dependent asthmatics? *Am J Respir Crit Care Med.* 2000;162:1-3.

49. Honda M, Orii F, Ayabe T, et al. Expression of glucocorticoid receptor beta in lymphocytes of patients with glucocorticoid-resistant ulcerative colitis. *Gastroenterology.* 2000;118:859-66.

50. Strickland I, Kisich K, Hauk PJ, et al. High constitutive glucocorticoid receptor beta in human neutrophils enables them to reduce their spontaneous rate of cell death in response to corticosteroids. *J Exp Med.* 2001;193:585-94.

51. Otto C, Reichardt HM,Schutz G. Absence of glucocorticoid receptor-beta in mice. *J Biol Chem.* 1997;272:26665-8.

52. Hauk PJ, Goleva E, Strickland I, et al. Increased glucocorticoid receptor Beta expression converts mouse hybridoma cells to a corticosteroid-insensitive phenotype. *Am J Respir Cell Mol Biol.* 2002;27:361-7.

53. Madan AP,DeFranco DB. Bidirectional transport of glucocorticoid receptors across the nuclear envelope. *Proc Natl Acad Sci U S A.* 1993;90:3588-92.

54. Pratt WB,Toft DO. Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev.* 1997;18:306-60.

55. Pratt WB. The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. *J Biol Chem.* 1993;268:21455-8.

56. Smith DF,Toft DO. Steroid receptors and their associated proteins. *Mol Endocrinol.* 1993;7:4-11.

57. Ratajczak T, Carrello A, Mark PJ, et al. The cyclophilin component of the unactivated estrogen receptor contains a tetratricopeptide repeat domain and shares identity with p59 (FKBP59). *J Biol Chem.* 1993;268:13187-92.

58. Hutchison KA, Dittmar KD,Pratt WB. All of the factors required for assembly of the glucocorticoid receptor into a functional heterocomplex with heat shock protein 90 are preassociated in a self-sufficient protein folding structure, a "foldosome". *J Biol Chem.* 1994;269:27894-9.

59. Tsai MJ,O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem.* 1994;63:451-86.

60. Hutchison KA, Scherrer LC, Czar MJ, et al. Regulation of glucocorticoid receptor function through assembly of a receptor-heat shock protein complex. *Ann N Y Acad Sci.* 1993;684:35-48.

61. Kang KI, Devin J, Cadepond F, et al. In vivo functional protein-protein interaction: nuclear targeted hsp90 shifts cytoplasmic steroid receptor mutants into the nucleus. *Proc Natl Acad Sci U S A.* 1994;91:340-4.

62. Raaka BM, Finnerty M, Sun E,Samuels HH. Effects of molybdate on steroid receptors in intact GH1 cells. Evidence for dissociation of an intracellular 10 S receptor oligomer prior to nuclear accumulation. *J Biol Chem.* 1985;260:14009-15.

63. Czar MJ, Galigniana MD, Silverstein AM,Pratt WB. Geldanamycin, a heat shock protein 90-binding benzoquinone ansamycin, inhibits steroid-dependent

translocation of the glucocorticoid receptor from the cytoplasm to the nucleus. *Biochemistry*. 1997;36:7776-85.

64. Galigniana MD, Scruggs JL, Herrington J, et al. Heat shock protein 90-dependent (geldanamycin-inhibited) movement of the glucocorticoid receptor through the cytoplasm to the nucleus requires intact cytoskeleton. *Mol Endocrinol.* 1998;12:1903-13.
65. Akner G, Wikstrom AC,Gustafsson JA. Subcellular distribution of the glucocorticoid receptor and evidence for its association with microtubules. *J Steroid Biochem Mol Biol.* 1995;52:1-16.
66. Czar MJ, Lyons RH, Welsh MJ, Renoir JM,Pratt WB. Evidence that the FK506-binding immunophilin heat shock protein 56 is required for trafficking of the glucocorticoid receptor from the cytoplasm to the nucleus. *Mol Endocrinol.* 1995;9:1549-60.
67. Perrot-Applanat M, Cibert C, Geraud G, Renoir JM,Baulieu EE. The 59 kDa FK506-binding protein, a 90 kDa heat shock protein binding immunophilin (FKBP59-HBI), is associated with the nucleus, the cytoskeleton and mitotic apparatus. *J Cell Sci.* 1995;108 (Pt 5):2037-51.
68. Galigniana MD, Radanyi C, Renoir JM, Housley PR,Pratt WB. Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus. *J Biol Chem.* 2001;276:14884-9.

69. Davies TH, Ning YM,Sanchez ER. A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. *J Biol Chem*. 2002;277:4597-600.

70. Oakley RH, Webster JC, Sar M, Parker CR, Jr.,Cidlowski JA. Expression and subcellular distribution of the beta-isoform of the human glucocorticoid receptor. *Endocrinology*. 1997;138:5028-38.

71. de Castro M, Elliot S, Kino T, et al. The non-ligand binding beta-isoform of the human glucocorticoid receptor (hGR beta): tissue levels, mechanism of action, and potential physiologic role. *Mol Med*. 1996;2:597-607.

72. Inoue A, Yanagisawa M, Kimura S, et al. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci U S A*. 1989;86:2863-7.

73. Noske W, Hensen J,Wiederholt M. Endothelin-like immunoreactivity in aqueous humor of patients with primary open-angle glaucoma and cataract. *Graefes Arch Clin Exp Ophthalmol*. 1997;235:551-2.

74. Kallberg ME, Brooks DE, Garcia-Sanchez GA, Komaromy AM, Szabo NJ,Tian L. Endothelin 1 levels in the aqueous humor of dogs with glaucoma. *J Glaucoma*. 2002;11:105-9.

75. Cioffi GA,Sullivan P. The effect of chronic ischemia on the primate optic nerve. *Eur J Ophthalmol*. 1999;9 Suppl 1:S34-6.

76. Lepple-Wienhues A, Becker M, Stahl F, et al. Endothelin-like immunoreactivity in the aqueous humour and in conditioned medium from cultured ciliary epithelial cells. *Curr Eye Res*. 1992;11:1041-6.

77. Prasanna G, Dibas A, Tao W, White K,Yorio T. Regulation of endothelin-1 in human non-pigmented ciliary epithelial cells by tumor necrosis factor-alpha. *Exp Eye Res*. 1998;66:9-18.

78. Taniguchi T, Haque MS, Sugiyama K, Okada K, Nakai Y,Kitazawa Y. Effects of endothelin A and B receptors on aqueous humor dynamics in the rabbit eye. *J Ocul Pharmacol Ther*. 1996;12:123-30.

79. Prasanna G, Dibas A, Hulet C,Yorio T. Inhibition of Na(+)/K(+)-atpase by endothelin-1 in human nonpigmented ciliary epithelial cells. *J Pharmacol Exp Ther*. 2001;296:966-71.

80. Sugiyama K, Haque MS, Okada K, Taniguchi T,Kitazawa Y. Intraocular pressure response to intravitreal injection of endothelin-1 and the mediatory role of ETA receptor, ETB receptor, and cyclooxygenase products in rabbits. *Curr Eye Res*. 1995;14:479-86.

81. Granstam E, Wang L,Bill A. Effects of endothelins (ET-1, ET-2 and ET-3) in the rabbit eye; role of prostaglandins. *Eur J Pharmacol*. 1991;194:217-23.

82. Wordinger RJ,Clark AF. Effects of glucocorticoids on the trabecular meshwork: towards a better understanding of glaucoma. *Prog Retin Eye Res*. 1999;18:629-67.

83. Kanse SM, Takahashi K, Warren JB, Ghatei M,Bloom SR. Glucocorticoids induce endothelin release from vascular smooth muscle cells but not endothelial cells. *Eur J Pharmacol.* 1991;199:99-101.

84. Roubert P, Viossat I, Lonchampt MO, et al. Endothelin receptor regulation by endothelin synthesis in vascular smooth muscle cells: effects of dexamethasone and phosphoramidon. *J Vasc Res.* 1993;30:139-44.

85. Kato H, Hayashi T, Koshino Y, et al. Autocrine production of endothelin-1 participates in the glucocorticoid-induced Ca2+ influx into vascular smooth muscle cells. *Biochem Biophys Res Commun.* 1995;208:82-8.

86. Morin C, Asselin C, Boudreau F,Provencher PH. Transcriptional regulation of pre-pro-endothelin-1 gene by glucocorticoids in vascular smooth muscle cells. *Biochem Biophys Res Commun.* 1998;244:583-7.

CHAPTER II

DEXAMETHASONE REGULATES ENDOTHELIN-1 AND ENDOTHELIN RECEPTORS IN HUMAN NON-PIGMENTED CILIARY EPITHELIAL (HNPE) CELLS

Xinyu Zhang, Raghu R. Kridhnamoorthy, Ganesh Prasanna, Santosh Narayan,

Abbot F. Clark, and Thomas Yorio

Experimental Eye Research 76 (2003) 261-272

SUMMARY

Endothelin-1 (ET-1) lowers intraocular pressure (IOP) in animal models by regulating aqueous humor dynamics through both inflow and outflow mechanisms. Moreover, ET's concentration is elevated in glaucoma patients and in animal models of glaucoma. Glucocorticoid therapy often can lead to increase IOP in susceptible individuals including patients with primary open angle glaucoma (POAG). In this study, we examined the effects of dexamethasone (Dex), a frequently used anti-inflammatory glucocorticoid, on the synthesis and release of endothelin-1 and on the expression of endothelin receptors in human non-pigmented ciliary epithelial (HNPE) cells, an established source for ET-1 in the anterior chamber. As measured by ET-1 immunoreactivity, ET-1 was concentration-dependently increased following 24 hr Dex treatment, with a maximum concentration (100 nM) causing a 3-fold increase of ET-1 release. Western blot analysis of HNPE cells showed the expression of endothelin receptor A (ET_A) and endothelin receptor B (ET_B) with approximate molecular weights of 40 kDa. Dex treatment decreased ET_A receptor expression at all Dex doses, but up-regulated ET_B receptors with 10 nM Dex having the greatest effect. Quantitative PCR demonstrated that Dex also increased the mRNA of pre-pro-ET-1 (ppET-1) and ET_B but decreased the mRNA of ET_A. RU486, a glucocorticoid receptor antagonist, was able to block Dex's actions on ET release and ET_B receptor expression, but did not block its action on ET_A receptor expression. Endothelin receptors were minimally expressed in HNPE cells as determined in binding experiments (B_{max}: ET_A 17, ET_B 25 fmol/mg membrane protein). However Dex treatment stimulated a dramatic increase in ET_B

receptor density while decreasing ET_A receptors (B_{max}: ET_A 11, ET_B 116 fmol/mg membrane protein). The regulation of endothelin and its receptors could be a novel mechanism associated with glucocorticoid's effects on intraocular pressure. The increase in ET-1 and disproportionate regulation in ET receptor expression by Dex could promote dysregulation in ET's mechanism on both inflow and outflow, thus affecting aqueous humor dynamics in the anterior chamber of the eye.

Key words: Dexamethasone, Endothelin-1, Endothelin Receptor A, Endothelin Receptor B, Ciliary Epithelium, Aqueous Humor Dynamics, Intraocular Pressure, Glucocorticoid Receptor, RU486

INTRODUCTION

Glucocorticoids, potent immunosuppressants and anti-inflammatory agents, are associated with primary open-angle glaucoma (POAG). It has been reported that POAG patients have elevated levels of the endogenous glucocorticoid, cortisol, in their blood (Ray et al., 1977; Rozsival et al., 1981; Schwatz et al., 1987; McCarty and Schwartz, 1991) and aqueous humor (Rozsival et al., 1981). Over 90% of POAG patients, compared with 35% of general population, are considered glucocorticoid responders and produce an elevated intraocular pressure following topical ocular administration of glucocorticoids (Becker, 1965; Lewis et al., 1988). The ability of both natural and synthetic glucocorticoids to act on a target tissue and elicit specific responses is dependent on the presence of the glucocorticoid receptors, which function as ligand-dependent transcription factors (Evans, 1988). Classically the actions of glucocorticoids are mediated by the activated glucocorticoid receptor binding to glucocorticoid response elements in the targeted genes. The activated glucocorticoid receptor is also capable of binding other transcription factors and thereby regulating their function (Jonat et al., 1990; Samples et al., 1993; Ray and Prefontaine, 1994).

The effects of glucocorticoids on the trabecular meshwork have been studied as a model system to better understand the pathogenic mechanisms involved in ocular hypertension. Glucocorticoids affect aqueous humor outflow resistance through morphological and activity changes in trabecular meshwork, including alterations in cell size and cytoskeletal organization (Wilson et al., 1993; Clark et al., 1997), extracellular matrix production (Johnson et al., 1990; Steely et al., 1992), extracellular

metalloproteinase activity (Samples et al., 1993; Snyder et al., 1993), phagocytosis (Johnson et al., 1997), cell adhesion molecules (McCartney et al., 1994; Underwood et al., 1999), and enhanced expression of the glaucoma gene, *MYOC* (Polansky et al., 1997; Clark et al., 2001). McCarty and Schwartz (1982) reported that rabbit iris-ciliary body contained high-affinity receptors for dexamethasone and the iris-ciliary body glucocorticoid receptor was present in nearly twice the concentration as compared to the rabbit liver receptor. Similarly the glucocorticoid receptor was also detected in human ocular tissues, including non-pigmented ciliary epithelium, trabecular meshwork, corneal epithelium and endothelium, and anterior lens epithelium (Strokes et al., 2000). The action of glucocorticoids on other ocular tissues may offer insight into glucocorticoid-induced ocular hypertension and its significance to glaucoma.

Endothelin-1 is also implicated in glaucoma pathology and optic neuropathy (Orgul et al., 1996; Cioffi and Sullivan, 1999; Yorio et al., 2002) as its concentration is elevated in glaucoma patients and in animal models of glaucoma (Noske et al., 1997; Kallberg et al., 2002). HNPE cells have been shown to be a source for ET, as they endogenously synthesize and release ET-1 (Lepple-Weinhues et al., 1992; Prasanna et al., 1998). Some of ET-1's action on the regulation of intraocular pressure can be explained by paracrine effects on the ciliary muscle and trabecular meshwork resulting in increased aqueous humor outflow (Erickson-Lamy et al., 1991), while autocrine effects have been linked to inhibition of Na^+/K^+-ATPase and a decrease in aqueous humor formation (Taniguchi et al., 1996; Prasanna et al., 2001). The diverse biological activities of ET-1 appear to be mediated through different receptor subtypes, ET_A and ET_B receptors (Pang and Yorio,

1997; Yorio et al., 2002). In the cardiovascular system, many factors influence ET-receptor expression profile, including actions of cytokines, growth factors, vasoactive agents, flow sheer stress (Rubanyi and Polokoff, 1994), chronic heart diseases (Zolk et al., 1999), and the action of glucocorticoids (Kanse et al., 1991; Roubert et al., 1993; Kato et al., 1995; Morin et al., 1998). Little is known of the interactions between glucocorticoids and endothelin in the eye. The current study focuses on the interaction of endothelin with glucocorticoids in cultured human non-pigmented ciliary epithelial (HNPE) cells.

MATERIAL AND METHODS

Cell Culture

SV-40 transformed human ciliary epithelial cells (HNPE: passage 15-30) (a gift of Dr. Miguel Coca-Prados) (Martin-Vasallo et al., 1989) were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Gibco, Grand Island, NY) and 44 mM $NaHCO_3$. For treatments, HNPE cells were grown on either 100 mm culture dishes or 6-well culture plates (35 mm diameter/well). The culture media was then changed to serum-free (SF) DMEM and treated for 24 hours with the glucocorticoid agonist, dexamethasone (dissolved in ethanol) (Sigma Chemical, St Louis, MO), at a final concentration range of 1 to 100 nM or with an equivalent volume of vehicle (ethanol). RU486 (dissolved in ethanol) (BIOMOL Research Labs, Inc., Plymouth Meeting, PA), an antagonist of the glucocorticoid receptor, was used at concentration of 1 μM to block the

effect of dexamethasone in some of the experiments. The antagonist was added 30 minutes prior to the addition of dexamethasone.

Extraction and Measurement of ET-1 in the Culture Media

Endothelin-1 extraction and measurement were done according to the procedures described by Prasanna et al. (1998). HNPE cells were grown on 6-well cultured plates and treated with Dex (1, 10, 100 nM), RU486 (1 μM) + Dex (100 nM), RU486 (1 μM) or TNFα (10 nM) (Pepro Tech Inc. Rocky Hill, NJ) in 1 ml of SF-DMEM for 24 hours, and the culture media were collected after treatments. The medium samples were acidified with 1 ml of 1% trifluoroacetic acid (TFA: v/v in water) for 10 minutes, then applied to pre-treated Sep-Pak C_8 cartridges (125 Å; Waters Corp., Milford, MA). Elution was carried out by application of solvent A (40% methanol + 0.5% TFA; v/v) (2 × 1 ml) and solvent B (90% methanol + 0.5% TFA; v/v) (2 × 1 ml). The eluents (containing ET-1) in solvent B were then dried under a nitrogen stream. For measurement of endothelin-1 released in the culture media, a commercially available radioimmunoassay (RIA) kit for ET-1 was used (Peninsula Laboratories, Belmont, CA). Briefly, on the first day, the dried eluents were reconstituted with 200 μl of radioimmunoassay buffer, and 0.1 ml of rabbit anti-ET-1 was added to each 0.1 ml of reconstituted sample. Samples were stored overnight at 4°C. On the following day, the samples were treated with 0.1 ml of [^{125}I]ET-1 (10-15,000 cpm/tube) overnight at 4°C. On the third day, the samples were treated with 0.1 ml of goat anti-rabbit IgG serum and 0.1 ml of normal rabbit serum for 90 minutes, and then added to 0.5 ml of RIA buffer and centrifuged at 1700Xg for 20 minutes at 4°C.

The supernatant was aspirated and the remaining pellet was counted in a gamma counter. The sample ET-1 concentrations were obtained from a standard curve.

Membrane preparation and Western Blotting

The isolation of plasma membrane was performed as previously described (Dibas et al., 1996). HNPE cells grown to confluence on 100 mm dishes were washed and scraped in PBS buffer (0.2 g/l KCl, 8 g/l NaCl, 2.16 g/l $Na_2HPO_4 \cdot 7H_2O$). After a brief centrifugation, the cells were resuspended in homogenization buffer (32 mM Tris-HCl, 16 mM sodium pyrophosphate, 1.6 mM EDTA, 0.8 mM EGTA, 0.64 mM NH_4 molybdate, add fresh 3.2 mM DTT) containing protease inhibitors (20 μg/ml leupeptin, 20 μg/ml aprotinin, 20 μg/ml soybean trypsin inhibitor and 40 μg/ml PMSF). The samples were quickly sonicated and the homogenate was centrifuged briefly to sediment unbroken cells and nuclei. The supernatant was centrifuged at 100,000Xg for 35 minutes at 4°C. The resultant pellet was dissolved in homogenization buffer containing proteinase inhibitors, Triton-X 100 (0.1%) and NP-40 (0.1%) by gently aspirating several times and incubating on ice for 30 minutes. Protein concentration of the membranes was determined using the BCA reagent and bovine serum albumin as the protein standard (Sigma Chemical, St Louis, MO). Membrane protein (60 μg) from HNPE was mixed with SDS-sample buffer (4X: 62.5 mM Tris, pH6.8, 0.1% (v/v) glycerol, 2% SDS, 0.05% 2-β mercaptoethanol and 0.005% (w/v) bromophenol blue), and incubated at 100°C for 5 minutes. SDS-PAGE was performed on 7.5% gels according to the procedures described by Laemmli (1970) using a Bio-Rad electrophoresis unit. Protein was then transferred to nitrocellulose membranes (Schleicher & Schuell Inc. Keene, NH) according to methods

of Towbin et al. (1979) in transfer buffer (192 mM Glysine, 20% methanol and 25 mM Tris pH 8.3) at room temperature overnight using Bio-Rad electrophoretic transfer unit. The blots were blocked in 10% non-fat milk in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20) for 1 hour before probing with a 1:200 dilution of rabbit polyclonal ET_A or ET_B antibodies (Alomone Labs, Jerusalem, Israel) for 1 hour. Non-specific bands in the immunoblot for ET_A and ET_B receptors were identified by pre-incubating the anti-ET_A or anti-ET_B antibodies with their respective receptor peptides (Alomone Labs, Jerusalem, Israel) at a ratio of 1 μg peptide per 1 μg antibody at 37°C for 1 hr before probing blots. These receptor peptides are the fractions (about 1.8 kDa) of ET receptors and were used to produce these antibodies. Following this incubation period, the blots were washed 3X for 5 minutes each in10 ml of TBST, and probed with 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ) for 30 minutes. After 3 washes with TBST, the blots were developed using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric analysis of the bands was done using the Scion image analysis software (NIH). The quantification of band intensity is represented as a percentage to the value of its corresponding control band in the same membrane and the intensity for ET_A and ET_B are presented as a mean percentage in four separate experiments.

Membrane preparation and [^{125}I]ET-1 binding

The binding assay protocol was modified from Bolger et al. (1990) and the lab protocol of Dr. Masashi Yanagisawa (Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas TX). HNPE cells were grown in 150 mm

culture dishes and homogenized in homogenization buffer containing proteinase inhibitors and centrifuged at 100,000Xg for 35 minutes as described previously. The supernatants were discarded and the pellets were resuspended in binding buffer (25 mM Hepes pH 7.4, 135 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, and 1.1 mM $MgSO_4$) containing protease inhibitors (5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml soybean trypsin inhibitor and 10 μg/ml PMSF) by gently aspirating. The homogenates were centrifuged at 100,000Xg for another 35 minutes. The supernatants were discarded and the pellets homogenized in binding buffer containing 0.7 μg/ml aprotinin and 1.6 μg/ml PMSF using a polytron stirrer (Talboys Engineering Corp., Emerson, NJ). Protein concentration was determined using the BCA protein assay, and the membrane faction concentration diluted in binding buffer to 1 μg/μl. 50 μg membrane protein was used for each binding reaction. [^{125}I]ET-1 (NEN Life Science Product. Inc., Boston, MA) binding was performed in polypropylene tube in a total assay volume of 100 μl containing 25 μl of [^{125}I]ET-1 ranged from 0.025 to 2.4 nM, 50 μl of membrane faction, 25 μl of competition reagent or buffer at 37°C for 1 hour. Binding was terminated by adding 10 ml of cold wash buffer (10 mM Tris-HCl and 150 mM NaCl pH 7.4 at 4°C) and rapid vacuum filtration over glass fiber filters (No. 30, Schleicher & Schuell Keene, NH) pre-soaked with 1% w/v BSA. Filters were washed with 10 ml of wash buffer twice and the bound radioactivity was quantitated in a gamma counter. Non-specific binding was defined as filter-bound radioactivity measured in the presence of 1 μM unlabeled ET-1 (Sigma Chemical, St Louis, MO) before addition of [^{125}I] ET-1. The binding of ET_A receptor was determined by pre-treating with 0.5 μM sarafatoxin S6C (Peninsula Lab Inc.

Belmont, CA), an ET_B agonist, to block the binding of [^{125}I]ET-1 to ET_B receptors. ET_B receptor binding was defined as the total specific [^{125}I]ET-1 binding minus the amount of ET_A receptor binding. Estimates of the equilibrium dissociation constant (Kd) and maximun number of binding sites (Bmax) were obtained using unweighted linear regression analysis of data transformed by the method of Scatchard (1949).

RNA isolation and Quantitative Polymerase Chain Reaction

Total cellular RNA was isolated from HNPE cells using the Trizol B reagent (Life Technologies, Rockville, MD). cDNA was synthesized from 5 μg of total RNA from each sample using random primers and AMV Reverse Transcriptase (Promega, Madison, WI) in a reaction volume of 50 μl at 42°C for 30 min. QPCR primers for ppET-1, ET_A and ET_B (Table 2) were designed from their respective cDNA sequence using GeneJockey II program (BioSOFT, Ferguson, MO). These primers are designed spanning different exons. β-actin served as an internal control to account for variability in the initial concentration and quality of the total RNA and in the conversion efficiency of the reverse transcription reaction. QPCR amplification of each sequence was performed with 2.5 μl of cDNA sample in a total volume of 25 μl using SYBR Green PCR Core regents (PE Applied Biosystems, Foster City, CA). QPCR reactions were performed for 50 cycles of denaturation at 94°C for 30 sec, annealing at 60°C (for β-actin and ET_A) or 58°C (for ppET-1 and ET_B) for 30 sec and extension at 72°C for 30 sec in an Cepheid Smart Cycler (Cepheid, Sunnyvale, CA). Immediately after the real-time PCR run, the melting curves were generated to detect the melting temperatures of the specific products (data not shown). The QPCR product purity was also confirmed by analyzing the QPCR

products by 2% agarose gel electrophoresis. The authenticity of QPCR products were confirmed by DNA sequencing and a BLAST search of the sequence through NCBI (http://www.ncbi.nim.nih.gov) (data not shown). Quantification of relative RNA level was achieved by using the comparative C_T method (as described in PE Biosystems User Bulletin#2: http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf.) QPCR data is presented as the mean percentage to the value of its corresponding untreated control in three separate experiments.

RESULTS

Dexamethasone Effects on ET-1 Concentrations

The effect of Dex on ET-1 release into the culture medium from HNPE cells was determined using a radioimmunoassay for ET-1 following cells treated with or without Dex. We used the cytokine TNF-α as a positive control, as we previously demonstrated that TNF-α increases ET-1 release in these cells (Prasanna et al., 1998). TNF-α treatment significantly elevated ET-1 concentration in the media as compared to that observed in control HNPE cells. Interestingly, 24 hr Dex treatment also increased ET-1 releasing into the media compared to control (Fig. 1A) (control (11 ± 1.5 pg/ml media/mg protein) = 100 ± 14%; TNF-α = 361 ± 38%; Dex 1 nM = 129 ± 20%; Dex 10 nM = 294 ± 30%; Dex 100 nM = 316 ± 38%, Mean ± S.E. of 9 experiments). Moreover, the Dex response was blocked by pretreatment with RU486 (1 μM), a glucocorticoid receptor antagonist (Fig. 1B) (control = 100 ± 14%; Dex 100 nM = 316 ± 38%; RU486 + Dex 100 nM = 178 ±

33%; RU486 = 108 ±18%, Mean ± S.E. of 9 experiments), suggesting Dex-treated ET-1 release was through glucocorticoid receptor activation.

Effects of Dexamethasone on ET_A and ET_B Receptor Protein Expression

HNPE cells have been shown to be a source for ET-1 (Prasanna et al., 1998; Lepple-Weinhues et al., 1992), however ET receptor expression has been less examined. In order to investigate ET receptor expression under control and Dex treatment conditions, we carried out Western Blotting. Western blot analysis revealed that both ET_A and ET_B receptors are present in HNPE cells. Anti-ET_A recognized two bands with the sizes of approximate 60 kDa and 40 kDa (Fig. 2A), and ET_A receptor peptide was able to block the recognition of the 40 kDa band, but was unable to block recognition of the 60 kDa band (Fig. 2B). Anti-ET_B recognized two bands with one of 50 kDa and one at approximately 40 kDa (Fig. 2D), and the ET_B receptor peptide completely blocked the lower band, but was unable to block the top band (Fig. 2E). The top bands (60 kDa or 50 kDa) therefore were being recognized as nonspecific bands by anti-ET_A or anti-ET_B antibody. The ET_A and ET_B receptor sizes were approximately 40 kDa with ET_B slightly smaller than ET_A. These molecular weights are similar to the sizes described by the manufacturer of the anti-ET_A and anti-ET_B antibodies (Alomone Labs).

Treatment with Dex for 24 hours produced a concentration-dependent decrease in the protein levels of the ET_A receptors (Fig. 2C, lanes 2,3,4), but significantly increased the protein levels of the ET_B receptors at 10 nM Dex (Fig. 2F, lane 3). RU486 treatment did not block Dex -induced alteration of protein expression of the ET_A (Fig. 2C, lane 5), but completely blocked Dex action on ET_B receptor expression (Fig. 2F, lane 5). It was

interesting that RU486 alone decreased ET_A receptor expression at high concentration RU486 100 nM and 1 μM (Fig. 2H, lanes 4,5).

Endothelin Receptor Binding Characteristics in HNPE Cells Treated with Dex

In order to verify and better quantify the ET receptor density changes, [^{125}I]ET-1 binding to its receptors was performed in HNPE cells. Competition [^{125}I]ET-1 binding experiments found that 1μM ET-1 was able to block all of the [^{125}I]ET-1 binding to its ET receptors (data was not shown). Therefore we used 1 μM ET-1 to determine non-specific binding. Since Western blot analysis found 10 nM Dex appeared to consistently influence both ET_A and ET_B receptor expression, we used the 10 nM Dex dose to investigate Dex's effect on ET receptor density in these binding experiments. Under control conditions, HNPE cells appear to have a relatively low ET binding activity (total ET receptor 41 ± 0.81, ET_A 17 ± 0.5, ET_B 25 ± 0.36 fmol/mg membrane protein, mean ± SE of 4 experiments). However, the total ET receptor density increased following 10 nM Dex treatment with the largest component of the increase attributed to the increases in ET_B receptor density (total ET receptor 126 ± 17, ET_A 11 ± 1.9, ET_B 116 ± 16 fmol/mg membrane protein, mean ± SE of 4 experiments) (Fig. 3). Table 1 summarizes the effect of 10 nM Dex on ET binding. Dex treatment did not significantly change the affinity of ET-1 for the ET_A receptor (control 0.63 ± 0.05 versus Dex 0.49 ± 0.1 nM, mean ± SE of 4 experiments), but significantly modified the affinity of ET-1 binding to the total ET receptor population (control 1.14 ± 0.08 versus Dex 4.25 ± 0.16 nM, mean ± SE of 4 experiments), which could reflect the predominant changes that occurred in the ET_B receptor.

Dexamethasone Effects on mRNA Expression of prepro-ET-1, ET_A and ET_B receptors in HNPE Cell

Since there were protein expression changes in ET-1, ET_A and ET_B receptors following Dex treatment, we next determined if Dex treatment also regulated the mRNA expression of ET-1 and its receptors. We carried out Quantitative PCR (QPCR) analysis for ppET-1, ET_A and ET_B receptors. Dex treatment for 24 hours significantly increased the mRNA levels of ppET-1 at 10 nM and 100 nM Dex (Fig 4A, lanes 3,4), but decreased the mRNA amount of the ET_A receptor in a concentration-dependent manner (Fig 4B, lanes 2,3,4), while significantly increasing the mRNA levels of the ET_B receptor with 10 nM Dex having the greatest effect (Fig 4C, lanes 3, 4). RU486 at 1μM completely blocked the Dex effects on the ppET-1 and ET_B receptor (Fig 4A and C, lane 5), but not on the ET_A receptor (Fig 4B, lane 5). RU486 alone also decreased the mRNA levels of the ET_A receptor (Fig 4B, lane 6).

DISCUSSION

Treatment of HNPE cells with dexamethasone (Dex), a potent synthetic glucocorticoid, caused a concentration-dependent increase in ET-1 mRNA and peptide levels. A maximal three fold stimulation of ET-1 release in the medium was observed at a dose of 100 nM Dex for 24 hrs, similar in magnitude to the induction seen with TNFα (10 nM) reported by our laboratory (Prasanna et al., 1998). RU486, an antagonist of glucocorticoids, has been shown to block glucocorticoid regulation of pre-pro-ET-1 mRNA and ET-1 release (Calderon et al., 1994; Morin et al., 1998). In the current study,

the stimulationary action of Dex on HNPE cell was blocked by the co-incubation of RU486 (1 μM), showing an involvement of glucocorticoid receptors in this response of ET-1.

How glucocorticoids regulate ET-1 expression is not clear. The actions of glucocorticoids can be mediated by the activated glucocorticoid receptor binding to glucocorticoid response elements in the targeted genes or binding other transcription factors and thereby regulating their function (Jonat et al., 1990; Samples et al., 1993; Ray and Prefontaine, 1994). Alternatively, glucocorticoid responses may involve glucocorticoid receptor activation of a primary response gene whose gene product in turn secondarily activates other genes (Gotoh et al., 1997; Shepard et al., 2001). The basal transcription level of the ET-1 encoding gene depends on two critical regions in its promoter: a GATA motif and an AP-1 consensus (Lee et al., 1990). There has been no report of a glucocorticoid response element for the ET-1 promoter. Interestingly, the GATA family members have a highly homologous DNA binding domain that contains two zinc fingers reminiscent of those of the glucocorticoid receptor (Omichinski, et al., 1993; Raich and Romeo, 1993). It has also been reported that glucocorticoid receptors bind a member of GATA family and interfere with its function before any interaction with DNA (Chang et al., 1993). Glucocorticoid receptors may indirectly regulate ET-1 gene expression by interacting with GATA family members. Recently some other transcription factors such as Vezf1/DB1, hypoxia-induced factor (HIF-1) were also demonstrated to be able to activate the transcription of ET-1 (Aitsebaomo et al., 2001; Kakinuma et al., 2001; Yamashita et al., 2001). The induction of (HIF)-1 alpha in heart

failure as an adaptation against impaired energy metabolism alternatively causes an elevation of cardiac ET-1 gene expression (Kakinuma et al, 2001). Whether there is a novel molecular mechanism of up-regulation of ET-1 as an adaptation in response to Dex treatment in HNPE cells is unknown. Glucocorticoids have been reported to increase ET-1 gene expression in vascular smooth muscle cells (Morin et al., 1998) and in bronchial epithelial cells (Aubert et al., 1997), and also stimulate ET-1 peptide secretion (Kato et al., 1995; Roubert et al., 1993; Kanse et al., 1991). In the present study, we demonstrated that Dex treatment for 24 hrs increased both ET-1 mRNA and peptide levels. Further experiments are needed to delineate the pathways that may be involved in the response to Dex treatment in HNPE cells.

Both quantitative PCR and Western blot analysis indicated that Dex concentration-dependently decreased the ET_A receptor but increased the ET_B receptor with 10 nM Dex having the greatest effect. RU486 has been shown to reverse the effect of glucocorticoids on ET receptor numbers in vascular smooth muscle cells (Provencher et al., 1995). In this study, RU486 blocked Dex's effects on the ET_B but not ET_A receptor expression. It also appears that RU486 may function as a partial agonist, as it had its own action on ET_A receptor expression. In fact, RU486, a classical antagonist for both progesterone and glucocorticoid receptors, interferes with steroid-mediated activation, but often confers an agonist activity to steroid receptors (Beck et al., 1993; Sartorius et al., 1994; Liu et al., 2002). Recently, it has been reported that RU486 can exhibit partial agonist activity and the N terminus of glucocorticoid receptor is a critical determinant for RU486-mediated agonist activity (Schulz et al., 2002). Shepard et al. (2001) also reported that RU486

functions as a partial agonist at myocilin/*TIGR* expression in trabecular meshwork cells. Such observations are important in evaluating the responses seen with glucocorticoids in the presence of RU486, and could account for the inability of RU486 to block Dex-mediated decrease in ET_A receptors.

ET-1 has been known to regulate its biological effects using two subtype receptors, the ET_A and ET_B receptors (Pang and Yorio, 1997; Yorio et at., 2002). Both subtypes are present in the ciliary body (Ripodas et al., 1998). In this study, we observed the expression of both mRNAs and proteins for ET_A and ET_B receptors in isolated HNPE cells. The ET_A and ET_B receptor proteins had molecular weights of appoximately 40 kDa, although ET_B receptors were slightly smaller than ET_A receptors. Using an autoradiography technique, Ripodas (1998) reported that ciliary body ET_A and ET_B receptors had molecular weights of 52 kDa and 34 kDa and suggested that the 34 kDa band is a proteolytic degradation product of the 52 kDa band. We also identified immunoreactive bands at about 60 kDa or 50 kDa using anti-ET_A or ET_B receptor antibodies respectively. However, labeling of these higher molecular weight bands was not blocked by the addition of competing soluble ET_A and ET_B receptor peptides, demonstrating the upper bands in the immunoblots were non-specific antibody binding. There have been numerous reports of different molecular weights for ET_A and ET_B receptors that range between 50 kDa and 70 kDa (Kondoh et al., 1990; Shannon and Hale, 1994; Freedman et al., 1997; Kurokawa et al., 1997), but other investigators have shown ET receptors with similar apparent molecular weights to what we have found (between 30 and 40 kDa) (Masuda et al., 1989; Bednar et al., 1991; Takasuka et al.,

1994). The apparent differences in reported molecular weights could be attributed to the source of antibody, species or tissue specificity, post-translation modification, result of difference *in vivo* proteolytic processing, or ultimately the result of the inaccuracy inherent in the estimation of the molecular weight of membrane proteins by SDS-PAGE.

[^{125}I]ET-1 binding experiments supported Western Blot analysis data in that Dex treatment regulated ET_A and ET_B receptor expression. HNPE cells had a much lower density of ET receptors, but had a similar binding affinity for ET-1 as compared to that found in other tissues (Bolger et al., 1990). Dex treatment did not change the receptor affinity but dramatically increased ET_B receptor density while decreasing ET_A receptor density. Sarafatoxin S6C, a specific agonist for the ET_B receptor, was able to compete for [^{125}I]ET-1 binding to the ET_B receptor. The remaining [^{125}I]ET-1 binding sites appeared to be ET_A receptors. Although there have been some reports about another endothelin receptor subtype, ET_C receptors (Karne et al., 1993), the ET_C receptor appears to be specific for ET-3 and is only been reported in amphibians (Karne et al., 1993). The high affinity binding of [^{125}I]ET-1 seen in this study strongly suggests that ET_A and ET_B receptors are responsible for this binding. The observations that ET_A receptors were down-regulated, but ET_B receptors were up-regulated by Dex demonstrate that additional mechanisms for ET receptor regulation exist besides agonist-induced homologous receptor down-regulation. Indeed glucocorticoids and chronic heart failure have been reported to induce the regulation of ET receptors in addition to a regulation of ET-1 (Zolk et al., 1999; Borcsok et al., 1998). Although the magnitudes were different, both Western Blotting and ligand binding experiments showed a similar trend in Dex's regulation of

ET_A and ET_B receptors. Those differences in the magnitude may be attributed to different sensitivities of both techniques and the extent of the extraction of membrane proteins for Western Blotting.

Changes in ET receptor expression and concomitant increases in ET-1 release by Dex could contribute to the Dex response in ocular tissues. In normal situations, increases in ET-1 in the anterior segment may have beneficial effects by contracting ciliary smooth muscle (Wiederholt, 1998) and increasing aqueous humor outflow (Erickson-Lamy et al., 1991) while inhibiting Na^+, K^+-ATPase in non-pigmented ciliary epithelial cells through ET_B receptors and decreasing aqueous humor formation (Prasanna et al., 2001). Such effects could contribute to the functional response in the decrease in IOP after intravitreal administration of ET (Sugiyama et al., 1995; Granstam et at., 1991). However in the presence of dexamethasone, there are changes in the morphology and activity of trabecular meshwork with concomitant increases in the deposition of extracellular matrix materials (Wordinger and Clark, 1999). The increase in synthesis and release of ET-1 from non-pigmented ciliary epithelial cells and contraction of ciliary smooth muscle by ET-1 in attempt to reduce IOP could also contract the trabecular meshwork and reduce the intertrabecular space and consequently increase outflow resistance and IOP as a maladaptation. Although ET_B receptors could play a role in clearance of ET-1 (Paasche et al., 2001; Bremnes et al., 2000), ET-1 has been shown to increase inducible NO synthase 2 and NO concentration in ocular tissues (Prasanna et al., 2000), which can have a further damaging effect on the eye (Neufeld, 1999). Such changes in ET receptor densities and

the increase in ET release could thus play a role in Dex-induced ocular hypertention and glaucoma, however this awaits further experimentation.

In conclusion, we have described a novel signaling pathway for dexamethasone in HNPE cells that regulates the release of ET-1 and the expression of ET receptors. Such actions could have consequences on aqueous humor inflow/outflow and intraocular pressure that are associated with endothelin and/or glucocorticoids.

ACKNOWLEDGEMENTS

This work was supported by a grant from NEI/NIH (Grant number: EY11979) and the Texas Higher Education Coordinating Board Advanced Technology Program. The authors would like to thank Dr. Michael Martin and Dr. Jerry Simecka for their expert technical assistance with the binding studies and quantitative PCR respectively.

REFERENCES

Aitsebaomo, J., Kingsley-Kallesen, M.L., Wu, Y., Quertermous, T. and Patterson, C. (2001). Vezf1/DB1 is an endothelial cell –specific transcription factor that regulated expression of endothelin-1 promoter. J. Biol. Chem. 276, 39197-205.

Aubert, J.D., Juillerat-Jeanneret, L. and Leuenberger, P. (1997). Expression of endothelin-1 in human broncho-epithelial and monocytic cell lines: influence of tumor necrosis factor-alpha and dexamethasone. Biochem. Pharmacol. 53, 547-52.

Beck, C.A., Weigel, N.L., Moyer, M.L., Nordeen, S.K. and Edwards, D.P. (1993). The progesterone antagonist RU486 acquires agonist activity upon stimulation of cAMP signaling pathways. Proc. Natl. Acad. Sci. USA. 90, 4441-5.

Becker, B. (1965). Intraocular pressure responses to topical corticosteroids. Invest Ophthalmol. 4, 198-205.

Bednar, D.L., Stein, R.B., Garsky, V.M. and Williams, D.L. (1991). The bovine endothelin receptor has an apparent molecular weight of 43,000. Biochemica et Biophysica Acta 1092, 226-32.

Bolger, G.T., Liard, F., Krogsrud, R., Thibeault, D. and Jaramillo, J. (1990). Tissue specificity of endothelin binding sites. J. Cardiovasc. Pharmacol. 16, 367-75.

Borcsok, I., Schairer, H.U., Sommer, U., Wakley, G.K., Schneider, U., Geiger, F., Niethard, F.U., Ziegler, R. and Kasperk, C.H. (1998). Glucocorticoids regulate the expression of the human osteoblastic endothelin A receptor gene. J. exp. Med. 188, 1563-73.

Bremnes, T., Paasche, J.D., Mehlum, A., Sandberg, C., Bremnes, B. and Attramadal, H.

(2000). Regulation and intracellular trafficking pathways of the endothelin receptors. J. Biol. Chem. 275, 17596-604.

Calderon, E., Gomez-Sanchez, C.E., Cozza, E.N., Zhou, M., Coffey, R.G., Lockey, R.F., Prockop, L.D. and Szentivanyi, A. (1994). Modulation of endothelin-1 production by a pulmonary epithelial cell line. I. Regulation by glucocorticoids. Biochem. Pharm. 48, 2065-71.

Chang, T.J., Scher, B.M., Waxman, S. and Scher W. (1993). Inhibition of mouse GATA-1 function by the glucocorticoid receptor: possible mechanism of steroid inhibition of erythroleukemia cell differentiation. Mol. Endocrinol. 7(4): 528-42.

Cioffi, G.A. and Sullivan, P. (1999). The effect of chronic ischemia on the primate optic nerve. Eur. J. Pharm. 9 Suppl 1, S34-6.

Clark, A.F., Aboul-Ela, N. and English-Wright, S. (1997). Microfilament structure and microfilament protein expression in cultured dexamethasone-treated and glaucomatous human trabecular meshwork (TM) cells. Invest. Ophthalmol. Vis. Sci. 38, S489.

Clark, A.F., Steely, H.T., Dickerson, J.E., Jr. English-Wright, S., Stropki, K., McCartney, M.D., Jacobson, N., Shepard, A.R., Clark, J.I., Matsushima, H., Peskind, E.R., Leverenz, J.B., Wilkinson, C.W., Swiderski, R.E., Fingert, J.H., Sheffield, V.C. and Stone, E.M. (2001). Glucocorticoid induction of the glaucoma gene MYOC in human and monkey trabecular meshwork cells and tissues. Invest. Ophthalmol. Vis. Sci. 42, 1769-80.

Dibas, A., Mia, A.J. and Yorio, T. (1996). Is protein kinase C alpha (PKCα) involved in vasopressin-induced effects on LLC-PK_1 pig kidney cells? Biochem. Mol. Biol. Int. 39,

581-8.

Erickson-Lamy, K., Korbmacher, C., Schuman, J.S. and Nathanson, J.A. (1991). Effect of endothelin on outflow facility and accommodation in the monkey eye in vivo. Invest. Ophthalmol. Vis. Sci. 32, 492-5.

Evans, R.M. (1988). The steroid and thyroid hormone receptor superfamily. Science 240, 889-95.

Freedman, N.J., Ament, A.S., Oppermann, M., Stoffel, R.H., Exum, S.T. and Lefkowitz, R.J. (1997). Phosphorylation and desensitization of human endothelin A and B receptors: evidence for G protein-coupled receptor kinase specificity. J Biol. chem. 272,17734-17743.

Granstam, E., Wang, L. and Bill, A. (1991). Effects of endothelins (ET-1, ET-2, and ET-3) in the rabbit eye: role of prostaglandins. Eur. J. Pharmacol. 194, 217-23.

Gotoh, T., Chowdhury, S., Takiguchi, M. and Morin, M. (1997). The glucocorticoid-responsive gene cascade: activation of the rat arginase gene through induction of C/EBPbeta. J. Biol. Chem. 272, 3694-8.

Johnson, D.H., Bradley, J.M.B. and Acott, T.S. (1990). The effect of dexamethasone on glycosaminoglycans of human trabecular meshwork in perfusion organ cultured. Invest. Ophthalmol. Vis. Sci. 31, 2568-71.

Johnson, D.H., Gottanka, J., Flugel, C., Hoffman, F., Futa, R. and Lutjen-Drecoll, E. (1997). Ultrastructural changes in the trabecular meshwork of human eyes treated with glucocorticoids. Arch. Ophthalmol. 115, 375-83.

Jonat, C., Rahmsdorf, H.J., Park, K.K., Cato, A.C., Gebel, S., Ponta, H. and Herrlich, P.

(1990). Antitumor promotion and antiinflammation: down-regulation of AP-1 (fos/Jun) activity by glucocorticoid hormone. Cell 62, 1189-1204.

Kakinuma, Y., Miyauchi, T., Yuki, K., Murakoshi, N., Goto, K. and Yamaguchi, I. (2001). Novel molecular mechanism of increased myocardial endothelin-1 expression in the failing heart involving the transcriptional factor hypoxia-inducible factor-1 alpha induced for impaired myocardial energy metabolism. Circulation 103, 2387-94.

Kallberg, M.E., Brooks, D.E., Garcia-Sanchez, G.A., Komaromy, A.M., Szabo, N.J. and Tian, L. (2002). Endothelin1 levels in the aqueous humor of dogs with glaucoma. J Glaucoma 11, 105-9.

Kanse, S.M., Takahashi, K., Warren, J.B., Ghatei, M. and Bloom, S.R. (1991). Glucocorticoids induce endothelin release from vascular smooth muscle cells but not endothelial cells. Eur. J. Pharm. 199, 99-101.

Karne, S., Jayawickreme, C.K. and Lerner, M.R. (1993). Cloning and characterization of an endothelin-3 specific receptor (ET_C receptor) from *Xenopus laevis* dermal melanophores. J. Biol. Chem. 268,19126-33.

Kato, H., Hayashi, T., Koshino, Y., Oida, K., Kutsumi, Y., Nakai, T. and Miyabo, S. (1995). Autocrine production of endothelin-1 participates in the glucocorticoid-induced Ca2+ influx into vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 208, 82-8.

Kondoh, M., Miyazaki, H., Watanabe, H., Shibata, T., Yanagisawa, M., Masaki, T. and Murakami, K. (1990). Isolation of anti-endothelin receptor monoclonal antibodies for use in receptor characterization. Biochem. Biophys. Res. Commun. 172, 503-10.

Kurokawa, K., Yamada, H. and Ochi, J. (1997). Topographical distribution of neurons containing endothelin type A receptor in the rat brain. J. Comparative Neurology. 389, 348-60.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-5.

Lee, M.E., Bloch, K.D., Clifford, J.A. and Quertermous, T. (1990). Functional analysis of the endothelin-1 gene promoter. Evidence for an endothelial cell-specific cis-acting sequence. J. Biol. Chem. 265, 10446-50.

Lepple-Weinheus, A., Becker, M., Stahl, F., Berweck, S. and Hensen, J. (1992). Endothelin-like immunoreactivity in the aqueous humor and in conditioned medium from ciliary epithelial cells. Curr. Eyes Res. 10, 983-9.

Lewis, J.M., Priddy, T., Judd, J., Gordon, M.O., Kass, M.A., Kolker, A.E. and Becker, B. (1988). Intraocular pressure response to topical dexamethasone as a predictor for the development of primary open-angle glaucoma. Am. J. Ophthalmol. 106(5), 607-12.

Liu, Z., Auboeuf, D., Wong, J., Chen, J.D., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (2002). Coactivator/corepressor ratios modulate PR-mediated transcription by the selective receptor modulator RU486. Proc. Natl. Acad. Sci. USA 99, 7940-4.

Martin-Vasallo, P., Gnosh, S. and Coca-Prados, M. (1989). Expression of Na, K-ATPase alpha subunit isoforms in the human ciliary body and cultured ciliary epithelial cells. J. Cellular Physiol. 141: 243-252.

Masuda, Y., Miyazaki, H., Kondoh, M., Watanabe, H., Yanagisawa, M., Masaki, T. and Murakami, K. (1989). Two different forms of endothelin receptors in rat lung. FEBS

Letters. 257, 208-10.

McCartney, M.D., Wilson, K., Miggans, S.T. and Clark A.F. (1994). Effect of dexamethasone and tetrahydocortisol on cultured human trabecular meshwork cell microfilament and microtubule structure. Invest. Ophthalmol. Vis. Sci. 35, S2735.

McCarty, G.R. and Schwartz, B. (1982). Increased concentration of glucocorticoid receptors in rabbit iris-ciliary body compared to rabbit liver. Invest. Ophthalmol. Vis. Sci. 23,525-8.

McCarty, G.R. and Schwartz, B. (1991). Increased plasma noncortisol glucocorticoid acitvity in open-angle glaucoma. Invest. Ophthalmol. Vis. Sci. 32, 1600-8.

Morin, C., Asselin, C., Boudreau, F. and Provencher, P. H. (1998). Transcriptional regulation of pre-pro- endothelin-1 gene by glucocorticoids in vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 244, 583-7.

Neufeld, A.H. (1999). Nitric Oxide: A potential mediator of retinal ganglion cell damage in glaucoma. Surv. Ophthalmol. 43, S129-35.

Noske, W., Hensen, J. and Wiederholt, M. (1997). Endothelin-like immunoreactivity in aqueous humor of primary open-angle glaucoma and cataract patients. Grafes Arch. Clin. Exp. Ophthalmol. 235, 551-2.

Omichinski, J.G., Clore, G.M., Schaad, O., Felsenfeld, G., Trainor, C., Appella, E., Stahl, S.J. and Gronenborn, A.M. (1993). NMR structure of a specific DNA complex of Zn-containing DNA binding domain of GATA-1. Science. 261, 438-46.

Orgul, S., Cioffi, G.A., Wilson, D.J., Bacon, D.R. and Van Buskirk E.M. (1996). An endothelin-1 induced model of optic nerve ischemia in the rabbit. Invest. Ophthalmol.

Vis. Sci. 37, 1860-9.

Paasche, J.D., Attramadal, T., Sandberg, C., Johansen, H.K. and Attramadal, H. (2001). Mechanisms of endothelin receptor subtype-specific targeting to district intracellular trafficking pathways. J. Biol. Chem. 276, 34041-50.

Pang, I.H. and Yorio, T. (1997). Ocular actions of endothelins. Proc. Soc. Exp. Biol. Med. 215, 21-34.

Polansky, J.R., Fauss, D.J., Chen, P., Chen, H., Lutjen-Drecoll, E., Johnson, D., Kurtz, R.M., Ma, Z.D., Bloom, E. and Nguyen, T.D. (1997). Cellular pharmacology and molecular biology of the trabecular meshwork inducible glucocorticoid response gene product. Ophthalmologica 211, 126-39.

Prasanna, G., Dibas, A., Hulet, C. and Yorio, T. (2001). Inhibition of Na(+)/K(+)-ATPase by endothelin-1 in human nonpigmented ciliary epithelial cells. J. Pharmacol. Exp. Ther. 296, 966-71.

Prasanna, G., Krishnamoorthy, R., Hulet, C., Zhang, H., Zhang, X. and Yorio. T. (2000). Endothelin-1 induces nitric oxide synthase-2 expression in human non-pigmented ciliary epithelial cells. Exp. Eye. Res. 71, 535-9.

Prasanna, G., Dibas, A., Tao, W., White, K. and Yorio, T. (1998). Regulation of endothelin-1 in human non-pigmented ciliary epithelial cells by tumor necrosis factor-α. Exp. Eye Res. 66, 9-18.

Provencher, P.H., Saltis, J. and Funder, J.W. (1995). Glucocorticoids but not mineralocorticoids modulate endothelin-1 and angiotensin II binding in SHR vascular smooth muscle cells. J. Steroid Biochem. & Mol. Biol. 52, 219- 25.

Raich, N. and Romeo, P.H. (1993). Erythroid regulatory elements. Stem Cells 11, 95-104.

Ray, A. and Prefontaine, K.E. (1994). Physical association and functional antagonism between the p65 subunit or transcription factor NF-κB and the glucocorticoid receptor. Proc. Natl. Acad. Sci. 91, 752-6.

Ray, S., Mehra, K.S., Misra, S. and Singh, R. (1977). Plasma cortisol in glaucoma. Ann. Ophthalmol. 9: 1151-4.

Ripodas, A., De Juan, J.A., Moya, F.J., Fernandez-Cruz, A. and Fernandez-Durango, R. (1998). Identification of endothelin receptor subtypes in rat ciliary body using subtype-selective ligands. Exp. Eye Res. 66, 69-79.

Roubert, P., Viossat, I., Lonchampt, M.O., Chapelat, M., Schulz, J., Plas, P., Gillard-Roubert, V., Chabrier, P.E. and Braquet, P. (1993). Endothelin receptor regulation by Endothelin synthesis in vascular smooth muscle cells: effects of dexamethasone and phosphoramidon. J. Vasc. Res. 30, 139-44.

Rozsival, P., Hampl, R., Obenberger, J., Starka, L. and Rehak, S. (1981). Aqueous humor and plasma cortisol levels in glaucoma and cataract patients. Curr. Eye Res. 1, 391-6.

Rubanyi, G.M. and Polokoff, M.A. (1994). Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. Pharmacol. Rev. 46, 325-415.

Samples, J.R., Alexander, J.P. and Acott, T.S. (1993). Regulation of the levels of human trabecular meshwork matrix metalloproteinases and inhibitor by interleukin-1 and dexamethasone. Invest. Ophthalmol. Vis. Sci. 34, 3386-95.

Sartorius, C.A., Groshong, S.D., Miller, L.A., Powell, R.L., Tung, L., Takimoto, G.S. and Horwitz, K.B. (1994). New T47D breast cancer cell lines for the independent study of progesterone B- and A-receptors: only antiprogestin-occupied B-receptors are switched to transcriptional agonists by cAMP. Cancer Res. 54, 3868-77.

Scatchard G. (1949). The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. 51, 660-672.

Schulz, M., Eggert, M., Baniahmad, A., Dostert, A., Heinzel, T. and Renkawitz, R. (2002). RU486-induced glucocorticoid receptor agonism is controlled by the receptor N terminus and by corepressor binding. J. Biol. Chem. 277, 26238-43.

Schwartz, B., McCarty, G. and Rosner, B. (1987). Increased plasma free cortisol in ocular hypertension and open angle glaucoma. Arch. Ophthalmol. 105, 1060-5.

Shannon, T.R. and Hale, C.C. (1994). Identification of a 65 kDa endothelin receptor in bovine cardiac sarcolemmal vesicles. Eur. J. Pharm. 267, 233-8.

Shepard, A.R., Jacobson, N., Fingert, J.H., Stone, E.M., Sheffield, V.C. and Clark, A.F. (2001). Delayed secondary glucocorticoid responsiveness of MYOC in human trabecular meshwork cells. Invest Ophthalmol. Vis. Sci. 42, 3173-81.

Snyder, R.W., Stamer, W.D., Kramer, T.R. and Seftor, R.E.B. (1993). Corticosteroid treatment and trabecular meshwork proteases in cell and organ culture supernatants. Exp. Eye Res. 57, 461-8.

Steely, H.T., Browder, S.L., Julian, M.B., Miggans, S.T., Wilson, K.L. and Clark, A. F. (1992). The effects of dexamethsone on fibronection expression in cultured human trabecular meshwork cell. Invest. Ophthalmol. Vis. Sci. 33,2242-50.

Strokes, J., Noble, J., Brett, L., Phillips, C., Seckl, J.R., O'Brien, C. and Andrew, R. (2000). Distribution of glucocorticoid and mineralocorticoid receptors and 11ß-hydroxysteroid dehydrogenases in human and rat ocular tissues. Invest Ophthalmol. Vis. Sci. 41, 1629-38.

Sugiyama, K., Haque, M.S., Okada, K., Taniguchi, T. and Kitazawa, Y. (1995). Intraocular pressure response to intravitreal injection of endothelin-1 and the mediatory role of ET_A receptor, ET_B receptor, and cyclooxygenase products in rabbits. Curr. Eye Res. 14, 479-86.

Takasuka, T., sakurai, T., Goto, K., Furuichi, Y. and Watanabe, T. (1994). Human endothelin receptor ETB. J. Biol. Chem. 269,7509-13.

Taniguchi, T., Haque, M.S.R., Sugiyama, K., Okada, K., Nakai, Y. and Kitanawa Y. (1996). Effects of endothelin A and B receptors on aqueous humor dynamics in the rabbit eye. J. Ocul. Pharmacol Ther. 12, 123-30.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350-4.

Underwood, J.L., Murphy C.G., Chen, J., Franse-Carman, L., Wood, I., Epstein, D.L.and Alvarado, J.A. (1999). Glucocorticoids regulate transendothelial fluid flow resistance and formation of intercellular junctions. Am. J. Physiol. 277, C330-42.

Wiederholt M. (1998). Nitric Oxide and endothelin in aqueous humor outflow regulation. In: Haefliger I. Flammer J. ets. Nitric Oxide and Endothelin in the pathogenesis of Glaucoma Philadelphia: Lippincott-Raven Publishers, 168-77.

Wilson, K., McCartney, M.D., Miggans, S.T., and Clark, A.F. (1993). Dexamethasone induced ultrastructural changes in cultured human trabecular meshwork cells. Curr. Eye Res. 12, 783-93.

Wordinger, R.J. and Clark, A.F. (1999). Effects of glucocorticoid on the trabecular meshwork: towards a better understanding of glaucoma. Retinal and Eye Res. 18, 629-67.

Yamashita, K., Discher, D.J., Hu, J., Bishopric, N.H. and Webster, K.A. (2001). Molecular regulation of the endothelin-1 gene by hypoxia. Contributions of hypoxia-inducible factor-1, activator protein-1, GATA-2, AND p300/CBP. J. Biol. Chem. 276: 12645-53.

Yorio, T., Krishnamoorthy, R., and Prasanna, G. (2002). Endothelin: Is it a contributor to glaucoma pathophysiology? J. Glaucoma 11, 259-70.

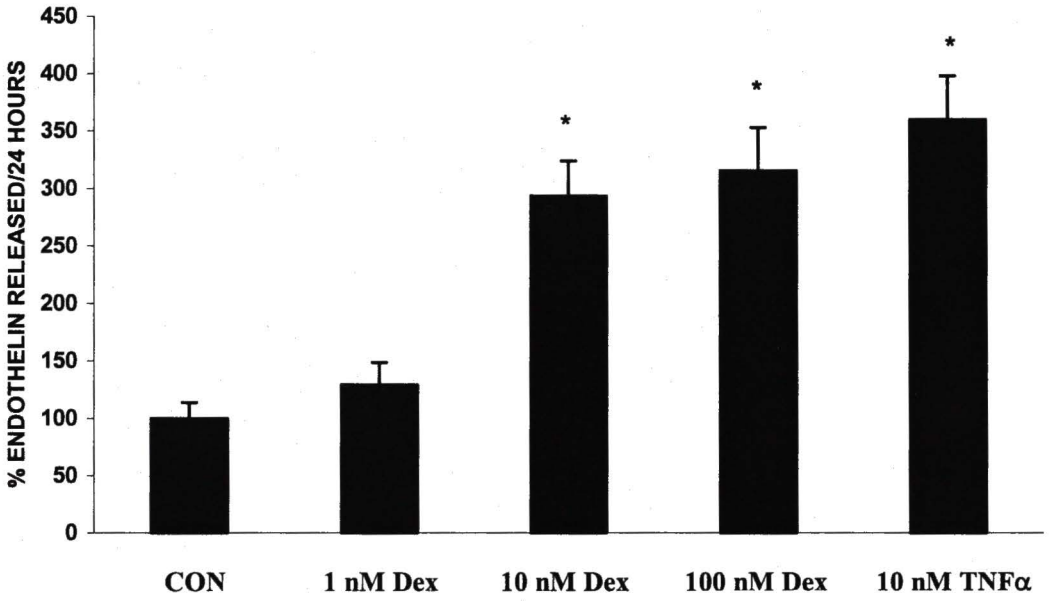
Zolk, O., Quattek, J., Sitzler, G., Schrader, T., Nickenig, G., Schnabel, P., Shimada, K., Takahashi, M. and Bohm, M. (1999). Expression of endothlin-1, endothelin-converting enzyme, and endothelin receptors in chronic heart failure. Circulation 99, 2118-23.

FIGURE 1. Effects of dexamethasone (Dex) and RU486, a glucocorticoid receptor antagonist, on endothelin-1 (ET-1) release from HNPE cells. ET-1 released into the culture media from HNPE cells grown in 6-well plates was measured using radioimmunoassay (RIA). **A)** HNPE cells were treated with no Dex (control), 1 nM, 10 nM, 100 nM Dex for 24 hrs. Tumor necrosis factor-α (TNF-α; 10 nM) was used as a positive control. **B)** HNPE cells were treated 24 hrs with no Dex (control), 100nM Dex, 1 μM RU486 + 100 nM Dex, or 1 μM RU486. HNPE cells were pre-treated with 1 μM RU486 for 30 minutes before the addition of 100 nM Dex. ET-1 released in control cells corresponded to 11.2 ± 1.5 pg / ml media / mg protein and was taken as 100 % (n = 9).

* Denotes statistical significance of mean % ET-1 released from Dex treatments versus that of control and ** denotes statistical significance of mean % ET-1 released from RU486 + Dex treatment versus that of Dex as determined by One-way ANOVA and Student-Newman-Keuls multiple comparison test at $p < 0.05$.

FIGURE 1

A


% ENDOTHELIN RELEASED/24 HOURS
450
400
350
300
250
200
150
100
50
0
*
*
*
CON
1 nM Dex
10 nM Dex
100 nM Dex
10 nM TNFα

B

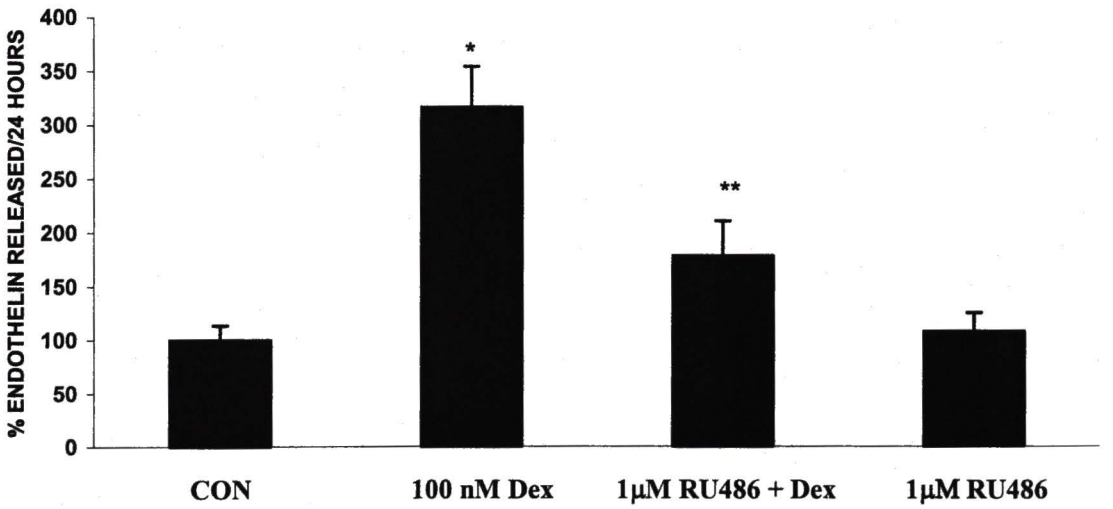

% ENDOTHELIN RELEASED/24 HOURS
400
350
300
250
200
150
100
50
0
*
**
CON
100 nM Dex
1μM RU486 + Dex
1μM RU486

FIGURE 2. Effects of dexamethasone (Dex) and RU486 on the protein expression of ET_A and ET_B receptors in HNPE cells as determined by Western Blotting. Panels A-F, HNPE cells were treated for 24 hours with: control (lane 1); 1 nM Dex (lane 2); 10 nM Dex (lane 3); 100 nM Dex (lane 4); 1 μM RU486 + either 100 nM Dex (Fig. 3A and 3B lane 5) or 10 nM Dex (Fig. 3D and 3E lane 5); 1 μM RU486 (lane 6); Panels G-H, HNPE cells were treated for 24 hours with: control (lane 1); RU486 1 nM (lane 2); 10 nM (lane 3), 100 nM (lane 4) and 1 μM (lane 5). Western blot analysis of ET_A receptor without (Fig. 3A), and with pre-incubation with ET_A receptor blocking peptide (Fig. 3B). Western blot analysis for ET_B receptor without (Fig. 3D), and with pre-incubation with ET_B receptor blocking peptide (Fig. 3E). Western Blotting for ET_A without pre-incubation with ET_A receptor blocking peptide (Fig. 3G). Both ET_A and ET_B can be detected as protein bands approximately 40 kDa. Densitometric analysis of the bands was done using the Scion image analysis software (NIH). The quantification of band intensity is represented as a percentage to the value of its corresponding control band in the same membrane and the intensity for ET_A (Fig. 3C), ET_B (Fig. 3F) and ET_A (Fig. 3H) are presented as a mean percentage in four separate experiments. *Denotes statistical significance of mean % ET receptor density from Dex or RU486 treatments versus that of control and ** denotes statistical significance of mean % of ET_B receptor density from RU486 + Dex treatments versus that of Dex treatment as determined by One-way ANOVA and Student-Newman-Keuls multiple comparison test at $p < 0.05$.

FIGURE 2

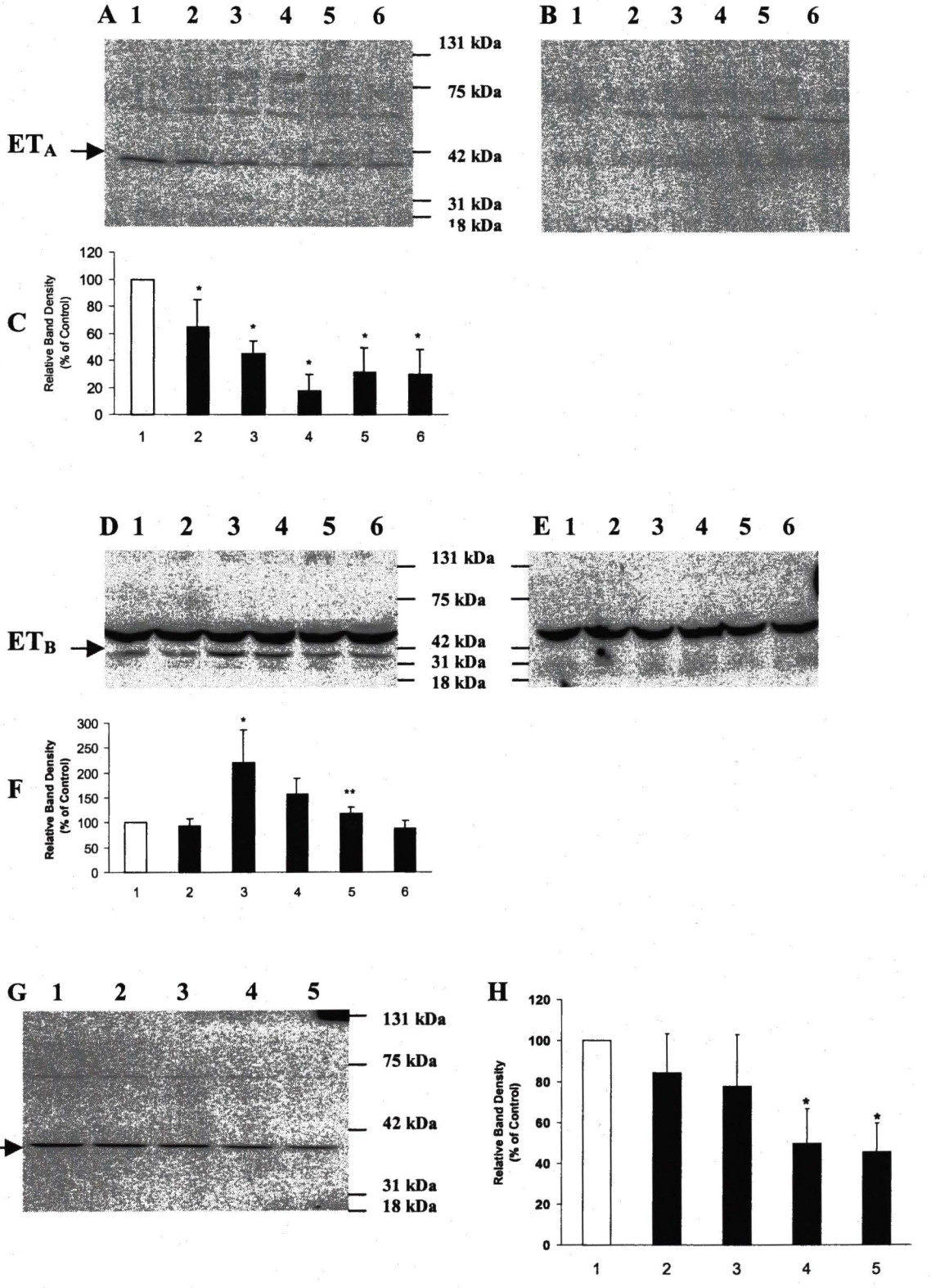

A 1 2 3 4 5 6
131 kDa
75 kDa
42 kDa
31 kDa
18 kDa
ET$_A$
B 1 2 3 4 5 6
C
Relative Band Density (% of Control)
120
100
80
60
40
20
0
1 2 3 4 5 6
D 1 2 3 4 5 6
131 kDa
75 kDa
42 kDa
31 kDa
18 kDa
ET$_B$
E 1 2 3 4 5 6
F
Relative Band Density (% of Control)
300
250
200
150
100
50
0
1 2 3 4 5 6
G 1 2 3 4 5
131 kDa
75 kDa
42 kDa
31 kDa
18 kDa
ET$_A$
H
Relative Band Density (% of Control)
120
100
80
60
40
20
0
1 2 3 4 5

FIGURE 3. Effect of dexamethasone (Dex 10 nM) on ET receptor density as determined by saturation plot of [^{125}I]ET-1 binding. Non-specific binding was determined by adding 1 μM of ET-1. Quantitation of ET_A and ET_B receptors was determined by sarafatoxin S6C (500 nM), an ET_B selective agonist. HNPE cells were treated for 24 hours with or without Dex (10 nM). 50 μg membrane protein was used for each binding reaction. * Denotes statistical significance of mean of ET receptor density from Dex treatment versus that of control in four experiments as determined by One-way ANOVA and Student-Newman-Keuls multiple comparison test at $p<0.01$.

FIGURE 3

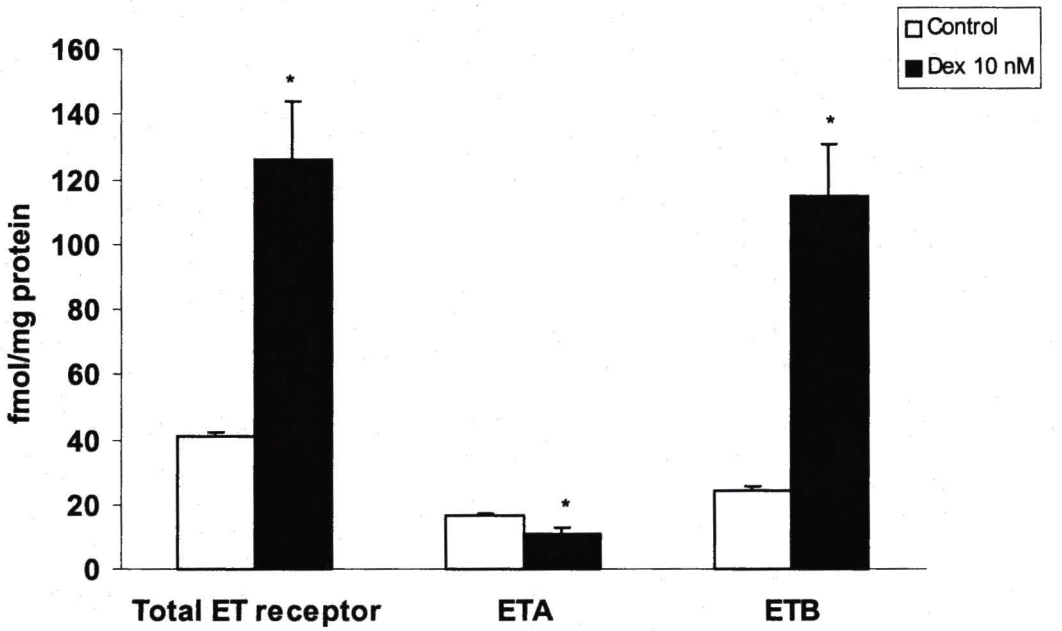

Control
Dex 10 nM
fmol/mg protein
160
140
120
100
80
60
40
20
0
*
*
*
Total ET receptor
ETA
ETB

TABLE 1

Summary of the Binding Results of ET-1 in Control and Dexamethasone treatment in HNPE cells

	Receptor Density (B_{max}) (fmol/mg membrane protein)	Kd (nM)
Control		
Total ET Receptor	41 ± 0.81	1.14 ± 0.08
ET_A	17 ± 0.50	0.63 ± 0.05
ET_B	25 ± 0.36	
Dexamethasone 10 nM		
Total ET Receptor	126 ± 17 *	4.25 ± 0.16†
ET_A	11 ± 1.9 ¶	0.49 ± 0.10
ET_B	116 ± 16 §	

NOTE: Estimates of the equilibrium dissociation constant (Kd) and maximum number of binding sites (B_{max}) were obtained using unweighted linear regression analysis. Statistic significance was determined by one-way ANOVA and Student-Newman-Keuls multiple comparison test. Values are expressed as mean ± S.D. (n = 4). For receptor density, * $p < 0.01$ control ET receptor versus Dex ET receptor, ¶ $p < 0.01$ control ET_A versus Dex ET_A, § $p < 0.01$ control ET_B versus Dex ET_B. For Kd, † $p < 0.001$ control ET receptor versus Dex ET receptor.

FIGURE 4. Dexamethasone effects on the mRNA expression of prepro-ET-1, ET_A and ET_B receptors in HNPE cells as determined by quantitative PCR. HNPE cells were treated for 24 hours with: vehicle control (lane 1); 1 nM Dex (lane 2); 10 nM Dex (lane 3); 1 μM RU486 + 100 nM Dex (Fig 4A and B, lane 5) or 1 μM RU486 + 10 nM Dex (Fig 4C, lane 5); 1 μM RU486 (lane 6). β-actin was used as an internal control. QPCR data is presented as the mean percentage of mRNA levels to the value of its corresponding control in three separate experiments. * Denotes statistical significance of mean % ppET-1 or ET receptor mRNA from Dex or RU486 treatment versus of control and ** denotes statistical significance of mean % from RU486 + Dex treatments versus that of Dex treatment as determined by One-way ANOVA and Student-Newman-Keuls multiple comparison test at $p<0.05$.

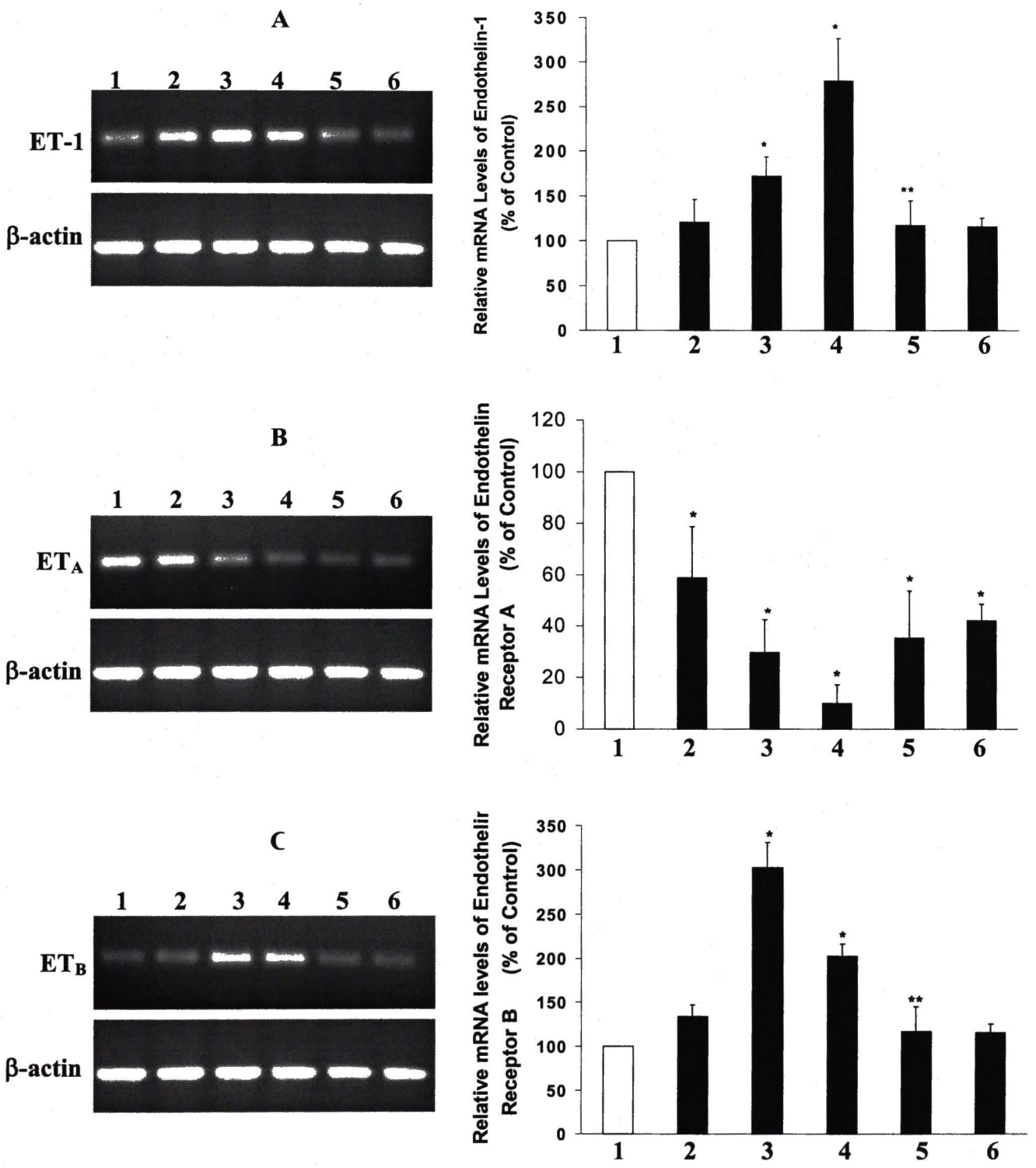
TABLE 2

Quantitative PCR Primer Sequences and Expected Product Sizes

Gene	Sense	Anti-Sense	Spacing
ppET-1*	TATCAGCAGTTAGTGAGAGG	CGAAGGTCTGTCACCAATGTGC	180 bp
ET_A	GTTGAACAGAAGGAATGGCAGC	ATTCACATCGGTTCTTGTCC	181 bp
ET_B	TCACTGTGCTGAGTCTATGTGC	AGCAGATTCGCAGATAACTTCC	206 bp
β-actin	TGTGATGGTGGGAATGGGTCAG	TTTGATGTCACGCACGATTTCC	514 bp

NOTE: *ppET-1 represents pre-pro-endothelin-1.

FIGURE 4


A
1 2 3 4 5 6
ET-1
β-actin
Relative mRNA Levels of Endothelin-1 (% of Control)
350
300
250
200
150
100
50
0
1 2 3 4 5 6
B
1 2 3 4 5 6
ETA
β-actin
Relative mRNA Levels of Endothelin Receptor A (% of Control)
120
100
80
60
40
20
0
1 2 3 4 5 6
C
1 2 3 4 5 6
ETB
β-actin
Relative mRNA levels of Endothelir Receptor B (% of Control)
350
300
250
200
150
100
50
0
1 2 3 4 5 6

CHAPTER III

INTERACTIONS OF ENDOTHELIN-1 WITH DEXAMETHASONE IN PRIMARY CULTURED HUMAN TRABECULAR MESHWORK CELLS

Xinyu Zhang, Abbot F. Clark, and Thomas Yorio

Investigative Ophthalmology & Visual Science

(In Press)

Abstract

Purpose: Aqueous humor endothelin-1 (ET-1) concentrations are increased in patients with primary open-angle glaucoma (POAG) as well as in animal models of glaucoma. Glucocorticoids have also been associated with glaucoma as topical administration of glucocorticoids can increase intraocular pressure by increasing outflow resistance in the trabecular meshwork (TM) in some individuals. Recently, we have shown that dexamethasone (Dex), a synthetic glucocorticoid, can increase ET-1 release from human non-pigmented ciliary epithelial cells (HNPE), a source for aqueous ET-1. In the current study we address the downstream interaction of ET-1 with Dex in target TM cells, an action which may alter outflow resistance.

Methods: A normal primary human TM cell line (NTM) and a TM cell line (GTM) derived from a glaucomatous patient were used. These cells were treated with vehicle or Dex. The mRNA levels of prepro-ET-1, endothelin receptor A (ET_A), and endothelin receptor B (ET_B) were measured by quantitative RT-PCR (QPCR). The protein expression of ET_A and ET_B receptors were investigated by Western blotting using polyclonal anti-ET_A and anti-ET_B antibodies respectively on plasma membrane fractions. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization mediated by ET-1 was measured using the Fura-2, AM fluorescent probe technique as an index of ET receptor function. ET-1-stimulated nitric oxide release was measured using a Griess colorimetric nitric oxide synthase assay kit.

Results: Both normal and glaucomatous cultured TM cells expressed prepro-ET-1 mRNA less abundantly compared to HNPE cells, and Dex treatment had no effect on the mRNA

expression of the ET-1 gene. TM cells expressed mRNA of ET_A receptors as detected by QPCR, whereas the ET_B message was not clearly delineated. Western blot analysis showed that both ET_A and ET_B receptor proteins were present. The ET_A receptor was linked to calcium mobilization as ET-1 produced an increase in intracellular calcium release and this increase was blocked with a selective ET_A receptor antagonist. Dex failed to induce any change in the expression of the ET_A receptor in both normal and glaucomatous TM cells, and this was supported by a lack of a Dex's effect on the ET-1 induced calcium response. However, Dex treatment diminished ET_B receptor protein expression as well as produced a decrease in ET-1-stimulated nitric oxide (NO) release, a response mediated by ET_B receptors in TM cells.

Conclusion: The Dex-induced increase in ET-1 release from HNPE cells coupled to the downstream Dex-induced specific suppression of ET_B receptor protein expression and declines in ET-1 mediated increase in NO release in TM cells could increase contraction and decrease relaxation of TM and contribute to the declines in conventional aqueous humor outflow and increases in intraocular pressure that are seen with glucocorticoids.

Key Words: Endothelin-1, Endothelin Receptor A, Endothelin Receptor B, Trabecular Meshwork, Dexamethaonse, Calcium, Nitric Oxide, Outflow Resistance

INTRODUCTION

Glaucoma is commonly associated with elevated intraocular pressure (IOP) as seen in POAG, which results primarily from pathological changes in the aqueous humor outflow pathway. Endothelin-1, a potent vasoactive peptide, may contribute to the etiology of POAG, as aqueous ET-1 concentrations are increased in POAG [1] and in animal models of glaucoma.[2] Moreover, chronic administration of ET-1 can produce an optic neuropathy[3, 4, 5] and ET-1 has been proposed as a contributor of glaucoma pathophysiology.[6] In the anterior segment, HNPE cells have been shown to be a source for aqueous ET-1, as they endogenously synthesize and release ET-1.[7, 8]

Endothelin-1 has a variety of physiological and/or pathophysiological ocular functions depending on the receptor subtype present and the tissue involved. There are two major classes of endothelin receptors, ET_A and ET_B, which have been cloned and characterized in mammalian species.[9, 10] Most commonly, ET_A mediates ET-1-induced increase in intracellular calcium $[Ca^{2+}]_i$[11] and vasoconstriction,[12] while ET_B mediates vasodilation apparently through increases in NO production.[13] ET_B receptors are also involved in the clearance of circulating ET-1.[14, 15, 16, 17] Although it has been reported that ET-1 elicited $[Ca^{2+}]_i$ transients,[18] the expression and regulation of ET receptors in the trabecular meshwork as well as the downstream signaling events relating to outflow regulation are incompletely understood.

Glucocorticoids, potent immunosuppressants and anti-inflammatory agents, are associated with primary open-angle glaucoma (POAG). It has been reported that POAG patients have elevated levels of the endogenous glucocorticoid, cortisol, in their blood[19,

[20, 21] and aqueous humor.[19] Ocular administration of glucocorticoids produce an elevated intraocular pressure in some subjects.[22,23] It has been well established that glucocorticoids change the morphology and activity of trabecular meshwork, including altering cell size and cytoskeletal organization,[24, 25] increasing extracellular matrix production,[26, 27] decreasing extracellular metalloproteinase activity,[28] and enhancing expression of a glaucoma gene, *MYOC*.[29, 30] Overall, glucocorticoids increase extracellular matrix material deposition in the trabecular meshwork and subsequently increase outflow resistance and IOP. Recently we have shown that glucocorticoids can increase ET-1 release from HNPE cells[31] and the HNPE cells may represent the cell source for ET-1 in the aqueous. A novel signaling pathway for glucocorticoids involving ET-1 may be implicated in the increase in outflow resistance by glucocorticoids. We therefore investigated whether the down-stream actions of ET-1 on the target TM cells were affected by glucocorticoids.

MATERIAL AND METHODS

Cell Culture

A primary normal human TM cell line (NTM) from a 79 years old normal white male donor, and a primary glaucomatous human TM cell line (GTM) from a 79 years old POAG white male donor were used. These cells were isolated and propagated as previously described.[24, 25, 27, 30] The cells were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and penicillin/streptomycin/Glutamine (Gibco, Grand Island, NY) and 44 mM $NaHCO_3$. For experiment protocols, TM cells were grown confluent either on

100 mm culture dishes, 6-well culture plates (35 mm diameter/well), or 24-well culture plates (15 mm diameter/well). The culture media was then changed to 5% FBS DMEM and treated for 72 hours with an equivalent volume of vehicle (ethanol) or the glucocorticoid agonist, dexamethasone (dissolved in ethanol) (Sigma Chemical, St Louis, MO), at a final concentration of 100 nM. RU486 (BIOMOL Research Labs, Inc., Plymouth Meeting, PA) (1 μM), an antagonist of glucocorticoids receptors, was used in some experiments. HNPE cells and astrocytoma cells U373 MG were used as positive controls for ET-1 or ET-receptor expression.

RNA isolation and Quantitative Polymerase Chain Reaction

Total cellular RNA isolation and QPCR were performed as previously described.[31] QPCR primers for human ppET-1, ET_A, ET_B, S15, and myocilin (Fisher-Scientific Genosys, TX) are shown in Table 1. The constitutively expressed "housekeeping" gene S15, a small ribosomal subunit protein, was served as an internal control. Myocilin gene expression was used as an index of a glucocorticoid response. Myocilin primer pairs and QPCR amplification were designed according to Shepard et al.[32] The authenticity of QPCR products was confirmed by DNA sequencing and a BLAST search of the sequence through NCBI (http://www.ncbi.nim.nih.gov) (data not shown). Quantification of relative RNA concentrations was achieved by using the comparative C_T method (as described in PE Biosystems User Bulletin#2:

http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf.)

Plasma membrane isolation and Western Blotting analysis

The isolation of plasma membranes and Western Blotting were performed as previously described.[31] Briefly after Dex treatment, plasma membrane fractions were isolated from both NTM and GTM cells. Protein concentration was determined using bicinchoninic acid reagent (BCA). Western blotting for ET_A and ET_B receptors was performed using polyclonal anti-ET_A or anti- ET_B antibodies respectively, with secondary horseradish peroxidase-conjugated donkey anti-rabbit IgG, and visualized using an enhanced chemiluminescence (ECL) system. Non-specific bands in the immunoblots were indentified by pre-inbubating the anti- ET_A or anti-ET_B antibodies with their respective receptor peptides. Densitometric analysis of the bands was accomplished using the Scion image analysis software (NIH).

Measurement of intracellular Ca^{2+} mobilization

Dynamic video imaging was used to measure intracellular Ca^{2+} within single cells following a previously described method.[33] Both NTM and GTM cells were grown on 25-mm diameter converslips (Fisher Scientific, PA) inserted in 6-well culture plates. After washing three times with KRB buffer (in mM: 115 NaCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 24 $NaHCO_3$, 5 KCl, 5 glucose, and 25 HEPES, pH 7.4), cells were incubated with 3 μM fura-2, AM dye (Molecular Probes, Eugene, OR) in KRB buffer at 37°C for 30 min. Following this incubation period, cells were washed three times and were maintained in KRB buffer at 37°C. The intracellular $[Ca^{2+}]_i$ was measured with a Nikon fluorescent microscope using Imaging Metafluor software (Universal Imaging Co., West Chester, PA) and fluorescence signal ratios at 340/380 nm exitation wave lengths were collected

every 5 secs. ET-1 (100 nM) was added to the buffer and changes in fluorescence ratios were recorded. BQ610 (Peninsula Lab Inc. Belmont, CA), an antagonist of ET_A receptor, was used at concentration of 1 μM to determine whether ET-1 induced-Ca^{2+} mobilization was mediated through ET_A receptor. The antagonist was added 30 minutes prior to the addition of ET-1. In order to convert fluorescent ratios to calcium concentrations, maximum and minimum ratios of fluorescence were obtained by applying 1 μM calcium ionophore, 4-Bromo-A23187 (Calbiochem, CA), and 5 mM EGTA, respectively. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was then calculated by using the Grynkiewicz equation.[34]

Determination of nitrite accumulation in culture medium

Following treatment with Dex for 72 hrs, confluent NTM cells grown on 24-well culture plates were shifted to serum-free (SF) DMEM with 250 μl SF-DMEM each well, supplied with 100 nM Dex and additional 100 nM ET-1. After this 24 hour incubation, culture media were collected and NO was measured as nitrite, the final oxidation breakdown product of nitric oxide (NO) released into the culture medium using Griess colorimetric nitric oxide synthase assay kit (Calbiochem, San Diego, CA) according to the manufacture's protocol. Nitrite concentration in the culture medium was calculated from a constructed nitrite standard curve. An ET_B receptor antagonist, BQ788 (Peninsula Lab Inc. Belmont, CA) 1 μM, was added 30 minutes prior to the addition of ET-1 to determine the role of ET_B receptor on NO release induced by ET-1.

RESULTS

The mRNA Expression of prepro-ET-1, ET_A and ET_B receptors in NTM and GTM Cells

QPCR revealed that both normal and glaucomatous TM cells express prepro-ET-1 message, however the prepro-ET-1 mRNA level was apparently much lower in TM cells than that in HNPE cells (Fig. 1). Dex (100 nM) produced a significant increase in mRNA levels of prepro-ET-1 in HNPE cells while it had no effect on prepro-ET-1 mRNA levels in TM cells (Fig. 1)

Both NTM and GTM cells expressed abundant mRNA of ET_A receptors and Dex (100 nM) had no effect on the mRNA expression of ET_A receptors, although these cells responded to Dex (100 nM) by significantly increasing myocilin mRNA expression (Fig. 2). ET_B receptor message expression was not detected in both NTM and GTM cells under either control, Dex (100 nM), or a serum free overnight condition, while as expected, ET_B receptor mRNA was present in positive control cells tested, including brain astrocytoma and HNPE cells (data not shown).

Effects of Dexamethasone on ET_A and ET_B Receptor Protein Expression in NTM and GTM Cells

Since there was mRNA expression of ET_A receptors while ET_B receptor mRNA was not detected, we next determined if TM cells expressed ET_A and ET_B receptor proteins and if their levels were under the regulation by Dex. Western blot analysis revealed that both ET_A and ET_B receptor proteins were present in NTM and GTM cells. Anti-ET_A

antibody recognized an approximately 43 kDa band (Fig. 3A and 3D), and ET_A receptor peptide completely blocked the recognition of this band (Fig. 3B and 3E). Therefore this 43 kDa protein was the ET_A receptor. Treatment with Dex for 72 hours did not alter protein levels of the ET_A receptor (Fig. 3C and 3F).

Anti-ET_B antibody recognized three bands sized approximately 80 kDa, 65 kDa and 38 kDa (Fig. 4A and 4D). Competing ET_B peptide was able to completely block the recognition of only the lowest band while the other bands remained (Fig 4B and 4E). Therefore, the high molecular weight bands appear to be nonspecific bands, and the protein recognized with a size of 38 kDa is the ET_B receptor. Dex treatment for 72 hours significantly decreased the protein levels of ET_B receptors in both NTM and GTM cells (Fig. 4C and 4F). Treatment with RU486 (1μM), an antagonist of glucocorticoid receptors, did not block Dex-induced decrease of ET_B receptor protein and interestingly, RU486 alone, showed partial agonist activity as itself also decreased ET_B receptor amount (Fig. 4G and 4H).

Effects of Dexamethasone on ET-1-mediated Intracellular Calcium Mobilization

To determine whether ET_A receptor function was altered by Dex treatment, ET-1 effects on $[Ca^{2+}]_i$ were measured in both NTM and GTM cells under vehicle control and Dex treatment conditions. As expected, primary TM cells produced a significant increase in $[Ca^{2+}]_i$ in response to 100 nM ET-1, and this response showed a typical biphasic phenotype with a transient spike followed by a sustained plateau (Fig. 5A and 5D). This plateau was still maintained even after ET-1 was removed (data not shown). The initial rise in $[Ca^{2+}]_i$ peaked at Ca^{2+} concentrations well over 1 μM while baseline levels were

initially below 100 nM (see Table 2). Dex (100 nM) treatment for 72 hours did not alter ET's Ca^{2+} response phenotype in both NTM and GTM cells (Fig. 5B and 5E), and Dex did not change the basal levels of $[Ca^{2+}]_i$ nor the ET-1-induced peak response in $[Ca^{2+}]_i$ in these TM cells. Pre-incubation with BQ610, an antagonist of ET_A receptors, completely blocked ET-1 mediated $[Ca^{2+}]_i$ mobilization (Fig. 5C). This indicated that ET-1 mediated $[Ca^{2+}]_i$ mobilization occurred through activation of ET_A receptors in TM cells.

Effects of Dexamethasone on ET-1-induced Nitric Oxide Release from NTM cells

In order to determine whether the decrease of ET_B receptor amount by Dex caused a similar decrease in functional response in TM cells, the effect of Dex on ET-1-induced nitric oxide (NO) release was measured. A Griess colorimetric assay was used to detect the nitrite (the final oxidation product of NO) in culture medium. ET-1 (100 nM) significantly elevated nitrite concentrations in medium compared to that observed in control cells without ET-1 treatment, and BQ 788 1 μM, an ET_B receptor antagonist, completely blocked the elevation of nitrite by ET-1 (Fig. 6). This indicated that ET-1 mediated NO release occurred through activation of ET_B receptors in TM cells. Dex (100 nM) alone did not alter NO release compared to the vehicle control; however, Dex treatment significantly decreased the ET-1 mediated NO release compared to cells without Dex treatment. This was consistent with the finding that Dex decreased ET_B receptor expression in TM cells (as reported above) and that ET-1 stimulated NO release by activating ET_B receptors.

DISCUSSION

It was postulated that aqueous ET-1 could have consequence on the down-stream Dex effects on TM since it is known that ET-1 could contract TM and TM contraction increases outflow resistance while TM relaxation increases outflow facility.[35, 36] However it was not known if TM cells express ET-1 or if Dex treatment stimulates ET-1 expression. In this study, QPCR was able to detect weak prepro-ET-1 mRNA expression in both NTM and GTM cells. Dex treatment did not change prepro-ET-1 mRNA expression in these TM cells. Compared to TM cells, HNPE cells expressed about four fold higher basal levels of prepro-ET-1 mRNA in control cells, and Dex treatment caused a further three fold increase of prepro-ET-1 message. Upon Dex (100 nM) treatment, HNPE cells expressed over 10 fold higher levels of prepro-ET-1 mRNA compared to TM cells. We propose that NPE cells are the source of aqueous humor ET-1 and that released ET-1 may have downstream actions in TM. The finding that Dex treatment also enhanced ET-1 expression and release suggests that ET-1 may mediate Dex effects on target tissues such as TM cells.

ET-1 has diverse biological activities through different receptor subtypes, ET_A and ET_B receptors.[6, 37] Although ET-1 has significant actions on the regulation of IOP,[38] functional ET receptor expression in trabecular meshwork, a tissue regulating outflow resistance, is not certain. Previously, ET_A receptor mRNA but not ET_B receptor message in TM cells was identified by RT-PCR.[18] Presently, only ET_A receptor mRNA was detected by QPCR, whereas the ET_B receptor message was not clearly delineated.

However, Western blot analysis showed that both ET_A and ET_B receptor proteins were present. The reason for this apparent difference between message and protein could be due to the finding that ET_B mRNA has a short intracellular half-life as the 3' noncoding region contains an AUUUA motif implicated in selective destabilization of mRNA.[10] The finding that ET_B receptor expression was not readily detected could reflect instability of the ET_B mRNA in TM cells.

Western blotting revealed that the ET_A receptor protein had a molecular weight of 43 kDa, whereas the ET_B receptor was slightly less than 40 kDa. Previously we found ET_A and ET_B receptors had similar molecular weights in HNPE cells.[31] There have been numerous reports of different molecular weights for ET_A and ET_B receptors that range between 30 - 70 kDa.[39, 40, 41, 42, 43] The ET_A gene has a single transcreption start site, whereas the murine and bovine ET_B gene has been reported to have alternative transcriptional initiation sites.[44, 45] An ET_B splice variant from alternative RNA splicing was identified in various human tissues.[46] Moreover, a metalloproteinase cleavage site was also identified in the ET_B protein sequence.[47] Overall, the apparent differences in reported molecular weights could be attributed to the source of antibodies, species or tissue specificity, post-translation modification, different proteolytic processing, or ultimately the result of inaccuracy inherent in the estimation of the molecular weight of membrane proteins by SDS-PAGE.

QPCR and Western blot analysis demonstrated that 100 nM Dex treatment for three days did not alter ET_A receptor mRNA and protein levels. In contrast, Dex treatment decreased ET_B receptor protein levels. There are several mechanisms that could account

for the effect of glucocorticoids on the regulation and expression of ET_B target gene. Activated glucocorticoid-receptor complex can either bind to the glucocorticoid response element in target genes to regulate the transcription of these genes, or perhaps interfering with other transcription factors, such as NF-κB and AP-1, and thereby preventing the actions of these transcription factors.[48] Some other glucocorticoid responses may involve glucocorticoid receptor activation of a primary response gene whose gene product in turn secondarily activates other genes.[32] However the precise mechanism of glucocorticoid regulation of ET_B receptor expression remains unclear. There is also very little information available on the regulation of endothelin receptor gene expression. Therefore, further experiments are needed to delineate the pathways that may be involved in the regulation of ET_B receptor expression by Dex in TM cells.

RU486 was unable to block Dex's effects on the ET_B receptor protein. It also appears that RU486 may function as a partial agonist, as it had its own action on ET_B receptor protein level. In fact, RU486, a classical antagonist for both progesterone and glucocorticoids receptors, interferes with steroid-mediated activation but often confers an agonist activity to steroid receptors.[49, 50, 51] RU486 could also function as a partial agonist in TM cells and HNPE cells.[31, 32] This partial agonist activity may explain the inability of RU486 to block Dex-mediated decrease in ET_B receptors.

ET-1 can mediate intracellular calcium $[Ca^{2+}]_i$ and contraction of trabecular meshwork[35] via ET_A receptors.[18] This was supported by the finding that the ET_A receptor antagonist, BQ610, completely blocked this calcium mobilization. Dex treatment had no effects on ET_A mRNA and protein levels as detected by QPCR and Western Blotting, and

Dex also did not alter the function of ET_A receptors because both the baseline and ET-1-induced peaked increase of $[Ca^{2+}]_i$ was similar under Dex treatment and control conditions. This normal function of ET_A receptors is important in considering Dex's effect on aqueous humor outflow, particularly since Dex increases ET-1 release from HNPE cells and ET-1 targets normally functioning ET_A receptors on TM cells. Such as action could cause more intensive contraction of the TM.

The finding that TM cells expressed not only ET_A receptors but also ET_B receptors suggested a complex regulation function. ET_B receptors most commonly regulate vasodilatation or relaxation.[52] The signaling pathway coupled to ET_B receptors has been linked to nitric oxide (NO) production. ET-1 activates endothelial nitric oxide synthase (eNOS) and hence NO production via ET_B receptors in vascular endothelial cells.[13] ET-3 increases retinal blood flow through activation of ET_B receptors, which also is dependent on NO production.[53] ET-1 and ET-3 also enhance inducible nitric oxide synthase (iNOS) expression, and this is mediated by ET_B receptors in glial cells.[54] TM cells have been shown to express different isoforms of NOS and produce NO.[55, 56, 57] Presently we determined that the ET_B receptors were linked to NO production in NTM cells. We were able to detect basal levels of NO production in NTM cell culture media and showed that 100 nM ET-1 markedly increased NO release. An ET_B receptor antagonist BQ788 completely blocked this stimulation of NO by ET-1. These findings indicated that ET-1-induced NO release is mediated by ET_B receptors. Dex treatment decreased the ET_B receptor protein level and also reduced ET-1-induced NO release, further implicating ET_B in ET-1-induced NO release in TM cells. There are several reports that NO donors could

relax trabecular meshwork and lower IOP.[58, 59, 60] This decrease of ET_B receptor level and subsequent decrease of ET-1-induced release of NO by Dex could reduce NO mediated relaxation of TM. Moreover, ET_B receptors are known to mediate ET-1 clearance.[14, 15, 16, 17] The decrease of ET_B receptors in TM cells by Dex could also potentiate ET-1-mediated contraction of TM via ET_A receptors.

In conclusion, Dex-induced specific suppression of ET_B receptor protein expression and declines in ET-1 mediated increase in NO levels in TM cells with the concomitant increase of ET-1 release from HNPE cells by Dex could increase contraction and decrease relaxation of trabecular meshwork and reduce the intratrabecular space. This may exacerbate Dex effects on the outflow pathway leading to increased outflow resistance and consequently elevated intraocular pressure.

Acknowledgements

This work was supported by a grant from NEI/NIH (Grant number: EY11979) and the Advanced Technology Program, Texas Higher Education Coordinating Board. The authors would like to thank Dr. Jerry Simecka for his assistance with quantitative PCR, and Dr. Ganesh Prasanna, Dr. Raghu Krishnamoorthy, and Santosh Narayan for their helpful discussions.

References:

1. Noske W, Hensen J,Wiederholt M. Endothelin-like immunoreactivity in aqueous humor of patients with primary open-angle glaucoma and cataract. *Graefes Arch Clin Exp Ophthalmol.* 1997;235:551-2.
2. Kallberg ME, Brooks DE, Garcia-Sanchez GA, Komaromy AM, Szabo NJ,Tian L. Endothelin 1 levels in the aqueous humor of dogs with glaucoma. *J Glaucoma.* 2002;11:105-9.
3. Orgul S, Cioffi GA, Wilson DJ, Bacon DR,Van Buskirk EM. An endothelin-1 induced model of optic nerve ischemia in the rabbit. *Invest Ophthalmol Vis Sci.* 1996;37:1860-9.
4. Cioffi GA,Sullivan P. The effect of chronic ischemia on the primate optic nerve. *Eur J Ophthalmol.* 1999;9 Suppl 1:S34-6.
5. Oku H, Sugiyama T, Kojima S, Watanabe T,Azuma I. Experimental optic cup enlargement caused by endothelin-1-induced chronic optic nerve head ischemia. *Surv Ophthalmol.* 1999;44 Suppl 1:S74-84.
6. Yorio T, Krishnamoorthy R,Prasanna G. Endothelin: is it a contributor to glaucoma pathophysiology? *J Glaucoma.* 2002;11:259-70.
7. Lepple-Wienhues A, Becker M, Stahl F, et al. Endothelin-like immunoreactivity in the aqueous humour and in conditioned medium from cultured ciliary epithelial cells. *Curr Eye Res.* 1992;11:1041-6.

8. Prasanna G, Dibas A, Tao W, White K,Yorio T. Regulation of endothelin-1 in human non-pigmented ciliary epithelial cells by tumor necrosis factor-alpha. *Exp Eye Res*. 1998;66:9-18.
9. Arai H, Hori S, Aramori I, Ohkubo H,Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature*. 1990;348:730-2.
10. Sakurai T, Yanagisawa M, Takuwa Y, et al. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature*. 1990;348:732-5.
11. Marsden PA,Brenner BM. Nitric oxide and endothelins: novel autocrine/paracrine regulators of the circulation. *Semin Nephrol*. 1991;11:169-85.
12. Marsault R, Feolde E,Frelin C. Receptor externalization determines sustained contractile responses to endothelin-1 in the rat aorta. *Am J Physiol*. 1993;264:C687-93.
13. Hirata Y, Emori T, Eguchi S, et al. Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. *J Clin Invest*. 1993;91:1367-73.
14. Fukuroda T, Fujikawa T, Ozaki S, Ishikawa K, Yano M,Nishikibe M. Clearance of circulating endothelin-1 by ETB receptors in rats. *Biochem Biophys Res Commun*. 1994;199:1461-5.
15. Berthiaume N, Yanagisawa M, Labonte J,D'Orleans-Juste P. Heterozygous knock-Out of ET(B) receptors induces BQ-123-sensitive hypertension in the mouse. *Hypertension*. 2000;36:1002-7.

16. Bremnes T, Paasche JD, Mehlum A, Sandberg C, Bremnes B,Attramadal H. Regulation and intracellular trafficking pathways of the endothelin receptors. *J Biol Chem*. 2000;275:17596-604.

17. Paasche JD, Attramadal T, Sandberg C, Johansen HK,Attramadal H. Mechanisms of endothelin receptor subtype-specific targeting to distinct intracellular trafficking pathways. *J Biol Chem*. 2001;276:34041-50.

18. Tao W, Prasanna G, Dimitrijevich S,Yorio T. Endothelin receptor A is expressed and mediates the [Ca2+]i mobilization of cells in human ciliary smooth muscle, ciliary nonpigmented epithelium, and trabecular meshwork. *Curr Eye Res*. 1998;17:31-8.

19. Rozsival P, Hampl R, Obenberger J, Starka L,Rehak S. Aqueous humour and plasma cortisol levels in glaucoma and cataract patients. *Curr Eye Res*. 1981;1:391-6.

20. Schwartz B, McCarty G,Rosner B. Increased plasma free cortisol in ocular hypertension and open angle glaucoma. *Arch Ophthalmol*. 1987;105:1060-5.

21. McCarty GR,Schwartz B. Increased plasma noncortisol glucocorticoid activity in open-angle glaucoma. *Invest Ophthalmol Vis Sci*. 1991;32:1600-8.

22. Armaly MF,Becker B. Intraocular pressure response to topical corticosteroids. *Fed Proc*. 1965;24:1274-8.

23. Lewis JM, Priddy T, Judd J, et al. Intraocular pressure response to topical dexamethasone as a predictor for the development of primary open-angle glaucoma. *Am J Ophthalmol*. 1988;106:607-12.

24. Wilson K, McCartney MD, Miggans ST,Clark AF. Dexamethasone induced ultrastructural changes in cultured human trabecular meshwork cells. *Curr Eye Res*. 1993;12:783-93.

25. Clark AF, Wilson K, McCartney MD, Miggans ST, Kunkle M,Howe W. Glucocorticoid-induced formation of cross-linked actin networks in cultured human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 1994;35:281-94.

26. Johnson DH, Bradley JM,Acott TS. The effect of dexamethasone on glycosaminoglycans of human trabecular meshwork in perfusion organ culture. *Invest Ophthalmol Vis Sci*. 1990;31:2568-71.

27. Steely HT, Browder SL, Julian MB, Miggans ST, Wilson KL,Clark AF. The effects of dexamethasone on fibronectin expression in cultured human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 1992;33:2242-50.

28. Samples JR, Alexander JP,Acott TS. Regulation of the levels of human trabecular matrix metalloproteinases and inhibitor by interleukin-1 and dexamethasone. *Invest Ophthalmol Vis Sci*. 1993;34:3386-95.

29. Polansky JR, Fauss DJ, Chen P, et al. Cellular pharmacology and molecular biology of the trabecular meshwork inducible glucocorticoid response gene product. *Ophthalmologica*. 1997;211:126-39.

30. Clark AF, Steely HT, Dickerson JE, Jr., et al. Glucocorticoid induction of the glaucoma gene MYOC in human and monkey trabecular meshwork cells and tissues. *Invest Ophthalmol Vis Sci*. 2001;42:1769-80.

31. Zhang X, Krishnamoorthy RR, Prasanna G, Narayan S, Clark A,Yorio T. Dexamethasone regulates endothelin-1 and endothelin receptors in human non-pigmented ciliary epithelial (HNPE) cells. *Exp Eye Res*. 2003;76:261-72.

32. Shepard AR, Jacobson N, Fingert JH, Stone EM, Sheffield VC,Clark AF. Delayed secondary glucocorticoid responsiveness of MYOC in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 2001;42:3173-81.

33. Prasanna G, Dibas AI,Yorio T. Cholinergic and adrenergic modulation of the Ca2+ response to endothelin-1 in human ciliary muscle cells. *Invest Ophthalmol Vis Sci*. 2000;41:1142-8.

34. Grynkiewicz G, Poenie M,Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem*. 1985;260:3440-50.

35. Lepple-Wienhues A, Stahl F, Willner U, Schafer R,Wiederholt M. Endothelin-evoked contractions in bovine ciliary muscle and trabecular meshwork: interaction with calcium, nifedipine and nickel. *Curr Eye Res*. 1991;10:983-9.

36. Wiederholt M, Thieme H,Stumpff F. The regulation of trabecular meshwork and ciliary muscle contractility. *Prog Retin Eye Res*. 2000;19:271-95.

37. Pang IH,Yorio T. Ocular actions of endothelins. *Proc Soc Exp Biol Med*. 1997;215:21-34.

38. Sugiyama K, Haque MS, Okada K, Taniguchi T,Kitazawa Y. Intraocular pressure response to intravitreal injection of endothelin-1 and the mediatory role of ETA receptor, ETB receptor, and cyclooxygenase products in rabbits. *Curr Eye Res*. 1995;14:479-86.

39. Masuda Y, Miyazaki H, Kondoh M, et al. Two different forms of endothelin receptors in rat lung. *FEBS Lett.* 1989;257:208-10.

40. Bednar DL, Stein RB, Garsky VM,Williams DL, Jr. The bovine endothelin receptor has an apparent molecular weight of 43,000. *Biochim Biophys Acta.* 1991;1092:226-32.

41. Takasuka T, Sakurai T, Goto K, Furuichi Y,Watanabe T. Human endothelin receptor ETB. Amino acid sequence requirements for super stable complex formation with its ligand. *J Biol Chem.* 1994;269:7509-13.

42. Shannon TR,Hale CC. Identification of a 65 kDa endothelin receptor in bovine cardiac sarcolemmal vesicles. *Eur J Pharmacol.* 1994;267:233-8.

43. Kurokawa K, Yamada H,Ochi J. Topographical distribution of neurons containing endothelin type A receptor in the rat brain. *J Comp Neurol.* 1997;389:348-60.

44. Cheng HF, Su YM, Yeh JR,Chang KJ. Alternative transcript of the nonselective-type endothelin receptor from rat brain. *Mol Pharmacol.* 1993;44:533-8.

45. Mizuno T, Saito Y, Itakura M, et al. Structure of the bovine ETB endothelin receptor gene. *Biochem J.* 1992;287 (Pt 1):305-9.

46. Shyamala V, Moulthrop TH, Stratton-Thomas J,Tekamp-Olson P. Two distinct human endothelin B receptors generated by alternative splicing from a single gene. *Cell Mol Biol Res.* 1994;40:285-96.

47. Saito Y, Mizuno T, Itakura M, et al. Primary structure of bovine endothelin ETB receptor and identification of signal peptidase and metal proteinase cleavage sites. *J Biol Chem.* 1991;266:23433-7.

48. Wordinger RJ,Clark AF. Effects of glucocorticoids on the trabecular meshwork: towards a better understanding of glaucoma. *Prog Retin Eye Res*. 1999;18:629-67.

49. Beck CA, Weigel NL, Moyer ML, Nordeen SK,Edwards DP. The progesterone antagonist RU486 acquires agonist activity upon stimulation of cAMP signaling pathways. *Proc Natl Acad Sci U S A*. 1993;90:4441-5.

50. Sartorius CA, Groshong SD, Miller LA, et al. New T47D breast cancer cell lines for the independent study of progesterone B- and A-receptors: only antiprogestin-occupied B-receptors are switched to transcriptional agonists by cAMP. *Cancer Res*. 1994;54:3868-77.

51. Schulz M, Eggert M, Baniahmad A, Dostert A, Heinzel T,Renkawitz R. RU486-induced glucocorticoid receptor agonism is controlled by the receptor N terminus and by corepressor binding. *J Biol Chem*. 2002;277:26238-43.

52. Miyauchi T,Masaki T. Pathophysiology of endothelin in the cardiovascular system. *Annu Rev Physiol*. 1999;61:391-415.

53. Mori F, King GL, Clermont AC, Bursell DK,Bursell SE. Endothelin-3 regulation of retinal hemodynamics in nondiabetic and diabetic rats. *Invest Ophthalmol Vis Sci*. 2000;41:3955-62.

54. Oda H, Murayama T, Sasaki Y, Okada T,Nomura Y. Endothelin enhances lipopolysaccharide-induced expression of inducible nitric oxide synthase in rat glial cells. *Eur J Pharmacol*. 1997;339:253-60.

55. Nathanson JA,McKee M. Alterations of ocular nitric oxide synthase in human glaucoma. *Invest Ophthalmol Vis Sci*. 1995;36:1774-84.

56. Geyer O, Podos SM,Mittag T. Nitric oxide synthase activity in tissues of the bovine eye. *Graefes Arch Clin Exp Ophthalmol.* 1997;235:786-93.

57. Matsuo T. Basal nitric oxide production is enhanced by hydraulic pressure in cultured human trabecular cells. *Br J Ophthalmol.* 2000;84:631-5.

58. Nathanson JA. Direct application of a guanylate cyclase activator lowers intraocular pressure. *Eur J Pharmacol.* 1988;147:155-6.

59. Wiederholt M, Sturm A,Lepple-Wienhues A. Relaxation of trabecular meshwork and ciliary muscle by release of nitric oxide. *Invest Ophthalmol Vis Sci.* 1994;35:2515-20.

60. Behar-Cohen FF, Goureau O, D'Hermies F,Courtois Y. Decreased intraocular pressure induced by nitric oxide donors is correlated to nitrite production in the rabbit eye. *Invest Ophthalmol Vis Sci.* 1996;37:1711-5.

Legends to figures:

Fig. 1. The mRNA expression of prepro-ET-1 in NTM and GTM cells as determined by QPCR.

(A) HNPE cells were used as positive controls and treated for 24 hours with vehicle control (lane 1); 1 nM Dex (lane 2); 10 nM Dex (lane 3); 100 nM Dex (lane 4). NTM cells vehicle control (lane 5); 100 nM Dex (lane 6); GTM cells vehicle control (lane 7); and 100 nM Dex (lane 8). S15 was used as an internal control. (B) QPCR data are presented as the mean ± SEM % to the vehicle control value of HNPE cells. Experiments were repeated three times for each culture cell line. * Denotes statistical significance from Dex treatment vs from vehicle control in HNPE cells and ** denotes statistical significance from vehicle control or Dex treatment in TM cells vs from vehicle control in HNPE cells as determined by One-way ANOVA and Student-Newman-Keuls multiple comparison test at $p < 0.05$.

Table 1

Quantitative PCR Primer Sequences and Expected Product Sizes

Gene	Sense	Anti-Sense	Spacing
ppET-1*	TATCAGCAGTTAGTGAGAGG	CGAAGGTCTGTCACCAATGTGC	180 bp
ET_A	GTTGAACAGAAGGAATGGCAGC	ATTCACATCGGTTCTTGTCC	181 bp
ET_B	TCACTGTGCTGAGTCTATGTGC	AGCAGATTCGCAGATAACTTCC	206 bp
Myocilin	GCCCATCTGGCTATCTCAGG	CTCAGCGTGAGAGGCTCTCC	82 bp
S15	TTCCGCAAGTTCACCTACC	CGGGCCGGCCATGCTTTACG	361 bp

NOTE: *ppET-1 represents pre-pro-endothelin-1.

Fig. 1

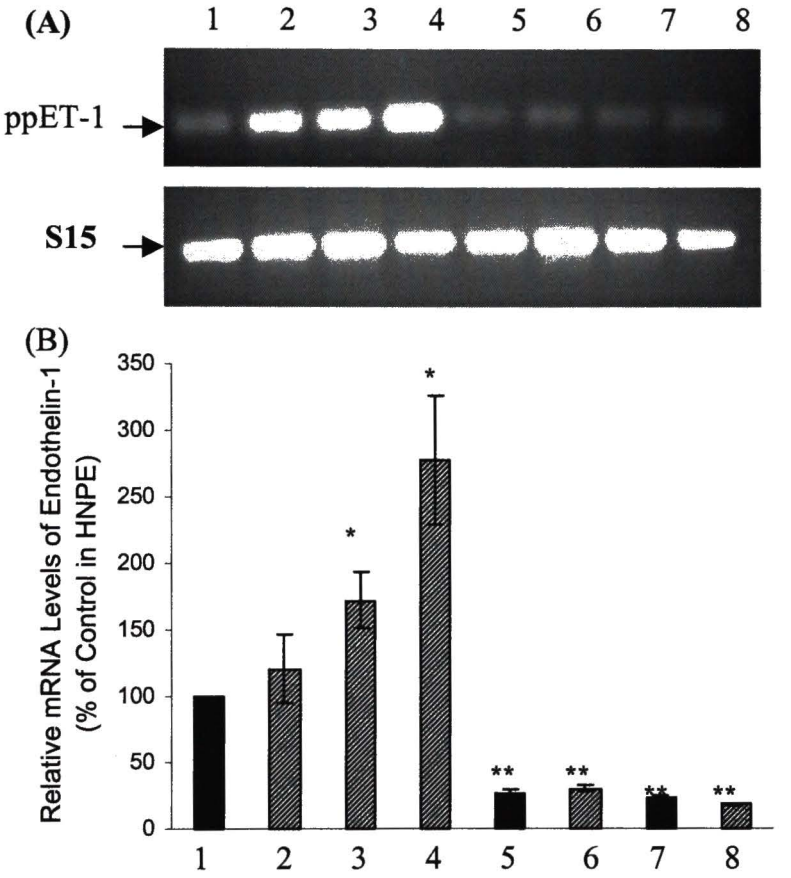

(A)
1 2 3 4 5 6 7 8
ppET-1
S15
(B)
Relative mRNA Levels of Endothelin-1
(% of Control in HNPE)
350
300
250
200
150
100
50
0
*
*
**
**
**
**
1 2 3 4 5 6 7 8

Fig. 2. Effects of dexamethasone on the mRNA expression of ET_A receptors in NTM and GTM cells as determined by quantitative PCR.

(A)-(C) NTM cells. (D)-(F) GTM cells. QPCR data are presented as the mean ± SEM % of mRNA levels of ET_A or myocilin from Dex treatment to the respective vehicle control values. * Denotes statistical significance of mean ± SEM % of myocilin from Dex treatment vs from control using student's t-test ($p < 0.001$). Experiments were repeated three times for each culture cell line.

Fig. 2

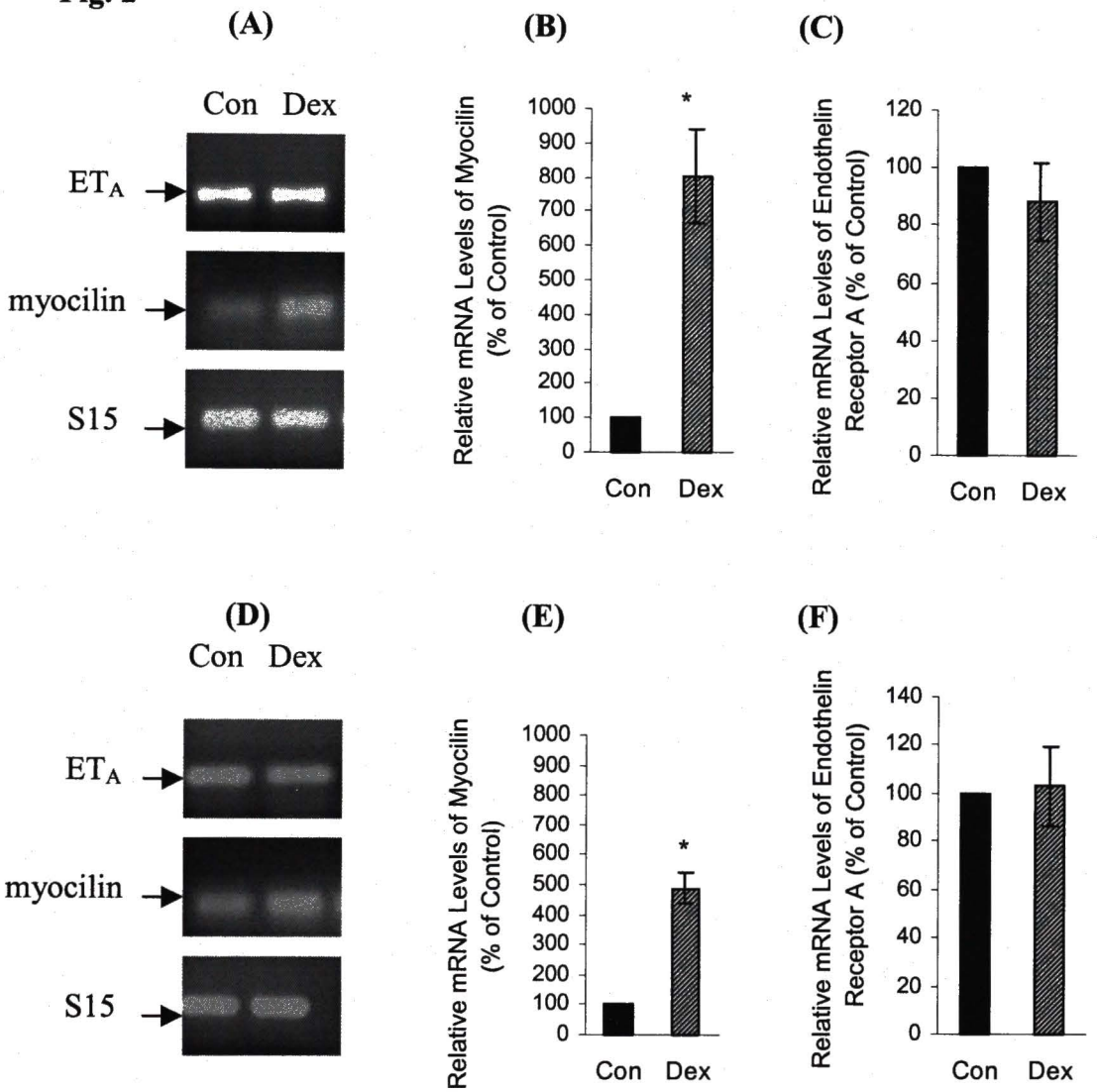

(A)
Con Dex
ET_A
myocilin
S15
(B)
Relative mRNA Levels of Myocilin (% of Control)
1000
900
800
700
600
500
400
300
200
100
0
*
Con Dex
(C)
Relative mRNA Levles of Endothelin Receptor A (% of Control)
120
100
80
60
40
20
0
Con Dex
(D)
Con Dex
ET_A
myocilin
S15
(E)
Relative mRNA Levels of Myocilin (% of Control)
1000
900
800
700
600
500
400
300
200
100
0
*
Con Dex
(F)
Relative mRNA Levels of Endothelin Receptor A (% of Control)
140
120
100
80
60
40
20
0
Con Dex

Fig. 3. Effects of dexamethasone on the protein expression of ET_A receptors in NTM and GTM cells as determined by Western Blotting. (A)-(C) NTM cells. (D)-(F) GTM cells. Western blot analysis of ET_A receptor using anti-ET_A receptor antibody without (A and D) and with pre-incubation with ET_A receptor peptide (B and E). The quantification of band intensity (C and F) is represented as mean ± SEM % to the value of its corresponding vehicle control band in the same blot. There was no statistically significant difference as determined by student's t-test in three repeated experiments for each culture cell line.

Fig. 3

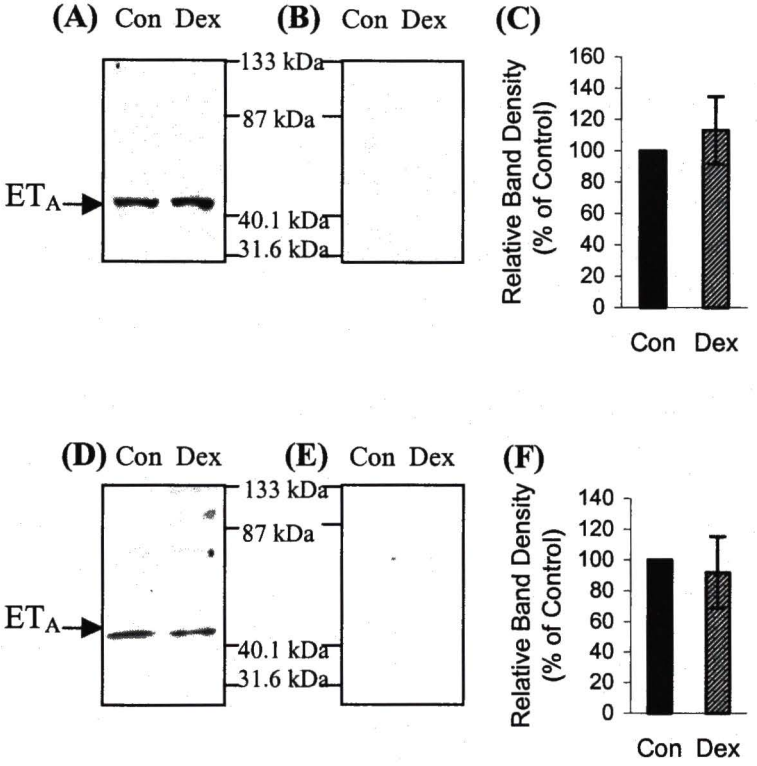

(A) Con Dex
(B) Con Dex
(C)
133 kDa
87 kDa
40.1 kDa
31.6 kDa
ET_A
Relative Band Density (% of Control)
160
140
120
100
80
60
40
20
0
Con Dex
(D) Con Dex
(E) Con Dex
(F)
133 kDa
87 kDa
40.1 kDa
31.6 kDa
ET_A
Relative Band Density (% of Control)
140
120
100
80
60
40
20
0
Con Dex

Fig. 4. Effects of dexamethasone on the protein expression of ET_B receptors in NTM and GTM cells as determined by Western Blotting. (A)-(C) NTM cells. (D)-(F) GTM cells. (G)-(H) the effects of RU486 on the action of Dex in ET_B receptor amount in NTM Cells. Western blot analysis of ET_B receptor using anti-ET_B receptor antibody without (A, D and G) and with pre-incubation with ET_B receptor peptide (B and E). The quantification of band intensity (C, F and H) is represented as mean ± SEM % to the value of its corresponding vehicle control band in the same blot. * Denotes statistical significance from Dex and/or RU486 treatment vs from vehicle control, and ** denotes statistical significance from RU486 treatment vs from Dex treatment as determined by student's t-test or One-way ANOVA and Student-Newman-Keuls multiple comparison test ($p \leq 0.001$). Experiments were repeated three times for each culture cell line.

Fig. 4

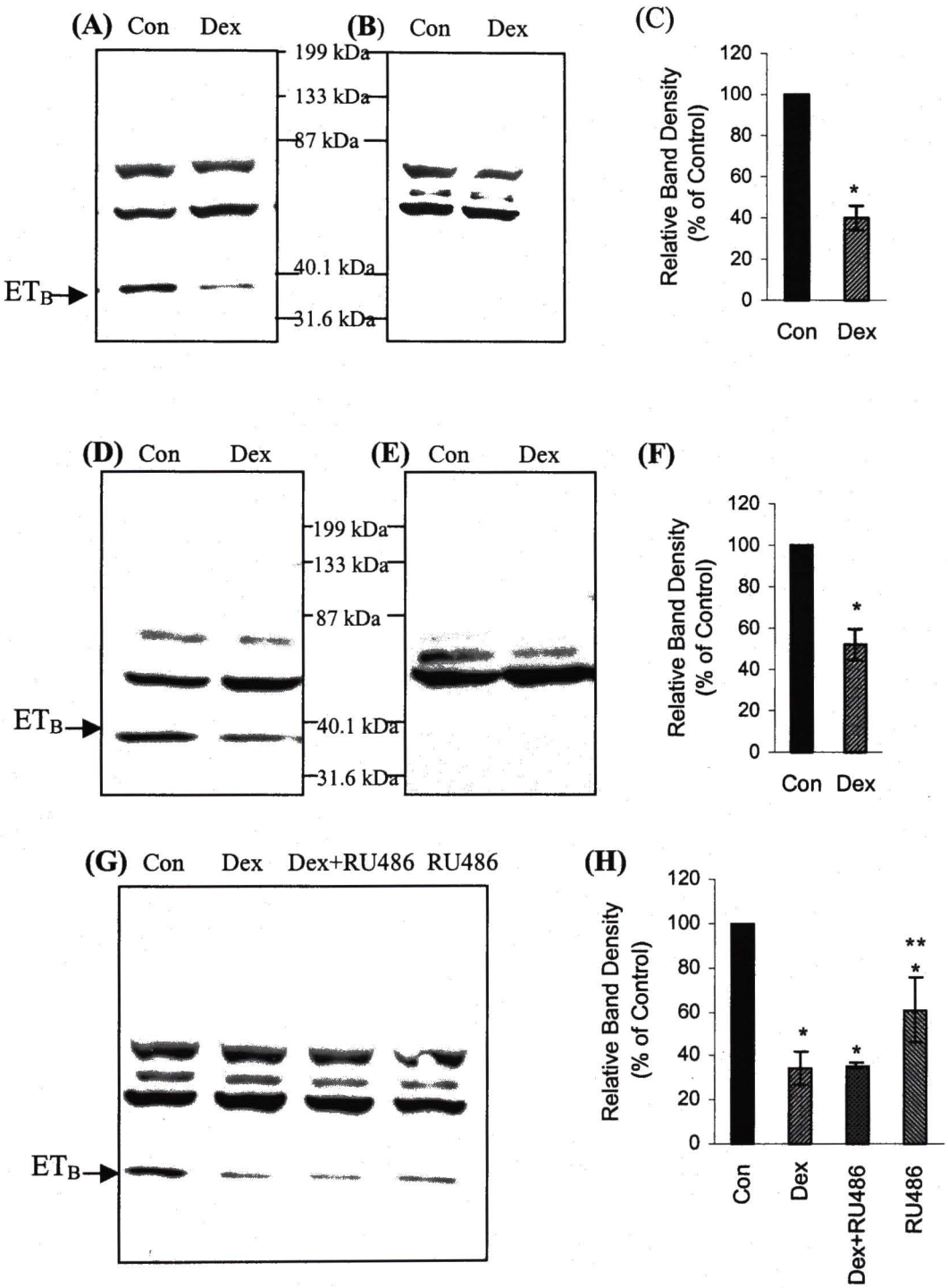

(A) Con Dex
(B) Con Dex
199 kDa
133 kDa
87 kDa
40.1 kDa
31.6 kDa
ETB
(C)
Relative Band Density (% of Control)
120
100
80
60
40
20
0
Con Dex
(D) Con Dex
(E) Con Dex
(F)
(G) Con Dex Dex+RU486 RU486
(H)
Dex+RU486
RU486

Fig. 5. Effects of dexamethasone on ET-1-mediated intracellular Ca^{2+} mobilization in NTM and GTM cells. NTM cells treated with vehicle (A) or 100 nM Dex (B). NTM cells pre-incubated with ET_A receptor antagonist, BQ610 1 μM (C). GTM cells treated with vehicle (D) or 100 nM Dex (E).

Fig. 5

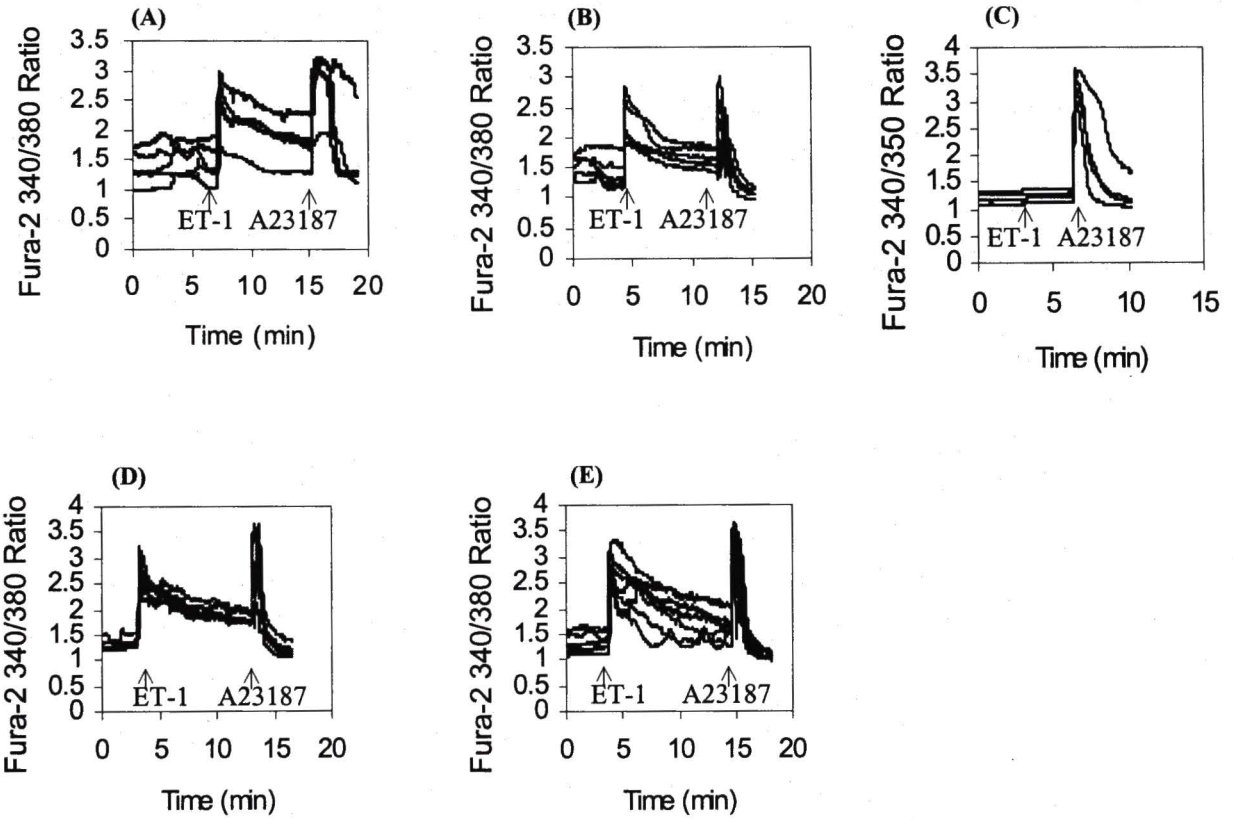

(A)
Fura-2 340/380 Ratio
3.5
3
2.5
2
1.5
1
0.5
0
ET-1
A23187
0
5
10
15
20
Time (min)
(B)
Fura-2 340/380 Ratio
3.5
3
2.5
2
1.5
1
0.5
0
ET-1
A23187
0
5
10
15
20
Time (min)
(C)
Fura-2 340/350 Ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
ET-1
A23187
0
5
10
15
Time (min)
(D)
Fura-2 340/380 Ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
ET-1
A23187
0
5
10
15
20
Time (min)
(E)
Fura-2 340/380 Ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
ET-1
A23187
0
5
10
15
20
Time (min)

Table 2

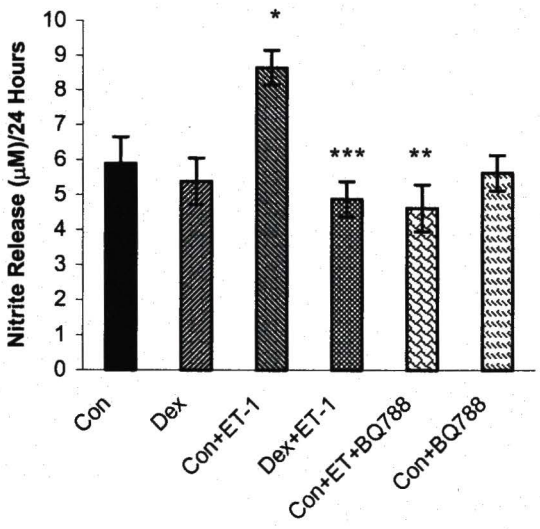
Summary of ET-1(100 nM) Mediated $[Ca^{2+}]_i$ Peak Response in TM Cells

Cell Line / Treatments		Baseline (Mean ± SEM nM)	Peak (Mean ± SEM nM)	Number of Cells (n)
NTM	Control	87.08 ± 9.45	1157.03 ± 164.67 *	51
NTM	Dex	73.54 ± 7.37	1183.34 ± 204.37 *	50
GTM	Control	45.70 ± 5.16	1295.64 ± 211.76 *	20
GTM	Dex	44.81 ± 6.98	1230.11 ± 218.71 *	20
NTM	BQ610	21.31 ± 2.60	23.16 ± 2.95	13

NOTE: * Statistic significance was determined by t-test at $p<0.001$ between baseline and ET-1 induced peak level.

Fig. 6. Effects of dexamethasone on ET-1-induced nitric oxide release from NTM cells. Data are represented as mean ± SEM μM of nitrite released in culture medium. * Denotes statistical significance from vehicle control + ET-1 (100 nM) treatment vs that from vehicle control, ** denotes statistical significance from vehicle control + BQ788 (1 μM) + ET-1 (100 nM) vs that from vehicle control + ET-1 (100 nM), and *** denotes statistical significance from Dex + ET-1 (100 nM) treatment vs that from vehicle control + ET-1 (100 nM) as determined by One-way ANOVA and Student-Newman-Keuls multiple comparison test ($p < 0.05$) in three repeated experiments on NTM cells.

Fig. 6


Nitrite Release (μM)/24 Hours
10
9
8
7
6
5
4
3
2
1
0
*
***
**
Con
Dex
Con+ET-1
Dex+ET-1
Con+ET+BQ788
Con+BQ788

CHAPTER IV

EXPRESSION AND TRANSLOCATION OF GLUCOCORTICOID RECEPTOR β AND ITS REGULATION OF GLUCOCORTICOID RESPONSIVENESS IN TRABECULAR MESHWORK CELLS

Xinyu Zhang, Abbot F. Clark, and Thomas Yorio

(To be submitted)

Abstract

Glucocorticoids (GC) can increase aqueous humor outflow resistance via morphological and biochemical changes in the trabecular meshwork (TM) that typically result in an increase in intraocular pressure (IOP). Glucocorticoids have also been associated with primary open angle glaucoma (POAG), however individual sensitivities to glucocorticoids differ considerably. About one in every three people in the general population is considered as a potential steroid responder while almost all POAG patients are steroid responders. The molecular mechanisms responsible for glucocorticoid sensitivities are unknown. The human glucocorticoid receptor (hGR) gene undergoes alternative splicing and generates two protein isoforms: a cytoplasmic alpha form (hGRα), which functions as a ligand-dependent transcription factor to regulate gene expression, and a beta isoform (hGRβ), which does not bind ligands and has been shown to attenuate hGRα action. We detect hGRβ not only in the cytoplasm but also in the nucleus in different TM cell lines. Most normal TM cell lines have relatively high amounts of hGRβ expression, especially in the nuclear region compared to glaucomatous TM cell lines. Moreover, overexpression of hGRβ in TM cells attenuates the induction of myocilin by dexamethaonse (Dex) treatment supporting the conclusion that hGRβ acts as a negative regulator of hGRα activity. As detected by coimmunoprecipitation, hGRβ can complex with heat shock protein 90 (hsp90). Interestingly, in the TM cells, which have high amount of hGRβ in the nucleus, hsp90 is also concentrated in the nucleus. When hGRβ is overexpressed in TM cells, both hGRβ and hsp90 accumulate in the nucleus, and 17AAG (17-allylamino, 17-demethoxygeldanamycin, a geldanamycin derivative), an

inhibitor of hsp90, blocks this nuclear accumulation of hGRβ and hsp90. Taken together, our results suggest that glucocorticoid responsiveness in TM cells is regulated by the expression of hGRβ in TM cells, and Hsp90 is a chaperon protein for nuclear transport of hGRβ.

Introduction

Glaucoma is the second leading cause of blindness in the world[1] and primary open angle glaucoma (POAG) is the most prevalent form of glaucoma that is generally associated with an elevated intraocular pressure (IOP).[2, 3] Glucocorticoids (GCs), potent immunosuppressants and anti-inflammatory reagents, are also associated with POAG.[4, 5, 6] Glucocorticoids change the morphology and biochemical activity of trabecular meshwork, including reorganize cytoskeletal structure,[7, 8, 9] increase extracellular matrix production;[10, 11, 12, 13] alter extracellular metalloproteinase activity;[14, 15] increase cell adhesion molecules;[16] and enhance the expression of myocilin, a glaucoma gene.[17, 18] Overall, glucocorticoids increase extracellular material deposition in trabecular meshwork and subsequently increase outflow resistance with a subsequent increase in IOP. However, individual sensitivities to glucocorticoids differ considerably. POAG patients have a much higher prevalence of glucocorticoid responsiveness than do normal subjects, with almost all POAG patients being considered GC responders, compared with one third of the normal population.[19] The molecular basis for the variance in GC responsiveness is unknown.

The first isolation and cloning of the human glucocorticoid receptor described two distinct isoforms termed hGRα and hGRβ, which were formed via alternative splicing and differed only at their carboxyl termini.[20] hGRα predominantly exists in the cytoplasm and undergoes ligand-dependent translocation to the nucleus,[21, 22, 23] where it regulates transcription of hormone-sensitive genes. In contrast, hGRβ is predominantly located in the nucleus, does not bind to glucocorticoids and antiglucocorticoids and lacks

transcriptional activity by itself.[20, 24] In addition, hGRβ main action appears to be in its ability to attenuate the gene transcription regulation of hGRα.[25, 24, 26, 27] The increased expression of hGRβ has been shown to be associated with glucocorticoid resistance in some diseases,[28, 29, 30, 31] with an extensive study in asthma.[32, 33, 34, 35, 36] Glucocorticoid receptors are capable of shuttling between cytoplasmic and nuclear compartments.[37, 38] Upon binding of a glucocorticoid, hGRα is translocated from the cytoplasm to the nucleus along microtubular tracks, requiring an association with heat shock protein 90 (hsp90), immunophilin, and the motor protein dynein.[39, 40] hGRβ can also complex with hsp90.[26, 41] However, no information is available concerning the translocation of hGRβ.

In the present study, we investigate the potential role of hGRβ in regulating glucocorticoid sensitivity in glaucoma. We demonstrate there is significant difference of expression of hGRβ between TM cell lines derived from normal subjects and that from POAG patients, consistent with the considerably different glucocorticoid sensitivity between these two groups. We also directly evaluated the negative activity of hGRβ in inhibiting the induction of myocilin, a glaucoma gene, by dexamethasone. Finally, we demonstrated, for the first time, that hsp90 is a chaperon for the nuclear import of hGRβ.

Materials and Methods

Cell Culture. Eleven human TM cell lines were generated as previously described.[10, 42, 43] Four primary normal TM cell lines (SNTM, SNTM153-00, SNTM302-00, NTM339-

02) and a stable transformed TM cell line (HTM-5) were derived from donors (ages 79 year, 58 year, 77 year, 6 day, and 18 year old respectively) with no reported history of glaucoma. The five primary glaucomatous TM cell lines (GTM956-99, SGTM152-99, GTM602-02, GTM626-02, GTM554-99) and a stable transfromed TM cell line (HTM-3) were derived from donors (ages 75, 79, 94, 78, 80, and 72 year old respectively) with a documented history of POAG. Early passages of TM cells were grown to confluence on 100 mm culture dishes or glass coverslips in 37°C and 5% CO_2 in DMEM (Gibco) supplemented with 10% FBS and penicilin/streptomycin/glutamate (Gibco) and 44 mM $NaHCO_3$. The effect of glucocorticoids on the hGRα and hGRβ receptors in TM cells was examined by addition of 100 nM dexamethasone (Dex, Sigma Chemical) for 3 days in 5% FBS DMEM.

Western Blot Analysis. The cytoplasmic and nuclear fractions were isolated as described previously.[39] SDS-PAGE was performed on 4-15% gradient gels (Bio-RAD). Membranes were then incubated with polyclonal anti-hGR SC-1003 (Santa Cruz) or anti-hGRβ PA3-514 (Affinity Bioreagents) at a dilution of 1:200 or 1:500 respectively. Membranes were also incubated with monoclonal anti-histone1 (Santa Cruz) at 1: 200 dilution as a control for separation of cytoplasmic and nuclear fractions. Immunoreactivity was detected by using enhanced chemiluminescence (ECL, Amershem Pharmacia).

Immunocytochemistry. TM cells were grown on glass coverslips to confluence, then fixed in 4% paraformaldehyde for 30 min, permeabilized in 0.2% Triton X-100 for 15 min, and incubated in 0.2M glycine for 30 min. After blocking for 20 min with 5% bovine serum albumin + 5% normal goat serum, these cells were incubated overnight at

4°C with either anti-hGRβ alone, or anti-hGRβ + anti-hsp90, or anti-hGRβ + anti-β-tubulin (Santa Cruz). Subsequently, the cells were treated for 1 h with either Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probe), or Alexa Fluor 594 goat anti-rabbit IgG + Alexa Fluor 488 goat anti-mouse IgG (Molecular Probe) at a dilution of 1:200. Finally the cells were incubated with 300 nM DAPI to stain nuclear regions. Images were viewed under a Nikon Diaphot Fluorescence Microscope and image analysis was performed using image analysis software Scanalytics IPLab. For confocal microscopy, Alexa Fluor 633 goat anti-rabbit IgG was used instead of Alexa Fluor 594 goat anti-rabbit IgG. Confocal immunofluorescence was performed using Zeiss LSM-410 Confocal Scanning Laser Microscope System.

Coimmunoprecipitation. Immunoprecipitation of hGRβ-hsp90 complex for cytoplasmic and nuclear fractions of TM cells was performed according to the methods described previously (Oakley, 1999). First anti-hsp90 antibody was used to immunprecipitate and anti-hGRβ antibody was used in immunoblotting, then we used the same protocol but reverse the sequence of antibody administration by using anti-hGRβ to run immunoprecipitation and using anti-hsp90 to perform immunoblotting.

Human GRβ Constructs and Transfection. hGRβ expression plasmid pCMX-hGRβ was prepared by modifying the plasmid pCMX-hGRα, which was initially modified from plasmid pCMX.[44] hGRα expression plasmid pCMX-hGRα was made using PCR to amplify hGRα cDNA with adding Asp718 site to 5' and Bam HI site to 3' and cloning this cDNA into Asp718/Bam HI sites of pCMX. hGRβ expression plasmid pCMX-hGRβ

was generated using PCR of the hGRβ receptor specific sequence with adding EcoRI site to 5' and Bam HI site to 3' and inserting this fragment into EcoRI/Bam HI sites of pCMX-hGRα. Identity of the inserted cDNA was confirmed by sequencing. Confluent TM cells were transfected using calcium phosphate reagent according to the manufacture's recommendations (BD Biosciences). Cells were switched to serum-free media 24 h post-transfection and cytoplasmic and nuclear lysates were isolated for Western Blotting for hGRβ, hsp90 or myocilin. In some experiments, the cells were imaged for hGRβ and hsp90 under confocal microscopy after transfection.

17AAG Treatment. For the study of nuclear translocation of hGRβ, Normal HTM-5 cells were transfected with hGRβ as stated above. 17AAG (17-allylamino, 17-demethoxygeldanamycin)[45] (gift from Dr. Thomas Mueller, Kosan Biosciences, Inc.), an inhibitorof hsp90 was added at a final concentration of 1 μM during transfection and post-transfection. Cells underwent immunocytochemistry analysis as stated above for hGRβ and hsp90 using confocal microscopy.

RESULTS

Expression of glucocorticoid receptor isoforms hGRα and hGRβ in cultured TM cell lines. Almost all known effects of glucocorticoids are mediated via the hGRα glucocorticoid receptor. The trabecular meshwork appears to be the main target of glucocorticoids in inducing ocular hypertension. It is known that trabecular meshwork cells express the glucocorticoid receptor (hGR),[46] however the isoforms of hGR have not

been specified in TM cells. To address this question, we examined the expression of hGRα and hGRβ in the cytoplasmic and the nuclear fractions of several cultured primary and transformed TM cell lines. Western Blotting only detected histone 1 in the nuclear fractions (Fig. 1) indicating we got clear separation of cytoplasmic and nuclear fractions. Both normal and POAG TM cell lines expressed hGRβ proteins as detected as a 90 kDa doublet protein by epitope-specific hGRβ protein in both cytoplasmic and nuclear fractions (Fig. 1). 100 nM Dex treatment for 72 h did not change the amount of hGRβ receptor expression. This data also demonstrated that the hGRβ isoform is not susceptible to hormone-induced down-regulation and persists in the cells despite glucocorticoid administration.

Western Blot analysis also detected the hGRα receptor protein at a molecular weight of 95 kDa in both normal and POAG TM cell lines. 100 nM Dex treatment caused hGRα translocation from the cytoplasmic to the nuclear fractions and also time-dependent homologous down-regulation of hGRα (Fig. 2).

Differential expression of hGRβ between normal and glaucomatous TM cell lines. Glucocorticoid responsiveness varies with a far higher prevalence among POAG patients than among normal subjects.[19] The molecular mechanism underlies the higher GC responsiveness among POAG patients is unknown. hGRβ has become a candidate for glucocorticoid resistance in some diseases, especially in asthma [47] based on the reports of its negative activity.[24, 25, 26, 27] We compared the expression and subcellular distribution of hGRβ between normal TM cell lines and POAG TM cell lines. Immunocytochemisty

detected hGRβ staining in both the cytoplasm and the nucleus, and 100 nM Dex treatment for 72 h had no effect on the expression of hGRβ in all these normal and POAG TM cell lines (Fig. 3, Fig. 4). However, four among the five normal TM cell lines (SNTM, HTM-5, SNTM302-00, and SNTM153-00) had relatively high amount of hGRβ staining and more concentrated in the nuclear regions, except one normal TM cell line (NTM334-02, 6 day old), which had low immunoreactivity of hGRβ in the nucleus. Whereas all POAG TM cell lines (GTM956-99, HTM-3, SGTM152-99, GTM602-02, GTM626-02, GTM554-99) expressed low amount of hGRβ, particularly lower in the nucleus. These immunostaining data indicated that most POAG TM cell lines had a lower expression of hGRβ, especially in the nucleus compared to normal TM cell lines.

Inhibitory effect of hGRβ on Dex-induced expression of myocilin. Since POAG TM cell lines had a relatively low expression of hGRβ, which is consistent with high sensitivity to glaucocorticoids among POAG patients, we directly tested the effect of overexpression of hGRβ in the expression of a glaucomatous gene, myocilin, which can be induced by Dex treatment. Transfected HTM-5 cells with pCMX-hGRβ increased the immunostaining of hGRβ as detected by confocal microscopy (Fig. 5A and 5B). This data showed that transfection with pCMX-hGRβ could overexpress hGRβ in HTM-5 cells and again, demonstrated the specificity of hGRβ antibody. Western Blot analysis also detected the increase in the amount of nuclear hGRβ after transfection with the hGRβ expression vector, while Dex treatment for 72 h did not change the amount of hGRβ detected (Fig. 5C). Whereas, 100 nM Dex treatment for 72 h significantly induced

the expression of myocilin in the cytoplasmic fractions, and this induction was inhibited by overexpression of hGRβ (Fig. 5C).

The association of hGRβ with microtubules. Conventional immunofluorescence staining of hGRβ using anti-hGRβ antibody in SNTM (normal) cells showed a fibrous distribution nature in the cytoplasm (Fig. 6A). It seems that the hGRβ receptor aligns itself with the cytoskeleton structure in these SNTM cells, whereas the immunostaining of hGRβ in SGTM152-99 (glaucomatous) cells showed a punctuate pattern (Fig. 6B). Further study of the cytoplasmic distribution pattern of hGRβ was performed by using a conventional immunofluorescence double staining for hGRβ (red) and β-tubulin (green). The staining of hGRβ was closely associated and in some points, colocalized with microtubule tracks in both SNTM cells (Fig. 7A) and SGTM152-99 cells (Fig. 7B). The punctuate staining of cytoplasmic hGRβ in SGTM 152-99 could reflect the low expression of hGRβ in this glaucomatous TM cell line. The association of hGRβ with microtubules could play a role in the transport of hGRβ to the nucleus.

The chaperon effects of hsp90 in nuclear transportation of hGRβ. Since hsp90 is known to be involved in the translocation of hGRα from the cytoplasm to the nucleus,[39, 48, 49, 50] and hGRβ is identical with hGRα through most sequences except the last 27 amino acids,[51] we were interested in the role of hsp90 in the cytoplasm to the nuclear transport of hGRβ. Using SNTM (normal) cells, co-immunoprecipitation studies of hGRβ using anti-hGRβ antibody demonstrated that hsp90 was associated with hGRβ as Western Blot analysis using anti-hsp90 was able to detect hsp90 protein in the nuclear

fraction (Fig. 8A). In addition, when reversed the sequence of antibodies, immunoprecipitation of hsp90, using anti-hsp90 antibody, was able to precipitate hGRβ as the immunoblot was able to detect hGRβ protein following immunoprecipitation (Fig. 8B). These data supported that hGRβ could complex with hsp90.

Confocal microscopy detected a high amount of hGRβ staining in the nucleus in SNTM (normal) cells (Fig. 9A). Interestingly, hsp90 also accumulated in the nucleus (Fig. 9B) and both hGRβ and hsp90 colocalized in the nucleus of these normal TM cells (Fig. 9C). In contrast, SGTM1529-99 (glaucomatous) cells had a much lower nuclear staining of hGRβ (Fig. 9D), and consequently, hsp90 was also lower in the nucleus (Fig. 9E) and there was not much colocalization of hGRβ and hsp90 in the nucleus in this POAG TM cells (Fig. 9F). Furthermore, transfection of HTM-5 cells with a pCMX-hGRβ vector produced a hGRβ overexpression and accumulation of hGRβ in the nucleus in these TM cells (Fig. 10A). Consequently, hsp90 was also concentrated and colocalized with hGRβ in the nucleus in the cells which overexpressed hGRβ, but not in the cells which were not transfected (Fig. 10B and 10C). This coincidence of nuclear localization of hGRβ and hsp90 and changes in their co-expression following hGRβ transfection suggested that hsp90 associates with hGRβ and is most likely the nuclear transport chaperon of hGRβ.

17AAG is an hsp90 inhibitor, which binds to hsp90 and inhibits hsp90 association with client proteins. Such as action by 17AAG simultaneously leads to degradation of these client proteins[45]. As reported above, HTM-5 cells transfected with pCMX-hGRβ

caused accumulation of both hGRβ and hsp90 in the nucleus in these cells which overexpressed hGRβ (Fig. 11A, B and C). However 1 μM 17AAG treatment completely blocked the nuclear import of hGRβ in these HTM-5 cells transfected with hGRβ expression vector. This data confirmed that hsp90 was a chaperon protein for the cytoplasm to the nuclear transport of hGRβ.

Discussion

The molecular basis of variable glaucocorticoid sensitivities as pertaining GC-induced ocular hypertention is poorly understood. There are some reports that hGRβ has a negative activity to glucocorticoid-regulated response[24, 25, 26, 27] and hGRβ becomes a potential candidate for GC-resistance in asthma.[47] We detected that hGRβ was expressed in cultured normal and POAG TM cell lines. Although the amino acid sequences of hGRα and hGRβ isoforms are identical except the carboxyl termini,[20] the anti-hGRβ antibody we used was specific for hGRβ, because this antibody only recognized protein bands with a molecular weight of 90 kDa, the size of hGRβ, and unlike hGRα, this receptor protein did not undergo ligand-dependent nuclear translocation and homologous down-regulation of receptors. Furthermore, when we transfected TM cells with an hGRβ expression vector, this antibody was able to detect the overexpression of hGRβ in these transfected cells. By using 4-15% gradient gels, we were able to detect the doublets of hGRβ pretein. The double bands of hGRβ protein would be due to an alternative translation site for the GR gene.[52]

By using two different techniques, Western Blotting and immunocytochemisty, we localized hGRβ in the cytoplasm and the nucleus in cultured primary and transformed TM cell lines. Some cell strains had high amount of hGRβ in the nucleus, while others had hGRβ staining in the cytoplasm but lower in the nucleus. Previous reports as to the subcellular localization of hGRβ have been varied. Oakley RH et al [53] showed that hGRβ was located in the nucleus, however other groups[54, 41] reported that hGRβ is present in the cytoplasm. The apparent differences in reported subcellular distribution of hGRβ could be attributed to tissue specificity, differential expression regulation, variable intracellular stability, or variance in nuclear transportation efficiency. In the current study, both cytoplasmic and nuclear hGRβ was detected and that the amount present in the nucleus can be influenced by overexpression.

The striking finding in this study was the significant difference of the hGRβ amount among normal versus glaucomatous cell lines. POAG TM cell lines had lower hGRβ receptor expression than normal TM cell lines. This is coincidence with the fact that in the normal population, there is a low rate of GC responders as compared to almost all of the POAG patients considered as GC responders.[19] These results suggest that low expression of hGRβ could lead to glucocorticoid sensitivity in TM cells. In order to directly test that the amount of hGRβ can regulate glucocorticoid responsiveness, we generated a hGRβ expression vector pCMX-hGRβ and increased cellular hGRβ amount in TM cells by transfecting this construct, followed by detecting the induction of myocilin expression after challenging these TM cells with dexamethsone. Although

mutant myoclin is associated with POAG,[55] the function of myocilin in trabecular meshwork remains unknown. The myocilin gene is known to be upregulated by glucocorticoids, like Dex, and can be used as an index of glucocorticoid responsiveness. We found that higher levels of hGRβ could indeed act to negatively regulate the activity of hGRα, as overexpression of hGRβ inhibited Dex-induced myocilin expression in TM cells. Our studies, therefore, identify a possible molecular mechanism by which glucocorticoid responsiveness is achieved as a result of the low level of the negative isoform receptor hGRβ.

Although there are intensive studies and many new findings on the nuclear translocation of hGRα,[39, 40, 56, 57, 58] the nuclear import of hGRβ is completely unknown. Currently we demonstrated that the cytoplasmic distribution of hGRβ was closely associated with microtubules in both normal and glaucomatous TM cell lines. This suggested that microtubules may play a role in the cytoplasmic to the nuclear translocation of hGRβ.

We also identified a chaperon protein, hsp90, as a requirement for the cytoplasmic to nuclear translocation of hGRβ. Several findings support this mechanism. We found that in the normal TM cell line SNTM, which had a high level of hGRβ in the nucleus, hsp90 was also concentrated in the nuclear region, while in the glaucomatous TM cell line SGTM152-99, hGRβ and hsp90 were both present at lower concentration in the nucleus. Transfection with the hGRβ construct in HTM-5 cells caused the overexpression and accumulation of hGRβ protein in the nucleus that also leaded to an increase in hsp90

level in the nucleus in these transfected HTM-5 cells. Most importantly, 17AAG, an inhibitor of hsp90, which blocks the binding of client proteins to hsp90 and prevents chaperon activity as well as promoting the degradation of client proteins,[45] completely blocked the accumulation of hGRβ in the nucleus in these transfected HTM-5 cells. Interestingly there was also a decrease in the accumulation of hGRβ in the cytoplasm. This could be attributed to the degradation of hGRβ in the cytosol under 17AAG treatment. The data indicated that accessibility of hGRβ to hsp90 was required for the nuclear import of hGRβ. Although like hGRα,[39] the nuclear transportation of hGRβ needs the association with hsp90, the transport machinery for hGRα or hGRβ may be not exactly same because the hGRα undergoes hormone-dependent nuclear translocation but hGRβ transports to the nucleus independent on hormones.[20] Further experiments are needed to delineate other components that may be involved in the regulation of the cytoplasm to nuclear translocation of hGRβ.

In conclusion, we have demonstrated a possible molecular mechanism by which glucocorticoid responsiveness is expressed in individuals susceptible to increase IOP following steroid therapy. The mechanism is related to the relative expression of the hGRβ isoform in the nucleus of TM cells. Furthermore, we have, for the first time, identified hap90 as a chaperon protein for the translocation of hGRβ from the cytoplasm to the nucleus.

FIGURE 1. Western Blot analysis of hGRβ in cytoplasmic and nuclear fractions from normal and glaucomatous TM cell lines in the presence and absence of Dex treatment. Immunoblot of hGRβ isoforms is performed by using an isoform-specific polyclonal antibody. TM cells were treated with 100 nM Dex or control vehicle for 72 h. Hela cell lysate was used as a positive control. Histone 1 was used as a nuclear marker. (A) Normal TM cell lines. (B) Glaucomatous TM cell lines.

FIGURE 1

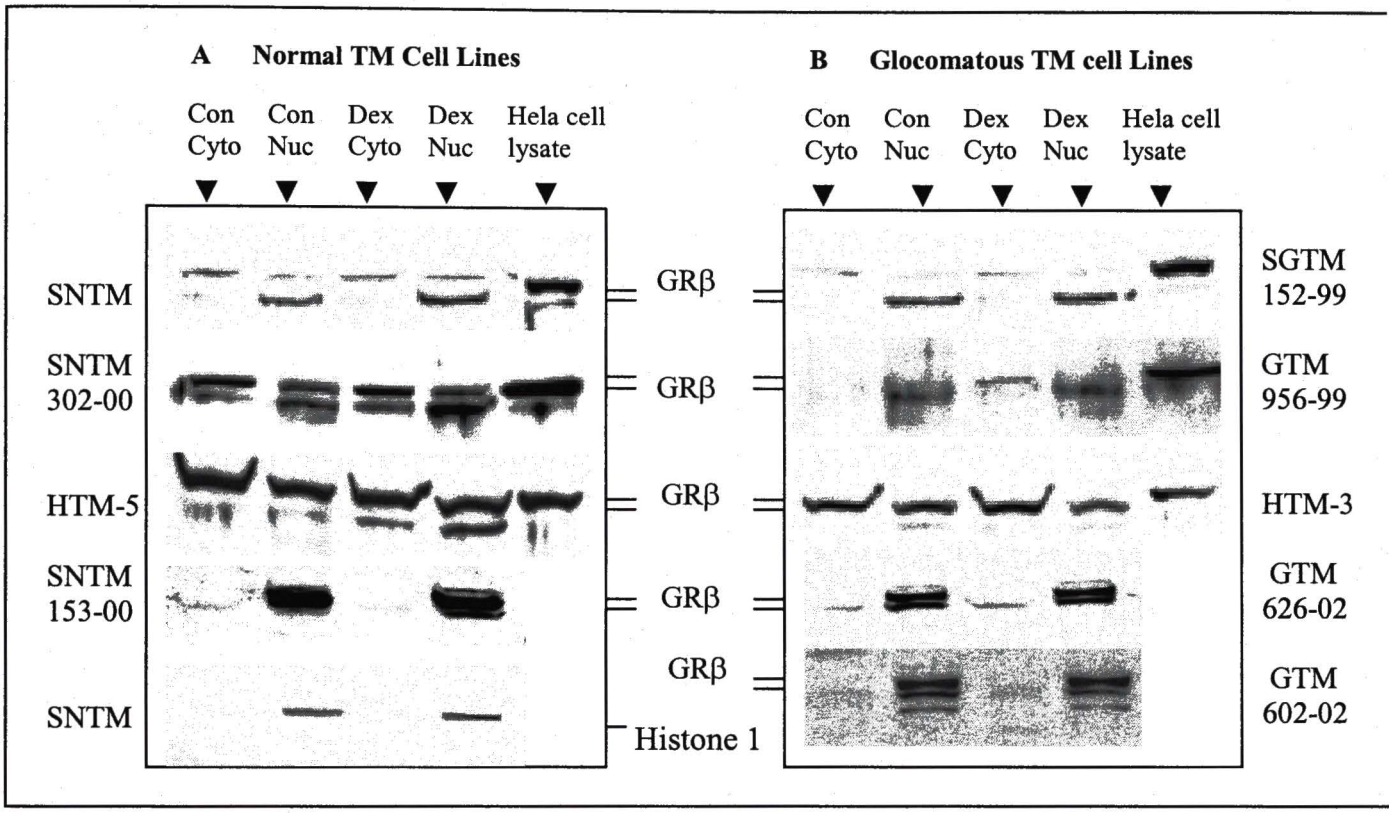

A Normal TM Cell Lines
Con Cyto
Con Nuc
Dex Cyto
Dex Nuc
Hela cell lysate
SNTM
SNTM 302-00
HTM-5
SNTM 153-00
SNTM
GRβ
GRβ
GRβ
GRβ
GRβ
Histone 1
B Glocomatous TM cell Lines
Con Cyto
Con Nuc
Dex Cyto
Dex Nuc
Hela cell lysate
SGTM 152-99
GTM 956-99
HTM-3
GTM 626-02
GTM 602-02

FIGURE 2. Western Blot analysis detecting the translocation of hGRα after Dex treatment for normal and glaucomatous TM cell lines using a polyclonal antibody against general GR. TM cells were treated with control vehicle or 100 nM Dex for 1, 2, 3, 4, 5 days, respectively as indicated.

FIGURE 2

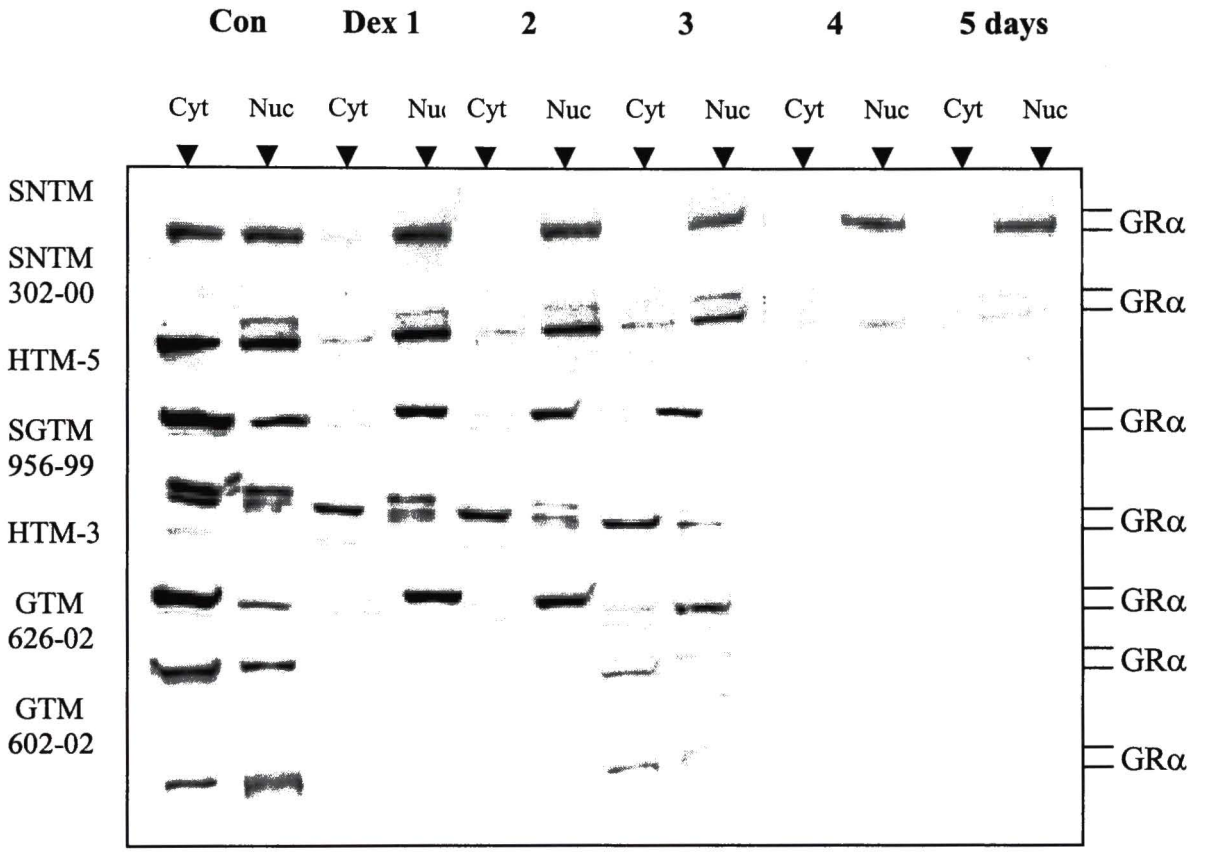

Con
Dex 1
2
3
4
5 days
Cyt
Nuc
SNTM
SNTM 302-00
HTM-5
SGTM 956-99
HTM-3
GTM 626-02
GTM 602-02
GRα
GRα
GRα
GRα
GRα
GRα
GRα

FIGURE 3. Immunocytochemistry analysis of hGRβ expression and distribution in normal TM cell lines. TM cells were treated with 100 nM Dex or control vehicle for 72 h. The negative control staining for hGRβ was performed using the rabbit pre-immune serum. The red color represents hGRβ staining and blue color is DAPI staining for nuclear regions.

FIGURE 3

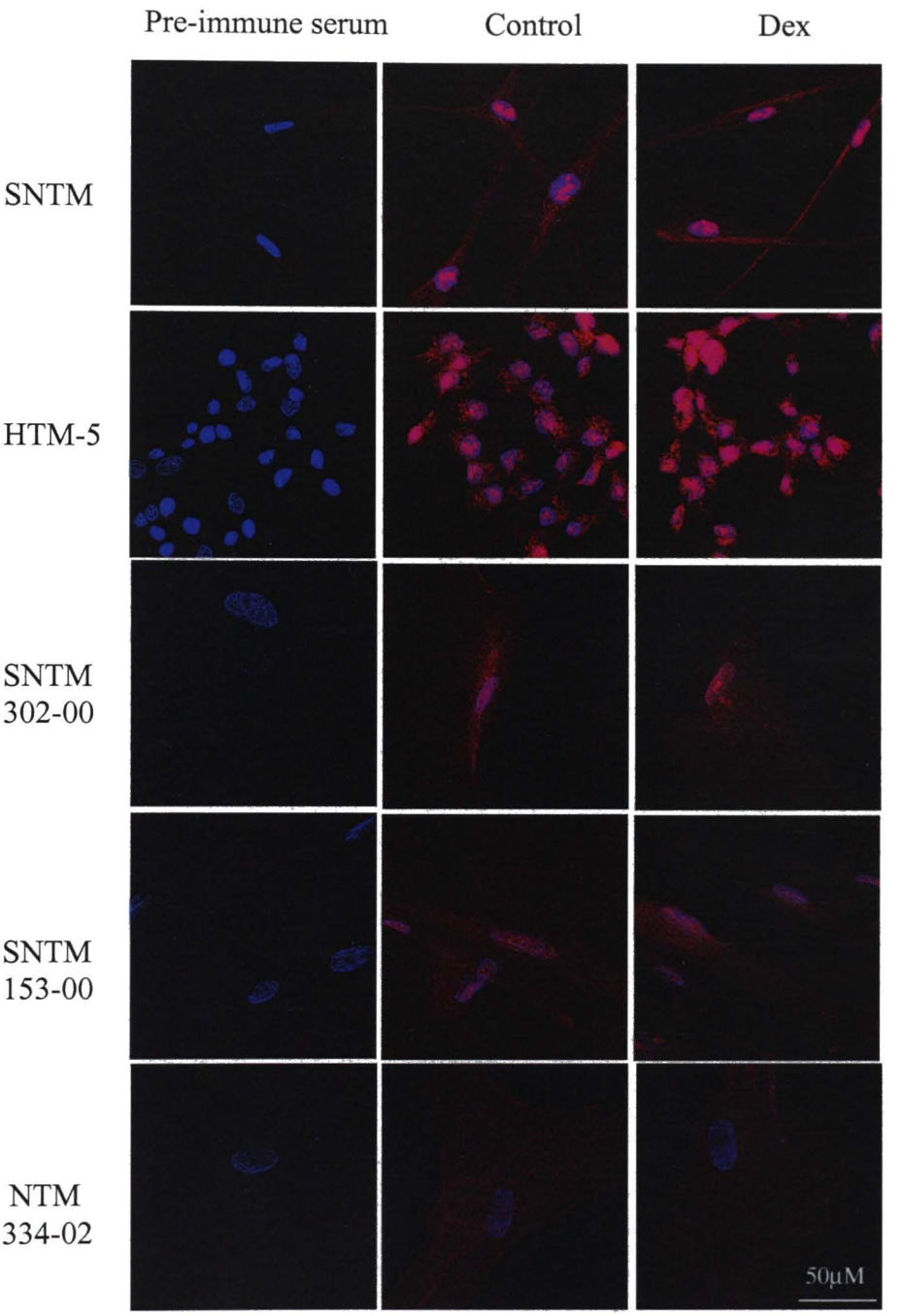

Pre-immune serum
Control
Dex
SNTM
HTM-5
SNTM 302-00
SNTM 153-00
NTM 334-02
50µM

FIGURE 4. Immunocytochemistry analysis of hGRβ expression and distribution in glaucomatous TM cell lines. TM cells were treated with 100 nM Dex or vehicle control for 72 h. The negative control staining for hGRβ was performed using rabbit pre-immune serum. The red color represents hGRβ staining and blue color is DAPI staining for nuclear regions.

FIGURE 4

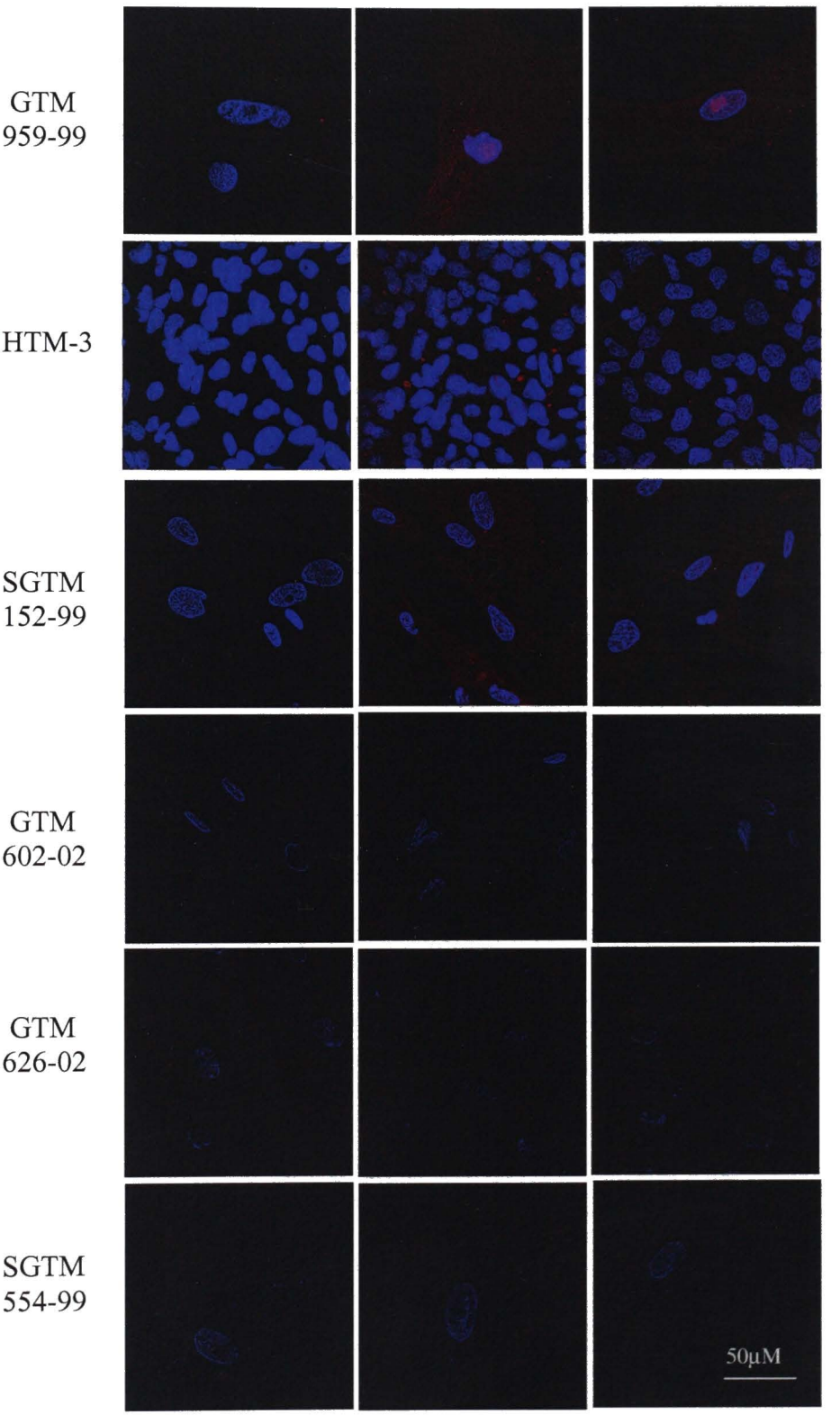

Pre-immune Serum
Control
Dex
GTM 959-99
HTM-3
SGTM 152-99
GTM 602-02
GTM 626-02
SGTM 554-99
50µM

FIGURE 5. Verification of the hGRβ antibody recognizing the hGRβ protein and detecting the effect of increased expression of hGRβ on Dex-induced expression of myocilin in transfected HTM-5 cells. Confocal microscopy detecting (A) Immunostaining of GRβ in HTM-5 cells transfected with control empty vector pCMX, (B) Immunostaining of GRβ in HTM-5 cells transfected with GRβ expression vector pCMX-GRβ. (C) Western Blot detecting the expression of hGRβ in the nuclear fraction and myocilin in the cytoplasmic fraction in transfected HTM-5 cells treated with control or 100 nM Dex in 5% FBS- DMEM for 72 h. Cells were transfected with pCMX and treated with vehicle control (lane 1), or Dex (lane 2), or transfected with pCMX-hGRβ and treated with Dex (lane 3). Histone 1 was used as a control for Western Blot for nuclear fraction and β-tubulin was used as a cytoplasmic fraction Western Blot control.

FIGURE 5

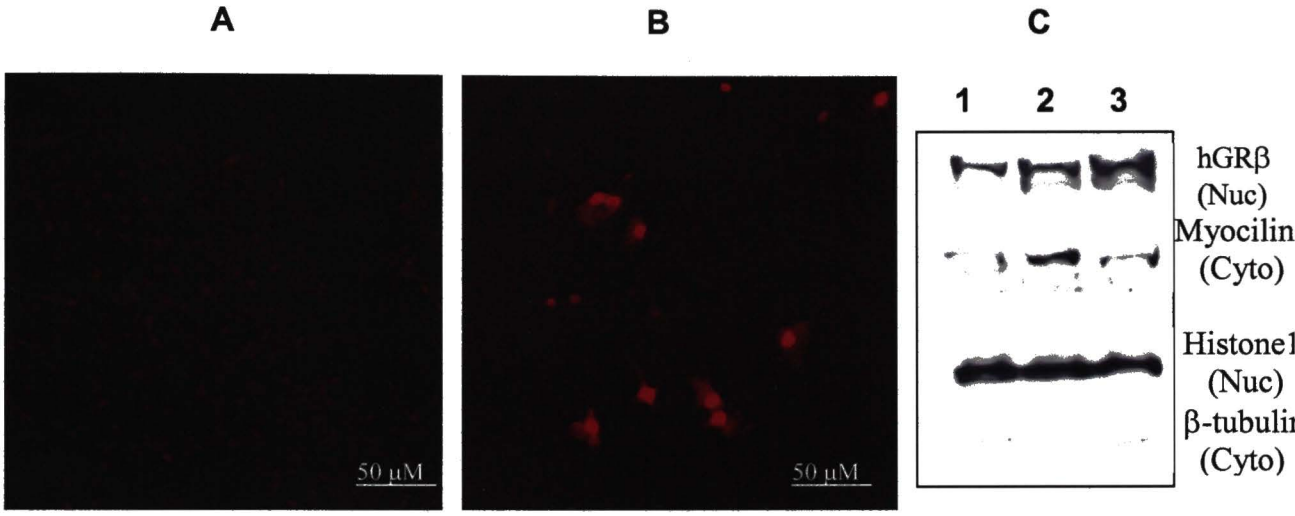
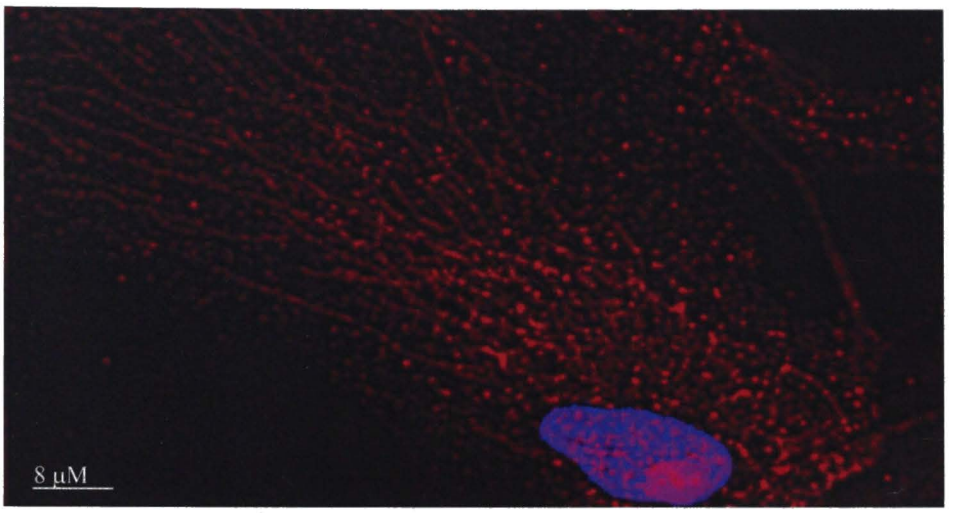

A
B
C
50 µM
50 µM
1
2
3
hGRβ
(Nuc)
Myocilin
(Cyto)
Histone1
(Nuc)
β-tubulin
(Cyto)

FIGURE 6. Illustration of hGRβ subcellular distribution pattern in the cytoplasm. (A) SNTM (normal), (B) SGTM152-99 (glaucomatous) cells were cultured to confluence without any treatment followed by fixation and precessing for immunocytochemisty as describe in Materials and Methods. Immunoreactivity was visualized under conventional immunofluorescence microscopy. Red color represents hGRβ staining and blue is DAPI staining for nucleus.

FIGURE 6

A SNTM



B SGTM 152-99

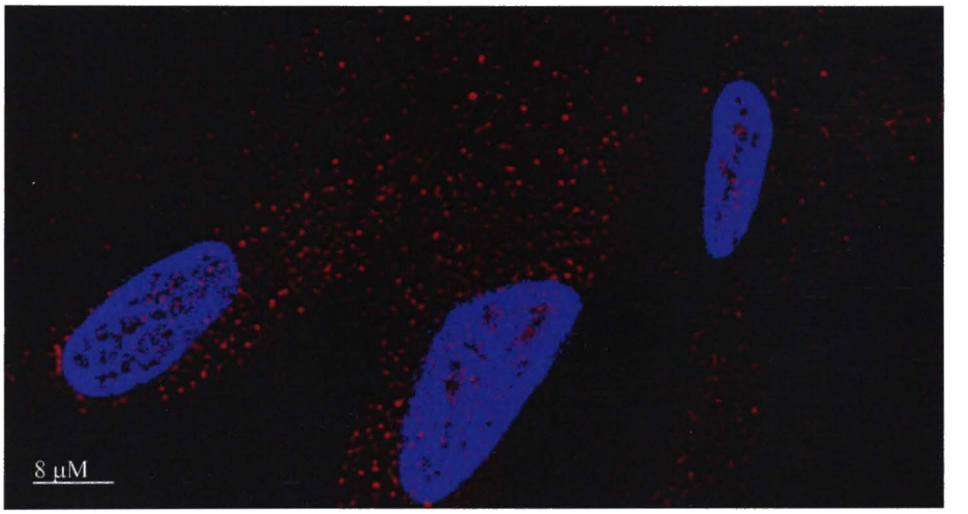
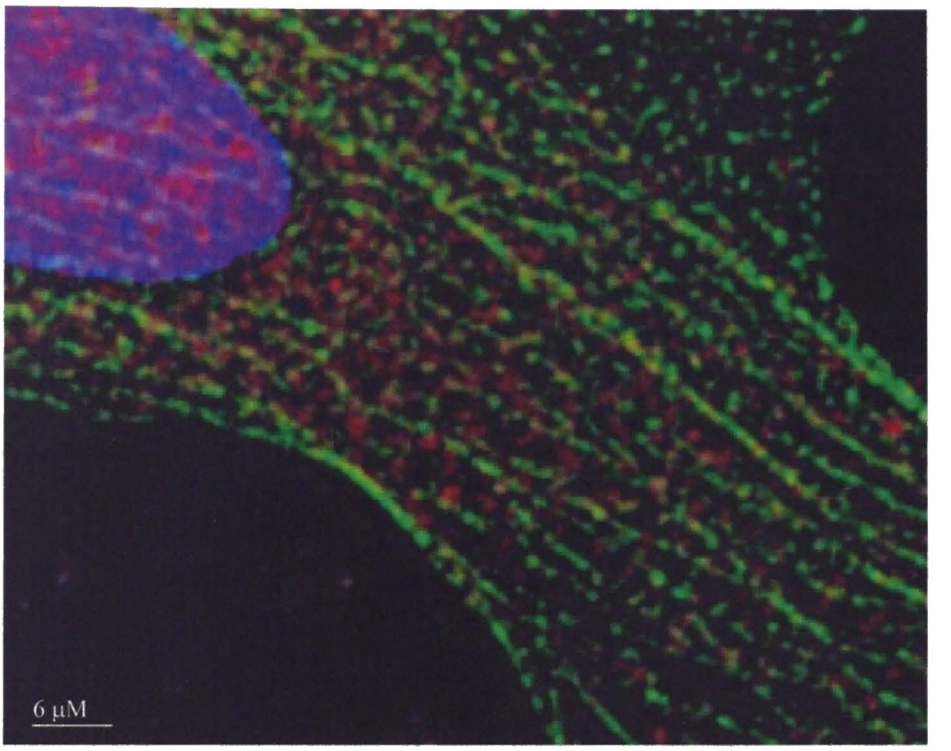


FIGURE 7. Double immunofluorescence microscopy detection of hGRβ and β-tubulin. (A) SNTM (normal), (B) SGTM152-99 (glaucomatous) cells were cultured to confluence without any treatment followed by fixation and double immunostaining for hGRβ (red) and β-tubulin (green) as described in Materials and Methods, to detect the association of hGRβ with microtubules. Blue color is DAPI staining for nucleus. Immunostaining was viewed under conventional immunofluorescence microscopy.

FIGURE 7

A SNTM



B SGTM
152-99

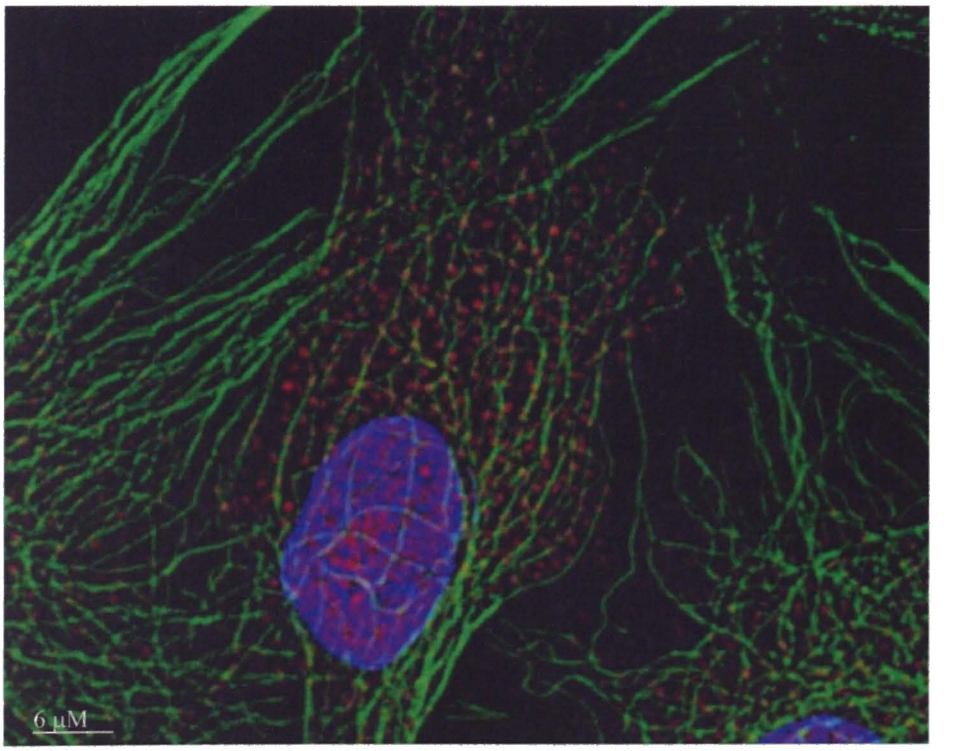


FIGURE 8. Coimmunoprecipitation of hGRβ and hsp90 to detect a hGRβ-hsp90 complex. SNTM (normal) cells were treated with Dex for 72 h followed by preparation of cytoplasmic and nuclear extracts. Coimmunopredipitation was performed as described in Materials and Methods. (A) Immunoprecipitation was carried by using hGRβ antibody and the pellets of immunprecipitation were resolved by Western Blotting analysis using hsp90 antibody. (B) Immnoprecipitiation was performed by using an hsp90 antibody and Western Blotting was used to detect hGRβ using hGRβ antibody. Hela cell lysates (Santa Cruz) was used positive control for Western Blot analysis.

FIGURE 8

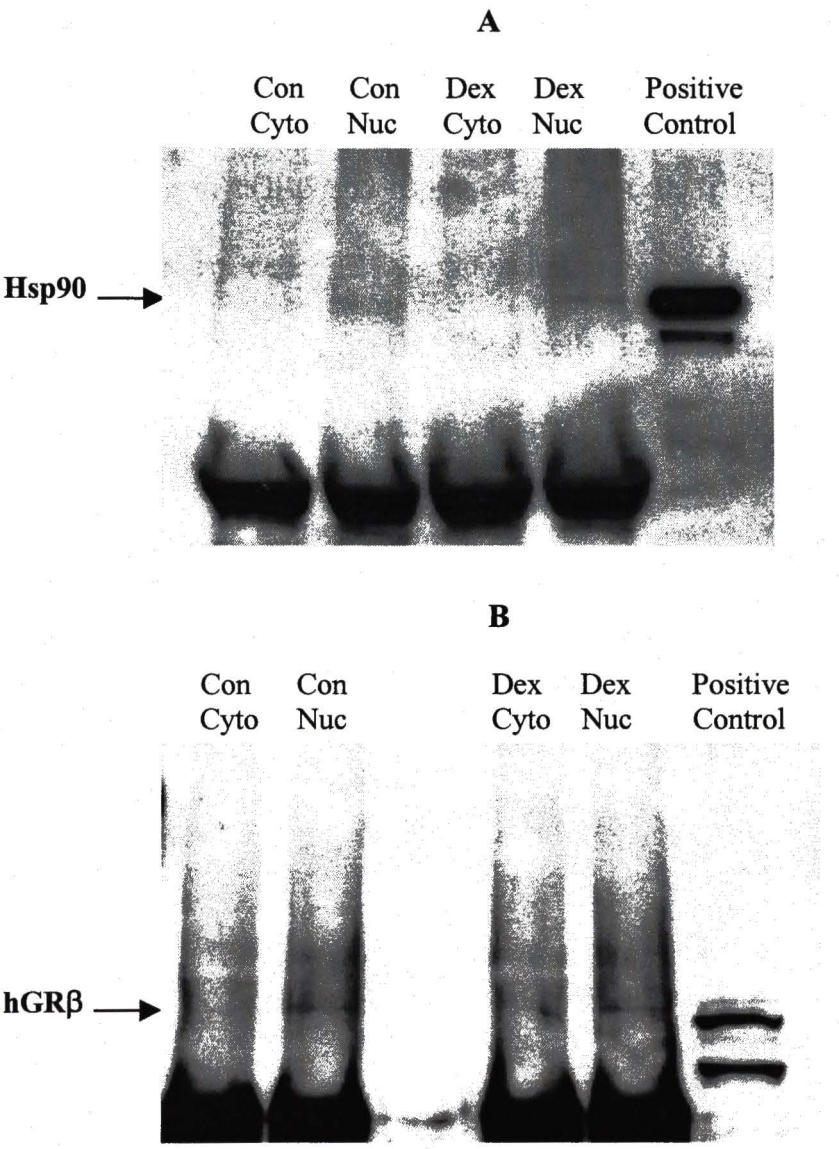

A
Con Cyto
Con Nuc
Dex Cyto
Dex Nuc
Positive Control
Hsp90
B
Con Cyto
Con Nuc
Dex Cyto
Dex Nuc
Positive Control
hGRβ

FIGURE 9. Double immunofluorescence microscopy detection of hGRβ and hsp90. TM cells were cultured to confluence without any treatment followed by fixation and double immunostaining for hGRβ (red) and hsp90 (green) to detect the colocalization of hGRβ with hsp90. (A) hGRβ staining in SNTM (normal) cells. (B) hsp90 staining in SNTM cells. (C) Overlay staining of hGRβ and hsp90 in SNTM cells. (D) hGRβ staining in SGTM152-99 (glaucomatous) cells. (E) hsp90 staining in SGTM152-99 cells. (F) Overlay staining hGRβ and hsp90 in SGTM152-99 cells. Immunostaining was viewed under confocal microscopy (Bar —— stands for 60 μM) .

FIGURE 9.

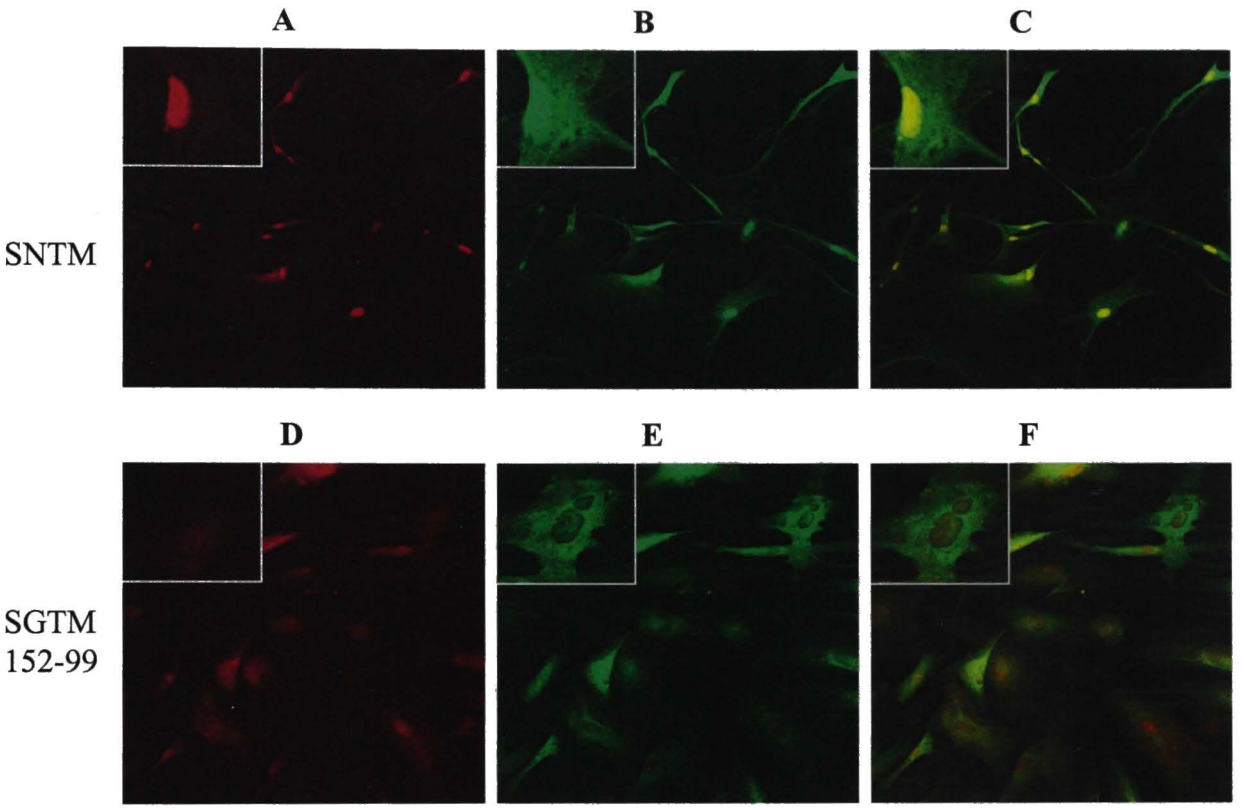


FIGURE 10. Confocal microscopy and Western Blot analysis of hGRβ and hsp90 in HTM-5 cells transfected with pCMX-GRβ. HTM-5 cells were transfected with hGRβ expression vector pCMX-GRβ followed by fixation and double immunostaining or isolation of nuclear fractions for Western Blotting analysis for colocalization of hGRβ and hsp90. In HTM-5 transfected with pCMX-GRβ, (A) hGRβ staining (red), (B) hsp90 staining (green), (C) Overlay staining of hGRβ and hsp90. (D) Western Blot analysis for hGRβ and hsp90 in nuclear fractions of HTM-5 transfected with pCMX (lane 1) and pCMX-hGRβ (lane 2). Histone 1 was used as a Western Blot control for the nuclear fraction (Bar ——— stands for 15 μM).

FIGURE 10

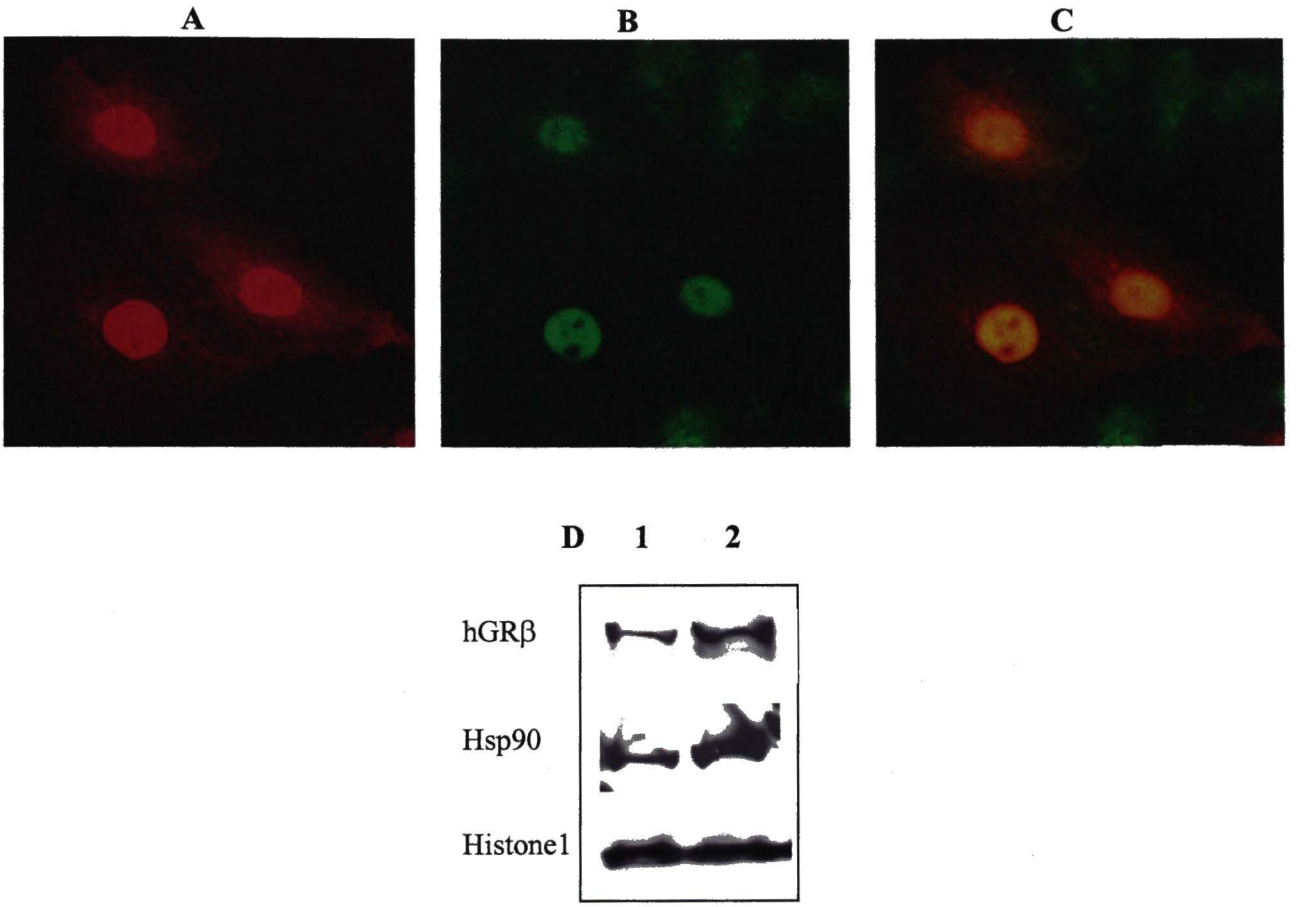
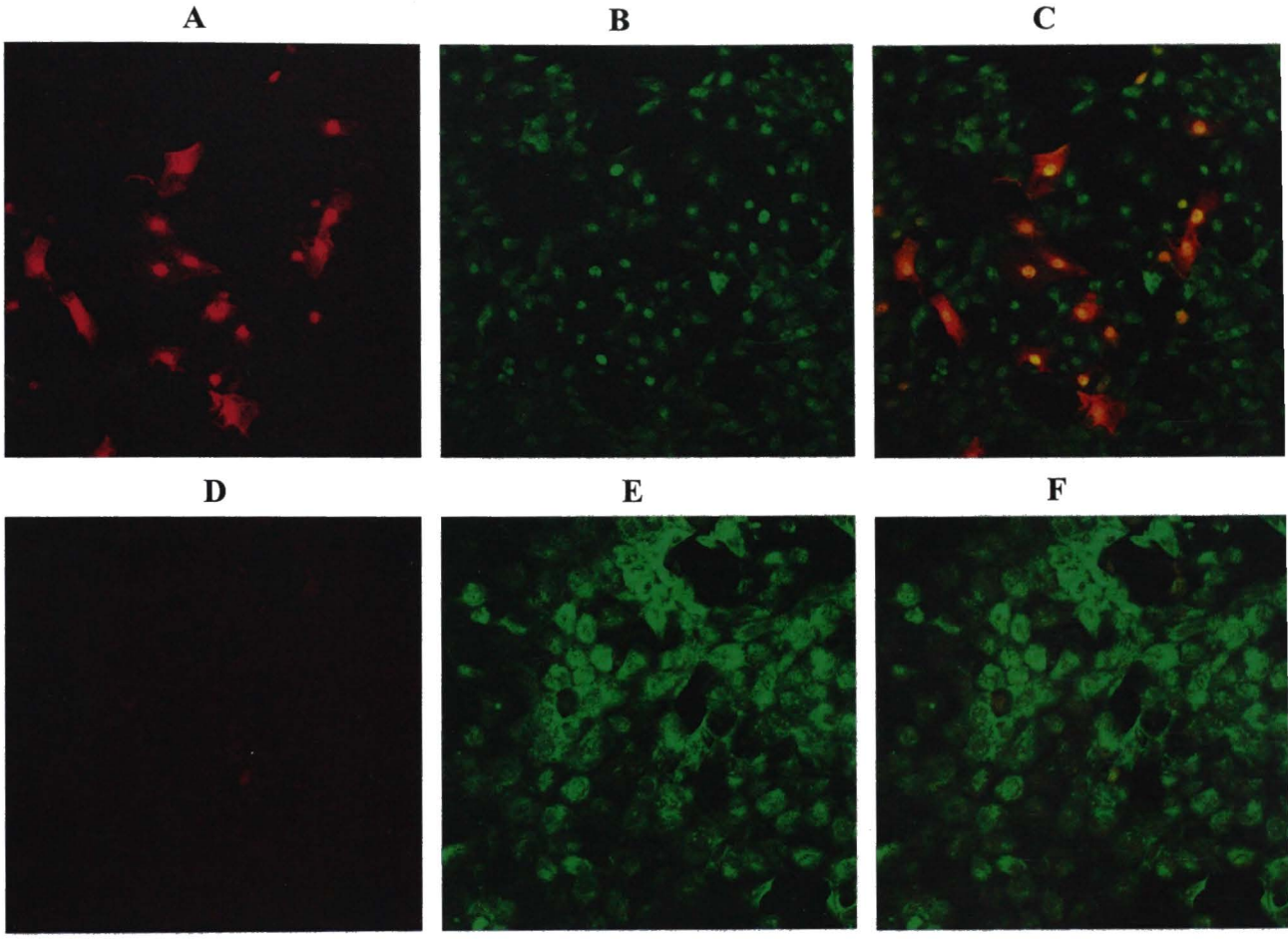


FIGURE 11. Effect of hsp90 on translocation of hGRβ from the cytoplasm to the nucleus in HTM-5 cells transfected with pCMX-GRβ. HTM-5 cells were transfected with hGRβ expression vector pCMX-GRβ and treated with an hsp90 inhibitor, 17AAG, for 20 h as described in Materials and Methods. Followed by fixation and double immunostaining for hGRβ and hsp90, cells were visualized under confocal microscopy to detect nuclear accumulation of hGRβ and hsp90. In HTM-5 cells transfected with pCMX-GRβ, (A) hGRβ staining (red), (B) hsp90 staining (green), (C) Overlay staining of hGRβ and hsp90. In HTM-5 transfected with pCMX-GRβ and treated with 17AAG, (D) hGRβ staining (red), (E) hsp90 staining (green), (F) Overlay staining of hGRβ and hsp90 (Bar _____ stands for 60 μM) .

FIGURE 11



References:

1. Quigley HA. Number of people with glaucoma worldwide. *Br J Ophthalmol.* 1996;80:389-93.

2. Anderson DR. Glaucoma: the damage caused by pressure. XLVI Edward Jackson memorial lecture. *Am J Ophthalmol.* 1989;108:485-95.

3. Sommer A, Tielsch JM, Katz J, et al. Racial differences in the cause-specific prevalence of blindness in east Baltimore. *N Engl J Med.* 1991;325:1412-7.

4. Rozsival P, Hampl R, Obenberger J, Starka L,Rehak S. Aqueous humour and plasma cortisol levels in glaucoma and cataract patients. *Curr Eye Res.* 1981;1:391-6.

5. Schwartz B, McCarty G,Rosner B. Increased plasma free cortisol in ocular hypertension and open angle glaucoma. *Arch Ophthalmol.* 1987;105:1060-5.

6. McCarty GR,Schwartz B. Increased plasma noncortisol glucocorticoid activity in open-angle glaucoma. *Invest Ophthalmol Vis Sci.* 1991;32:1600-8.

7. Clark AF, Wilson K, McCartney MD, Miggans ST, Kunkle M,Howe W. Glucocorticoid-induced formation of cross-linked actin networks in cultured human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 1994;35:281-94.

8. Wilson K, McCartney MD, Miggans ST,Clark AF. Dexamethasone induced ultrastructural changes in cultured human trabecular meshwork cells. *Curr Eye Res.* 1993;12:783-93.

9. Johnson D, Gottanka J, Flugel C, Hoffmann F, Futa R,Lutjen-Drecoll E. Ultrastructural changes in the trabecular meshwork of human eyes treated with corticosteroids. *Arch Ophthalmol.* 1997;115:375-83.

10. Steely HT, Browder SL, Julian MB, Miggans ST, Wilson KL,Clark AF. The effects of dexamethasone on fibronectin expression in cultured human trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 1992;33:2242-50.

11. Yun AJ, Murphy CG, Polansky JR, Newsome DA,Alvarado JA. Proteins secreted by human trabecular cells. Glucocorticoid and other effects. *Invest Ophthalmol Vis Sci.* 1989;30:2012-22.

12. Johnson DH, Bradley JM,Acott TS. The effect of dexamethasone on glycosaminoglycans of human trabecular meshwork in perfusion organ culture. *Invest Ophthalmol Vis Sci.* 1990;31:2568-71.

13. Hernandez MR, Weinstein BI, Dunn MW, Gordon GG,Southren AL. The effect of dexamethasone on the synthesis of collagen in normal human trabecular meshwork explants. *Invest Ophthalmol Vis Sci.* 1985;26:1784-8.

14. Samples JR, Alexander JP,Acott TS. Regulation of the levels of human trabecular matrix metalloproteinases and inhibitor by interleukin-1 and dexamethasone. *Invest Ophthalmol Vis Sci.* 1993;34:3386-95.

15. Snyder RW, Stamer WD, Kramer TR,Seftor RE. Corticosteroid treatment and trabecular meshwork proteases in cell and organ culture supernatants. *Exp Eye Res.* 1993;57:461-8.

16. Underwood JL, Murphy CG, Chen J, et al. Glucocorticoids regulate transendothelial fluid flow resistance and formation of intercellular junctions. *Am J Physiol.* 1999;277:C330-42.

17. Polansky JR, Fauss DJ, Chen P, et al. Cellular pharmacology and molecular biology of the trabecular meshwork inducible glucocorticoid response gene product. *Ophthalmologica.* 1997;211:126-39.

18. Clark AF, Steely HT, Dickerson JE, Jr., et al. Glucocorticoid induction of the glaucoma gene MYOC in human and monkey trabecular meshwork cells and tissues. *Invest Ophthalmol Vis Sci.* 2001;42:1769-80.

19. Lewis JM, Priddy T, Judd J, et al. Intraocular pressure response to topical dexamethasone as a predictor for the development of primary open-angle glaucoma. *Am J Ophthalmol.* 1988;106:607-12.

20. Hollenberg SM, Weinberger C, Ong ES, et al. Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature.* 1985;318:635-41.

21. Picard D,Yamamoto KR. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *Embo J.* 1987;6:3333-40.

22. Wikstrom AC, Bakke O, Okret S, Bronnegard M,Gustafsson JA. Intracellular localization of the glucocorticoid receptor: evidence for cytoplasmic and nuclear localization. *Endocrinology.* 1987;120:1232-42.

23. Cidlowski JA, Bellingham DL, Powell-Oliver FE, Lubahn DB,Sar M. Novel antipeptide antibodies to the human glucocorticoid receptor: recognition of

multiple receptor forms in vitro and distinct localization of cytoplasmic and nuclear receptors. *Mol Endocrinol.* 1990;4:1427-37.

24. Oakley RH, Sar M,Cidlowski JA. The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. *J Biol Chem.* 1996;271:9550-9.

25. Bamberger CM, Bamberger AM, de Castro M,Chrousos GP. Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. *J Clin Invest.* 1995;95:2435-41.

26. Oakley RH, Jewell CM, Yudt MR, Bofetiado DM,Cidlowski JA. The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanisms of action. *J Biol Chem.* 1999;274:27857-66.

27. Yudt MR, Jewell CM, Bienstock RJ,Cidlowski JA. Molecular origins for the dominant negative function of human glucocorticoid receptor beta. *Mol Cell Biol.* 2003;23:4319-30.

28. Honda M, Orii F, Ayabe T, et al. Expression of glucocorticoid receptor beta in lymphocytes of patients with glucocorticoid-resistant ulcerative colitis. *Gastroenterology.* 2000;118:859-66.

29. Liu Y, Song L,Li B. [The expression of glucocorticoid receptor beta messenger RNA in peripheral white blood cells of hormone-resistant nephrotic syndrome patients]. *Zhonghua Nei Ke Za Zhi.* 2001;40:725-8.

30. Derijk RH, Schaaf MJ, Turner G, et al. A human glucocorticoid receptor gene variant that increases the stability of the glucocorticoid receptor beta-isoform mRNA is associated with rheumatoid arthritis. *J Rheumatol.* 2001;28:2383-8.

31. Webster JC, Oakley RH, Jewell CM,Cidlowski JA. Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative beta isoform: a mechanism for the generation of glucocorticoid resistance. *Proc Natl Acad Sci U S A.* 2001;98:6865-70.

32. Adcock IM, Lane SJ, Brown CR, Peters MJ, Lee TH,Barnes PJ. Differences in binding of glucocorticoid receptor to DNA in steroid-resistant asthma. *J Immunol.* 1995;154:3500-5.

33. Leung DY, Hamid Q, Vottero A, et al. Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. *J Exp Med.* 1997;186:1567-74.

34. Hamid QA, Wenzel SE, Hauk PJ, et al. Increased glucocorticoid receptor beta in airway cells of glucocorticoid-insensitive asthma. *Am J Respir Crit Care Med.* 1999;159:1600-4.

35. Sousa AR, Lane SJ, Cidlowski JA, Staynov DZ,Lee TH. Glucocorticoid resistance in asthma is associated with elevated in vivo expression of the glucocorticoid receptor beta-isoform. *J Allergy Clin Immunol.* 2000;105:943-50.

36. Kraft M, Hamid Q, Chrousos GP, Martin RJ,Leung DY. Decreased steroid responsiveness at night in nocturnal asthma. Is the macrophage responsible? *Am J Respir Crit Care Med.* 2001;163:1219-25.

37. Madan AP,DeFranco DB. Bidirectional transport of glucocorticoid receptors across the nuclear envelope. *Proc Natl Acad Sci U S A.* 1993;90:3588-92.

38. Pratt WB,Toft DO. Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev.* 1997;18:306-60.

39. Galigniana MD, Radanyi C, Renoir JM, Housley PR,Pratt WB. Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus. *J Biol Chem.* 2001;276:14884-9.

40. Davies TH, Ning YM,Sanchez ER. A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. *J Biol Chem.* 2002;277:4597-600.

41. Hecht K, Carlstedt-Duke J, Stierna P, Gustafsson J, Bronnegard M,Wikstrom AC. Evidence that the beta-isoform of the human glucocorticoid receptor does not act as a physiologically significant repressor. *J Biol Chem.* 1997;272:26659-64.

42. Tripathi RC,Tripathi BJ. Human trabecular endothelium, corneal endothelium, keratocytes, and scleral fibroblasts in primary cell culture. A comparative study of growth characteristics, morphology, and phagocytic activity by light and scanning electron microscopy. *Exp Eye Res.* 1982;35:611-24.

43. Pang IH, Shade DL, Clark AF, Steely HT,DeSantis L. Preliminary characterization of a transformed cell strain derived from human trabecular meshwork. *Curr Eye Res*. 1994;13:51-63.

44. Umesono K, Murakami KK, Thompson CC,Evans RM. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell*. 1991;65:1255-66.

45. Workman P. Auditing the Pharmacological Accounts for Hsp90 Molecular Chaperone Inhibitors: Unfolding the Relationship between Pharmacokinetics and Pharmacodynamics. *Mol Cancer Ther*. 2003;2:131-8.

46. Stokes J, Noble J, Brett L, et al. Distribution of glucocorticoid and mineralocorticoid receptors and 11beta-hydroxysteroid dehydrogenases in human and rat ocular tissues. *Invest Ophthalmol Vis Sci*. 2000;41:1629-38.

47. Leung DY,Chrousos GP. Is there a role for glucocorticoid receptor beta in glucocorticoid-dependent asthmatics? *Am J Respir Crit Care Med*. 2000;162:1-3.

48. Raaka BM, Finnerty M, Sun E,Samuels HH. Effects of molybdate on steroid receptors in intact GH1 cells. Evidence for dissociation of an intracellular 10 S receptor oligomer prior to nuclear accumulation. *J Biol Chem*. 1985;260:14009-15.

49. Czar MJ, Galigniana MD, Silverstein AM,Pratt WB. Geldanamycin, a heat shock protein 90-binding benzoquinone ansamycin, inhibits steroid-dependent

translocation of the glucocorticoid receptor from the cytoplasm to the nucleus. *Biochemistry*. 1997;36:7776-85.

50. Galigniana MD, Scruggs JL, Herrington J, et al. Heat shock protein 90-dependent (geldanamycin-inhibited) movement of the glucocorticoid receptor through the cytoplasm to the nucleus requires intact cytoskeleton. *Mol Endocrinol.* 1998;12:1903-13.

51. Encio IJ,Detera-Wadleigh SD. The genomic structure of the human glucocorticoid receptor. *J Biol Chem*. 1991;266:7182-8.

52. Yudt MR,Cidlowski JA. Molecular identification and characterization of a and b forms of the glucocorticoid receptor. *Mol Endocrinol.* 2001;15:1093-103.

53. Noske W, Hensen J,Wiederholt M. Endothelin-like immunoreactivity in aqueous humor of patients with primary open-angle glaucoma and cataract. *Graefes Arch Clin Exp Ophthalmol.* 1997;235:551-2.

54. de Castro M, Elliot S, Kino T, et al. The non-ligand binding beta-isoform of the human glucocorticoid receptor (hGR beta): tissue levels, mechanism of action, and potential physiologic role. *Mol Med.* 1996;2:597-607.

55. Fingert JH, Stone EM, Sheffield VC,Alward WL. Myocilin glaucoma. *Surv Ophthalmol.* 2002;47:547-61.

56. Perrot-Applanat M, Cibert C, Geraud G, Renoir JM,Baulieu EE. The 59 kDa FK506-binding protein, a 90 kDa heat shock protein binding immunophilin

(FKBP59-HBI), is associated with the nucleus, the cytoskeleton and mitotic apparatus. *J Cell Sci.* 1995;108 (Pt 5):2037-51.

57. Yang J,DeFranco DB. Assessment of glucocorticoid receptor-heat shock protein 90 interactions in vivo during nucleocytoplasmic trafficking. *Mol Endocrinol.* 1996;10:3-13.

58. Owens-Grillo JK, Stancato LF, Hoffmann K, Pratt WB,Krishna P. Binding of immunophilins to the 90 kDa heat shock protein (hsp90) via a tetratricopeptide repeat domain is a conserved protein interaction in plants. *Biochemistry.* 1996;35:15249-55.

CHAPTER V

CONCLUSIONS

The purpose of this dissertation was to investigate the mechanisms of glucocorticoid-induced ocular hypertention. This study design was necessary to ascertain not only the responses of glucocorticoids in the anterior segment associated with regulation of intraocular pressure but also to define the key element of control mechanisms that trigger glucocorticoid responsiveness in primary open-angle glaucoma (POAG) patients. Previous studies addressing pathological responses to glucocorticoids have been studied only in trabecualr meshwork. We examined the glucocorticoid responsiveness in both human non-pigmented ciliary epithelial (HNPE) cells and trabecular meshwork (TM) cells and determined if there was cross-talk between glucocorticoids and endothelin-1 (ET-1) as both glucocorticoids and ET-1 are implicated in glaucoma pathology. In addition, for over forty years, it has remained unknown as to what aspects of the control system were responsible for the glucocorticoid responsiveness in POAG patients. We investigated the potential role of the glucocorticoid receptor beta (hGRβ) isoforms as a possible contributor of the glucocorticoid responsiveness in TM cells based on the reports of its negative activity and suspected function in glucocorticoid resistance in some other diseases.

In general, the results obtained from the three related studies delineated in this dissertation support the following interpretation of the principal findings that occur in intraocular pressure regulation after glucocorticoid administration.

The first investigation demonstrated that ET-1 was concentration-dependently increased following 24 h dexamethasone (Dex) treatment, with a maximum concentration (100 nM) causing a 3-fold increase of ET-1 release from cultured HNPE cells. Dex treatment also changed ET receptor expression with a decrease in ET_A receptors while increasing ET_B mRNA and protein expression. As HNPE cells are a source for aqueous humor ET-1 and ET-1 can act in an autocrine fashion to regulate aqueous humor formation in HNPE cells as well as targeting the down-stream TM in regulating aqueous humor outflow, ET-1 could have consequences on aqueous humor inflow and outflow in glaucoma as well as ocular hypertension seen with glucocorticoid therapy.

The second investigation demonstrated that Dex decreased ET_B receptor protein expression in both normal and glaucomatous TM cells and attenuated ET-1 mediated increase in nitric oxide (NO) release through ET_B receptors in TM cells, while having no effect on ET_A receptor expression and ET-1 mediated intracellular Ca^{2+} mobilization through ET_A receptors. Dex-induced specific suppression of ET_B receptor expression and decline in ET-1 mediated increase in NO levels in TM cells with a concomitant increase of ET-1 release from HNPE cells could result in enhanced contraction response and decrease in relaxation of trabecular meshwork, hence reducing the intratrabecular space. Such actions by ET-1 may exacerbate Dex effects on the outflow pathway leading to increased outflow resistance and consequently elevated intraocular pressure. Collectively, these studies suggest a novel signaling pathway for glucocorticoids in the anterior segment in regulation of aqueous humor outflow in part through their interactions with ET-1 and ET receptors (Figure 1). In these investigations, we also observed that RU486

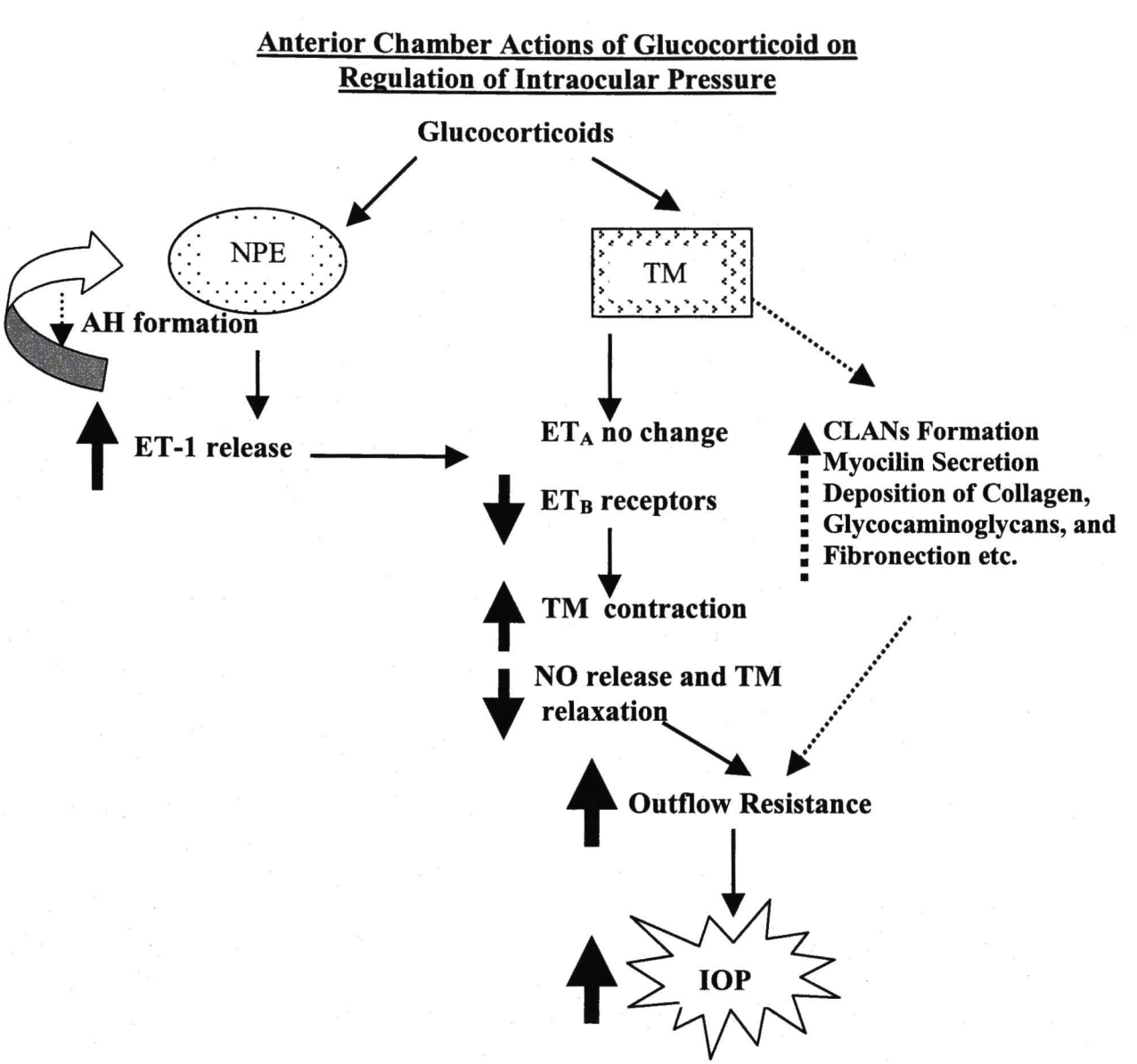


Figure 1. A novel signaling pathway for glucocorticoids in the anterior segment in the regulation of intraocular pressure (IOP). Glucocorticoids increase endothelin-1 (ET-1) release from non-pigmented ciliary epithelial (NPE) cells with the concomitant suppression of endothelin B (ET_B) receptors and decline in nitric oxide (NO) release from trabecular meshwork (TM) cells. Such effects would increase contraction and decrease relaxation of trabecular meshwork and reduce the intratrabecular space. ET-1 thus may exacerbate glucocorticoid effects on the outflow pathway leading to increased outflow resistance and consequently elevated intraocular pressure.

function as an glucocorticoid antagonist in those Dex response that resulted in enhanced expression whereas it was unable to block these Dex effects that resulted in decreased gene expression. In fact, RU486 acted as a partial agonist as itself produced a decrease in gene expression. The reason for this difference may be explained by the study that RU486 can recruit gene regulatory factors to an RU486-GRα receptor complex, and consequently limit the activities of these regulatory factors in the expression of their target genes. Additional studies need to be done to determine these mechanisms.

In the third investigation, we detected that most normal TM cell lines had relatively high amounts of hGRβ expression, especially in the nucleus compared to POAG TM cell lines. This is coincidence with the fact that in the normal population, there is a low rate of glucocorticoid responders as compared to almost all of the POAG patients. Moreover, overexpression of hGRβ in TM cells attenuated Dex-induced expression of myocilin, a glaucomatous gene. Therefore, our studies identify a possible molecular mechanism by which glucocorticoid responsiveness in POAG patients is achieved as a result of the low level of the negative isoform receptor hGRβ (Figure 2). Interestingly, in the TM cells, which have high amount of hGRβ in the nucleus, heat shock protein 90 (hsp90) was also concentrated in the nuclear regions, while in the glaucomatous TM cells, both hGRβ and hsp90 were present at lower concentrations in the nucleus. Transfection with hGRβ construct in TM cells caused the overexpression and accumulation of hGRβ protein in the nucleus that also led to an increase in hsp90 expression in the nucleus in these transfected TM cells. Most directly, 17AAG, an inhibitor of hsp90, which blocks the binding of client proteins to hsp90, completely blocked the accumulation of hGRβ and hsp90 in the

nucleus in these transfected TM cells. These studies suggest that hsp90 is a requirement for transport of hGRβ from the cytoplasm to the nucleus. Therefore, we have, for the first time, identified that hsp90 as a chaperon protein for the cytoplasm to nuclear translocation of hGRβ.

In summary, we have described a novel signaling pathway for glucocorticoids in anterior segments in regulation of intraocular pressure (IOP) through interaction with ET-1 and ET receptors. We have also identified that glucocorticoid responsiveness in TM cells is regulated by the expression of hGRβ in TM cells, and hsp90 is a chaperon protein for the nuclear translocation of hGRβ. The enhanced effects of ET-1 on TM cells by glucocorticoids coupled with an enhanced glucocorticoids responsiveness in glaucoma patients may explain the ocular hypertension seen with glucocorticoids therapy in POAG patients.

A Possible Molecular Mechanism Regulating Glucocorticoid Responsiveness in TM Cells

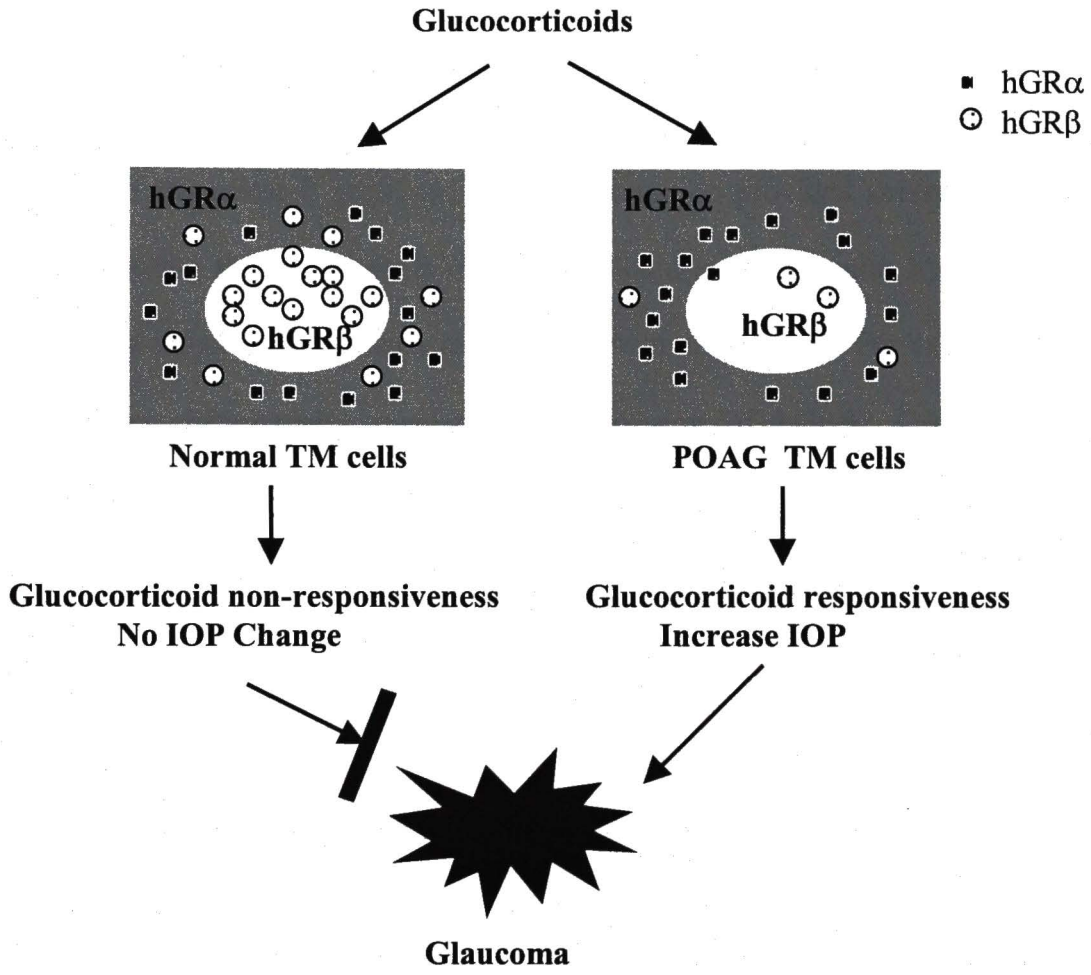


Figure 2. Glucocorticoid responsiveness pertaining to steroid-induced glaucoma is regulated by the expression of hGRβ in trabecular meshwork (TM) cells. In normal TM cells, high amount of negative hGRβ attenuates hGRα actions leading to glucocorticoid non-responsiveness, while in primary open-angle glaucoma patients, lower level of hGRβ attributes to enhanced glucocorticoid responsiveness.

CHAPTER VI

PROPOSAL OF FURTHER RESEARCH

Although the research presented in this dissertation provided several striking new findings regarding the mechanisms of glucocorticoid-induced ocular hypertension and glucocorticoid reponsiveness, many questions remain unanswered. For example, how do TM cell lines differentially regulate the expression of hGRβ? Can hGRβ, after overexpression, directly block steroid-induced ocular hypertension in an eye perfusion system? What are the other components, besides hsp90, required for the translocation of hGRβ from the cytoplasm to the nucleus? Listed below are several future investigations:

1) To test the hypothesis that the expression levels of the splicing factor or factors in TM cell lines are controls for creating hGRβ from GR pre-mRNA. hGRα and hGRβ isoforms are created from the same pre-mRNA transcript via alternative splicing. Different cell types can have very different ratios of hGRα to hGRβ. The factor or factors that control alternative splicing of GR pre-mRNA are of great importance. The family of serine-arginine-rich proteins known as SR proteins are major components for constitutive or alternative splicing processing in eukaryotic genes. Recently, a SR protein, SRp30 (serine-arginine-rich protein p30), has been reported to be necessary for alternative splicing of the GR pre-mRNA to create mRNA encoding hGRβ in neutrophils.[1] To test

this hypothesis, an investigation should be designed to detect the expression of SRp30 in normal and glaucomatous TM cell lines to determine if the expression of SRp30 is coincident with the expression of hGRβ receptor proteins. Further experiments could be conducted to detect the expression level of hGRβ receptor protein by altering the SRp30 expression in an *in vitro* TM cell system.

2) In the current investigations, we observed that overexpression of hGRβ inhibited the Dex-induced expression of myocilin. Although mutant myocilin is implicated in POAG, the function of myocilin remains unknown. A better way to exam the potential role of hGRβ in regulating glucocorticoid responsiveness in glaucoma is to directly test if hGRβ blocks the increase in intraocular pressure, which is seen following glucocorticoid administration. This experiment could be conducted by transferring adenovirus-mediated hGRβ gene into the isolated anterior segment in eye perfusion system, followed by a challenge with dexamethasone and measurement of intraocular pressure.

3) The efficiency of transport of hGRβ from the cytoplasm to the nucleus may be associated with the nuclear accumulation and stability of hGRβ in TM cells. Hsp90 was identified, for the first time, as a chaperon protein for nuclear import of hGRβ in the current investigations. Some other components may also be required for this retrograde nuclear transport. Several immunophilins and the motor protein dynein are involved in the nuclear translocation of hGRα. An investigation should be carried to determine the nuclear transport machinery of hGRβ, mainly targeting on immunophilins and motor

proteins. These experiments could be conducted using the procedures for hsp90 described in Chapter IV, including immunocytochemistry, co-immunoprecipitation, and the use of inhibitors.

Reference:

1. Xu Q, Leung DYM, and Kisich KO (2003). Serine-arginine-rich protein p30 directs alternative splicing of glucocorticoid receptor pre-mRNA to glucocorticoid receptor β in neutrophils. J. Biol. Chem. 29, 27112-27118.

HECKMAN
BINDERY, INC.
Bound-To-Please®
MAY 04
MANCHESTER, INDIANA 46962