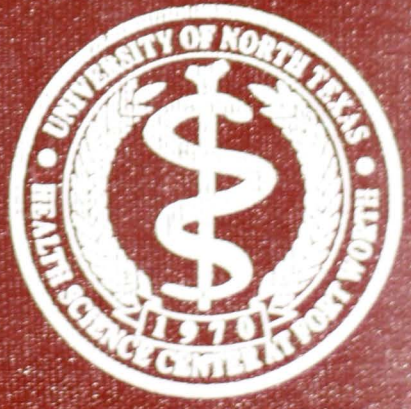
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Endothelin –1 Mediated Regulation of Extracellular Matrix Collagens –A role in Pathology of Primary Open Angle Glaucoma. Vidhya R. Rao, Doctor of Philosophy. (Pharmacology and Neuroscience), November, 2007, 157 pp., 3 tables, 18 figures.

SUMMARY

Primary Open Angle Glaucoma (POAG) is a progressive optic neuropathy characterized by loss of retinal ganglion cells, optic nerve degeneration and characteristic extracellular matrix (ECM) remodeling of the optic nerve head. An increase in collagen type I and VI is observed at the level of lamina cribrosa (LC), a distinct connective tissue region of optic nerve head in POAG subjects. Extensive ECM remodeling with enhanced collagen deposition observed in POAG is consistent with the pathology of fibrosis. Mechanisms contributing to ECM remodeling in POAG is not known. Endothelin-1(ET-1), a potent vaso-active peptide plays a key role in glaucoma pathology. Intra-vitreal administration of ET-1 in animal models results in optic neuropathy, RGC apoptosis, axonal transport block and ONA activation. An upregulation of ET-1 and ET_B receptors is observed in glaucomatous LC and animal models of glaucoma and ET-1 mediated detrimental effects in POAG appears to be mediated by ET_B receptors. ET-1 initiates and maintains enhanced collagen synthesis and deposition in various tissues under pathological conditions and is recognized as a potent profibrotic factor. In the present study we hypothesized that ET-1 increases extracellular matrix collagen deposition in lamina cribrosa and this change in ECM contributes to optic nerve fibrosis.

We have demonstrated that cells of lamina cribrosa (LC) cells, express functional ET_A and ET_B receptors. ET-1 increases intracellular calcium mobilization via ET_A receptors and increases NO release by mechanisms involving both ET_A and ET_B receptors. Consistent with POAG pathology we have observed an upregulation ET_B receptors in LC cells in response to chronic treatment with ET-1. LC cells also express prepro-ET-1, the primary gene transcript of ET-1. We have demonstrated for the first time that ET-1 exerts its profibrotic effects by enhancing collagen type I and type VI mRNA, protein synthesis, deposition and secretion in LC cells. ET-1 enhanced collagen deposition in LC cells appears to involve both ET_A and ET_B receptors, as both of the receptor antagonist, individually inhibit ET-1 mediated collagen synthesis. We have demonstrated that ET-1 also exerts its profibrotic effects *in vivo* by enhancing collagen deposition in rat optic nerve head. We have also observed an apparent decrease in ET-1 mediated collagen VI deposition in optic nerve heads of ET_B deficient transgenic rats suggesting that ET-1 mediated collagen VI synthesis involves ET_B receptor activation

In conclusion, endothlein-1 stimulates collagen synthesis and deposition both *in vitro* in LC cells as well as *in vivo* at the level of rat optic nerve head. ET-1 mediated increase in collagen synthesis at the level of optic nerve head could render a fibrotic mechanism that contributes to the progression of POAG.

Endothelin –1 Mediated Regulation of Extracellular Matrix Collagens –A role in Pathology of Primary Open Angle Glaucoma.

Vidhya Ramachandiran Rao

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Endothelin –1 Mediated Regulation of Extracellular Matrix Collagens –A role in Pathology of Primary Open Angle Glaucoma.

DISSERTATION

Presented to the Graduate Council of the

University of North Texas Health Science Center at Fort Worth

In Partial Fulfilment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The work is dedicated to all the human donors

and laboratory animals used in this study.

By

Vidhya Ramachandiran Rao

Fort Worth, Texas

November, 2007

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CHAPTER I

INTRODUCTION

Glaucoma is a leading cause of irreversible blindness affecting more than 66 million people worldwide (Quigley and Broman 2006). Primary open angle glaucoma (POAG) is the most prevalent form affecting more than two thirds of glaucoma subjects (Quigley 2005). Epidemiologic studies have identified several risk factors associated with POAG and include elevated intraocular pressure (IOP), age, ethnicity, familial history, systemic diseases such as hypertension and corticosteroid therapy (Boland and Quigley 2007). Elevated IOP is an important risk factor implicated in the incidence, prevalence and progression of the disease (Boland and Quigley 2007). Lowering IOP either by pharmacological or surgical intervention is the only therapy currently available in the management of disease (Marquis and Whitson 2005). Incidence and prevalence of POAG increases more than linearly with age (Quigley 1997). With the increase in aging population and life expectancy the incidence and prevalence of POAG is projected to double by 2020 (Friedman et al., 2004; Tuck and Crick 2003). A better understanding of the pathogenesis of POAG is therefore important in order to develop effective therapies necessary for treating the disease.

Primary open angle glaucoma – pathology.

POAG is defined as a progressive optic neuropathy characterized by structural change in the optic disc best described as excavation and by a characteristic visual field defect (Medeiros and Weinreb 2002; Weinreb 2004). The loss of retinal ganglion cells (RGCs) the major output neurons of the retina underlies the vision loss associated with glaucoma (Minckler 1989; Quigley 1995; Quigley 1989). The loss of retinal glanglion cells results in the segmental loss of the nerve fiber layer and thinning of the optic nerve (Sommer et al., 1991). Various mechanisms, including, the mechanical effect of IOP elevation, vascular dysregulation, distinct cellular responses of ganglion cells themselves to glaucomatous stimuli or contribution of other cell types including astrocytes, lamina cribrosa cells as well as abnormal effects of endogenous substances such as endothelin, glucocorticoids, glutamate, nitric oxide, and transforming growth factor beta have been implicated in the pathophysiology of primary open angle glaucoma (Fetchner et al., 1994; Neufeld et al., 1997; Wordinger at al., 1999; Vorwek et al., 1999). Excavation of the optic nerve head is a clinical hallmark of glaucoma and while the loss of RGC axons accompanied by thinning of optic nerve results in excavation, histopathological studies have demonstrated that the primary site of injury is at the level of the optic disc, particularly the lamina cribrosa (Ernest et al., 1968; Emery et al., 1974; Susana 1983; Hernandez 2000; Quigley 2005).

Lamina Cribrosa and POAG.

The lamina-cribrosa (LC) is a distinct connective tissue region of the ONH composed of perforated collagen sheets or lamellae, through which the RGC axons exit the eye (Anderson 1969; Birch et al., 1997). LC provides metabolic and mechanical support to the exiting nerve fibers against a pressure gradient established by the high IOP and the surrounding low intracranial pressure and diurnal IOP changes (Morgan et al., 1995; Brooks et al., 1999). Studies in non-human primates have demonstrated that raising IOP over a several minute period produces a backward movement of the LC, which returns to the baseline after the IOP is reduced to normal and this process is termed as compliance. Chronic raise in IOP however, results in reduced compliance, resulting in a posterior movement of LC suggesting that the LC is able to adapt to the changes in pressure (Burgoyne et al., 2005). The prominent difference between POAG and other optic neuropathies is the physical excavation of the optic disc (Quigley 2005). Marked disruption in the connective tissue architecture of the LC is observed in POAG subjects and suggests that the connective tissue of LC stretches backwards, collapses its successive plates and distorts at the scleral insertion to give the typical excavated appearance (Quigley and Green 1979 ; Miller and Quigley 1988). A significant population of POAG subjects do not have elevated IOP but still retain the characteristic excavation of the optic nerve head (optic disc) at the level of LC, suggesting an abnormal connective tissue response under normal IOP (Leibowitz et.al, 1980; Klein et al., 1992; Quigley et al., 1983). These changes in the LC have been associated with blockade of axonal transport, resulting in optic nerve degeneration and loss of RGCs by apoptosis

(Quigley et al., 1983; Sakugawa and Chihara 1985; Martin et al., 2003). An Increase in extracellular matrix components (ECM) including collagen type I, type IV, type VI, tenascin (a stress reactive protein) and elastin degeneration is observed in POAG (Hernandez et at., 1987, 2000; Morrison et al., 1989; Miller and Quigley 1988; Sawaguchi et. al, 1999). The composition of ECM renders resiliency and compliance to the LC and therefore allowing it to sustain changes in intraocular pressure (IOP) without the loss of structural integrity (Burgoyne et al., 2005; Morrison et al., 2005). Increase in ECM components, collagens in particular, results in tissue fibrosis and loss of structural integrity and tissue compliance (Varga et al., 2005). Changes in lamina cribrosa collagens in response to variance in IOP or release of endogenous agents such as profibrotic factors including endothelin-1 and transforming growth factor – beta (TGF-β), could therefore contribute to the glaucoma pathology.

Cells of Lamina Cribrosa and POAG.

Lamina cribrosa cells (LC cells) and ONH astrocytes (ONA) are the two primary types of cells identified in LC (Hernandez et. al., 1988). ONA form the glial columns surrounding the exiting axons while, the LC cells, unlike ONA, do not express glial fibrillary acidic protein (GFAP) and are located in the cribriform plates (Hernandez et al., 1988; Lambert et al., 2001). Both ONA and LC cells have been implicated in glaucoma pathology (Hernandez 2000). Reactive astrocytes or astrogliosis is a common pathologic feature of various neurologic disorders (Eddleston and Mucke 1993; Ridet, et al., 1997). Reactive astrocytes are characterized by enhanced GFAP expression and often appear to have migrated to the lamina cribrosa of glaucomatous subjects and in animal models of

glaucoma (Hernandez and Pena 1997; Varela and Hernandez 1997). *In vitro* cultures of LC cells appear to express neurotrophin receptors and to secrete neurotrophins and thus LC cells may serve as neurotophic support to the RGC axons (Lambert et al., 2004). LC cells share biochemical similarities to trabecular meshwork cells *in vitro* and *in vivo*, indicating that POAG involves both the trabecular meshwork and lamina cribrosa (Steely et al., 2000). Recent studies have shown that LC cells respond to mechanical stretch upregulating the expression of various extracellular protein molecules including collagen I, IV, VI, fibronectin, and elastin similar to that observed in glaucomatous subjects and in animal models of glaucoma (Kirwan, Fenerty et al., 2005). LC could therefore represent an important pro-fibrotic tissue prone to tissue fibrosis resulting in loss of structural integrity and collapse of LC.

Collagen type I, VI and POAG.

Collagen type I molecules are composed of three polypeptide chains, two identical α1 and a distinct α2 chain forming a triple helix that offers structural support to the ECM (Van der Rest 1992). Collagen VI is a heterotrimeric molecule made of three polypeptide chains, αI (VI) α2 (VI) and α3 (VI). The chains interact with cell receptors of the integrin type and serve to associate ECM to cells (Van der Rest 1992). Increases in collagen I and collagen VI in LC is characteristic of POAG and elevated IOP models of glaucoma. Elevated IOP models of glaucoma have demonstrated that the deposition of collagen I and VI at the optic nerve head to be an early event and are correlated linearly to the degree of IOP- induced injury (Jhonson et al., 2000; Guo et al., 2005; Morrison et al., 2005; Jhonson et al., 2007). The changes in collagens, principal structural components

of the ECM, could alter the biomechanical properties of LC, resulting in loss of structural integrity and contribute to the pathogenesis of POAG. Studies in transgenic mice with a targeted collagen type I mutation resulted in enhanced collagen type I deposition and developed an increase in intra ocular pressure with subsequent optic nerve damage. (Aihara et al., 2003; Mabuchi et al., 2005).

Collagen I, VI and Tissue Fibrosis.

Excess accumulation of collagen type I results in fibrosis leading to loss in normal structure and function of the respective tissue (Varga et al., 2005). Hepatic stellate cells, activated myofibroblasts of lungs, kidney and heart in response to profibrotic agents like transforming growth factor-β (TGF-β) and endothelin-1(ET-1) or mechanical stress secrete excess collagen type I and produce tissue fibrosis (Tsukada et,al., 2005; Phan 2002; Eddy 2000; Wakatsuki et.al., 2004). Associated with fibrotic tissues is the increase in collagen type VI also considered as a marker for tissue fibrosis (Groma 1998; Zeichen et.al., 1999; Specks et.al, 1995; Hatamochi et.al., 1996; Gerling et.al, 1997). Increase in collagen VI results in cutis laxa characterized by loss of elastic properties of the skin (Hatamochi et.al., 1996). Collagen VI surrounds normal cardaic myocytes and increases with heart failure indicating that it is an early marker of the disease (Mollnau et al., 1995). Soluble collagen VI inhibits apoptosis of serum starved fibroblasts, (Ruhl et.al., 1992). Collagen VI and Tennasin –X have a synergistic effect on increasing the rate of collagen fibril formation (Minamitani et al., 2004). Collagen VI also contributes to migration of U-87MG glioblastoma cells (Harumiya et al., 1995; Han et.al, 1995). Cyclic albumin drug carriers that selectively interact with collagen type VI, have been developed

for targeting anti fibrotic drugs to fibrotic tissues (Schuppan et.al., 2002). The finding that collagen VI increases in POAG (Morrison et al., 2000; Hernandez 2000) also suggests that a fibrotic mechanism may contribute to glaucoma pathology.

Endothelin-1 and POAG.

Endothelin-1 (ET-1) and its isoforms, ET-2 and ET-3, belong to a family of 21-amino acid vasoactive peptides which play a regulatory role in vascular homeostasis mediating their effects through seven trans-membrane G-protein coupled receptors, endothelin receptor A (ET_A) and endothelin receptor B (ET_B). Initially discovered for its potent vasoconstrictive activity, ET-1 has been implicated in normal physiology and pathophysiology of several systems, including the cardiovascular, renal, lungs, hepatic and central nervous system (Kedzierski and Yanagisawa 2001). ET-1 levels are 2-3 fold higher in aqueous humor than in plasma of the normal human eye (Lepple-Wienhues et al., 1992). Furthermore, POAG subjects have significantly higher levels of ET-1 in plasma and aqueous humor compared to their age matched controls (Sugiyama et al., 1995; Noske et al., 1997). Elevated ET-1 levels have also been observed in elevated IOP animal models of glaucoma (Prasanna et al., 2005; Kallberg et al., 2002). Intravitreal ET-1, when administered into various animal models results in loss of retinal ganglion cells by apoptosis, blocks axonal transport and activates optic nerve head astrocytes and contributies to optic neuropathy. (Stokely et al., 2002; Chauhan et al., 2004; Lau et al., 2006).

Endothelin-1 and Tissue fibrosis.

Endothelin may play an important role as a pro- fibrotic factor in initiating and maintaining fibrosis of various tissues and results in increases in collagen synthesis and extracellular matrix accumulation in several cell types including cardiac myocytes, fibroblasts and smooth muscle cells (Tsukada et al., 2005; Phan SH 2002; Eddy AA 2000; Wakatsuki et al., 2004; Sticherling). ET-1 is also capable of regulating the effects of other pro-fibrotic factors like, transforming growth factor-beta & connective tissue growth factor, where as inhibiting ET-1 activity using a dual ET_A and ET_B receptor antagonist prevents ET-1 mediated tissue fibrosis (Rodriguez-Vita et al., 2005; Clozel et al., 2005). The role of ET-1 in increasing extra cellular matrix components, in particular, the basement membrane components, fibronectin and collagen IV has been demonstrated in diabetic retinopathy (Khan et al., 2006). However the role of ET-1 as a profibrotic factor in glaucoma pathogenesis remains to be studied.

Significance.

POAG is accompanied by loss in structural integrity of LC tissue with an increase in collagen type I and VI and other extra cellular matrix components consistent with the morphology of other fibrotic tissues. The molecular mechanisms regulating the changes associated with ECM remodeling and associated structural changes however are not clear. ET-1, a potent vasoactive peptide and also a profibrotic factor, has been implicated in the POAG. The mechanisms by which ET-1 can mediate a fibrotic mechanism in POAG is outlined in Fig.1. Specific aims addressed in the proposal would help to understand the regulation of collagens, the key components of ECM in LC and determine

if ET-1 mediates fibrotic changes in ocular tissues thereby contributing to the pathology of POAG. The studies would also provide mechanisms by which one could alter the fibrotic process of the disease to help maintain the structural and functional integrity of LC.

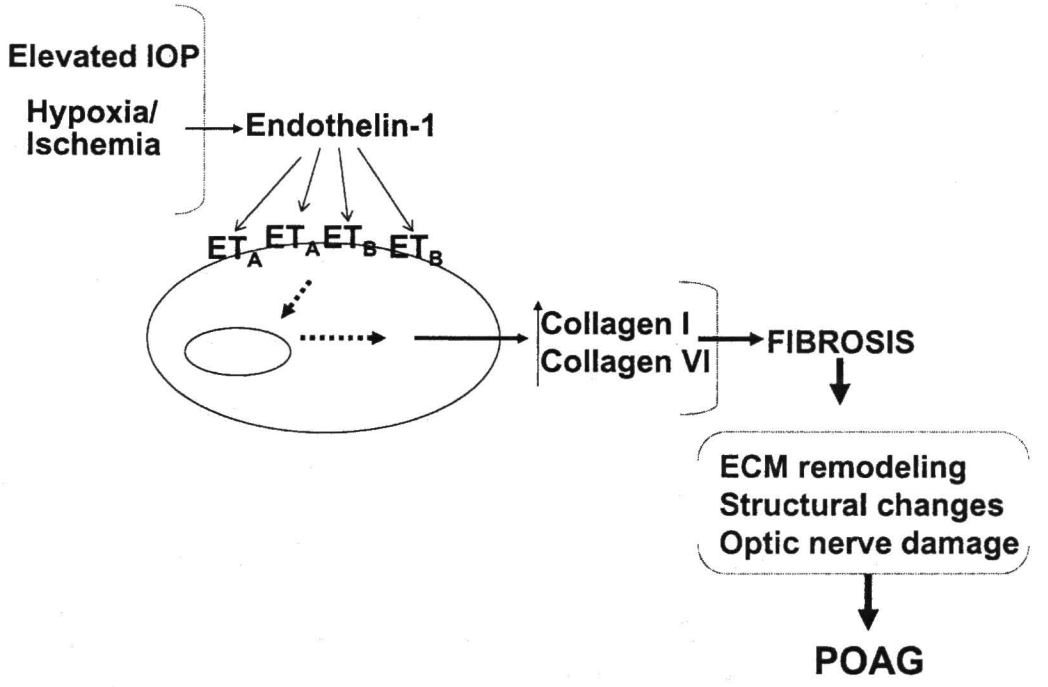

Fig.1. Proposed mechanism of ET-1 mediated fibrosis in pathology of POAG.

An outline of mechanism by which, ET-1 could potentially contribute to fibrosis in pathology of POAG. Elevated IOP, hypoxia/ischemica implicated in pathology of POAG results in elevated ET-1 levels at the optic nerve head. Elevated ET-1, further acts on resident cells of the optice nerve head including LC cells via ET_A and ET_B resulting in enhanced collagen synthesis and deposition. Enahnced collagen deposition including

collagen type I and type VI results in ECM remodeling, thereby alters the biomechanical properties of Lamina cribrosa (LC) a distinct connective tissue region in the optic nerve head. The changes in biomechanical properties further results in the structural changes including compression of connective tissue plates, backward bowing, collapse of LC and associated optic nerve damage and retinal ganglion cells loss observed in POAG.

RESEARCH DESIGN, RATIONALE AND METHODS

Primary Open Angle Glaucoma (POAG) is a progressive optic neuropathy characterized by loss of retinal ganglion cells, optic nerve degeneration and characteristic excavation of the optic disc. Elevated intra-ocular pressure (IOP) and age are important risk factors associated with progression of glaucoma. Extensive ECM remodeling and compromise in structural integrity of the LC is associated with excavation of the optic disc and ganglion cell loss. An increase in collagen type I, IV, VI, and elastin degeneration is observed in LC of POAG subjects and animal models of glaucoma. The changes in collagens could alter the biomechanical properties of the tissue and result in the loss of structural integrity. Pathophysiological changes in the optic nerve head astrocytes (ONA) and lamina cribrosa cells (LC) including, proliferation, hypertrophy, migration and extra cellular matrix regulation have been attributed to the changes observed in lamina cribrosa of POAG subjects. LC cells constitute a major cell type in LC that are characterized in vitro by their ability to secrete neurotrophins (Hernandez et. Al., 1988; Lambert et al., 2000) and exhibit pro-fibrotic properties resulting in enhanced extracellular matrix

synthesis (Kirwan et. al., 2005). Endothelin-1(ET-1), a potent vaso-active peptide, has been proposed to play a key role in glaucoma pathology. POAG subjects have elevated aqueous humor and plasma levels of ET-1. Intra-vitreal administration of ET-1 in animal models results in optic neuropathy, RGC apoptosis, axonal transport block and ONA activation resulting in their hypertrophy and proliferation. ET-1 is recognized as a potent pro-fibrotic factor in various cell type and tissues. In the present study we propose to study endothelin-1 mediated extra cellular matrix changes in lamina cribrosa. **The hypothesis to be tested in the present study is that, endothelin-1 through its receptors increases the deposition of collagen type I and type VI in LC cells *in vitro* and *in vivo* and confers a fibrotic mechanism to POAG.** The following specific aims were designed to address our hypothesis.

Specific Aim1: To determine the expression of endothelin-1, & endothelin receptors in LC cells:

a. To determine the expression of endothelin receptors ET_A and ET_B in LC cells by QPCR and Western blot.
b. To determine the functional properties of ET_A and ET_B receptors in LC cells by determining ET-1 mediated increase in intracellular calcium levels and release of nitric oxide.

Specific Aim 2: To determine the effect of endothelin-1 on collagen regulation in LC cells:

a. To determine endothelin-1 mediated increase in collagen I and VI expression by QPCR in LC cells

b. To determine endothelin-1 mediated increase in collagen I and VI by Western blot and immunocytochemistry in LC cells.

c. To determine the role of ET_A/ET_B receptor in ET-1 mediated increase in collagen I and VI in LC cells.

Specific Aim 3: To determine the effect of endothelin-1 on collagen regulation in rat optic nerve head following intra-vitreal ET-1injection.

a. To determine endothelin-1 mediated increase in collagen I and VI in rat optic nerve head by immunohistochemistry following intra-vitreal ET-1injection.

b. To determine the role of ET_A/ET_B receptor in ET-1 mediated increase in collagen I and VI in rat optic nerve head following intra-vitreal ET-1injection.

The following section outlines the rationale, research design, approach and methods employed to address the above specific aims, details of which have been included in individual chapters.

Specific Aim1: To determine the expression of endothelin-1 and endothelin receptors in LC cells:

a. To determine the expression of endothelin receptors ET_A and ET_B in LC cells by QPCR and Western blot.

b. To determine the functional properties of ET_A and ET_B receptors in LC cells by determining ET-1 mediated increase in intracellular calcium levels and release of nitric oxide.

Rationale:

ET-1 a potent vasoactive compound is implicated in glaucoma pathology (Yorio et al., 2002). ET-1 primarily acts as an autocrine and paracrine factor mediating its effects through G-protein coupled seven transmembrane ET_A and ET_B receptors. ET-1 mediated biphasic increases in intracellular calcium levels via Gq coupled ET_A receptors, and ET_B receptor mediated activation of nitric oxide synthase resulting in release of NO via Gi coupled mechanism are well characterized signaling mechanisms of ET-1 in various cell types (Rubanyi and Polokoff 1994). Effects of ET-1 have been characterized in various ocular cell types important in glaucoma pathology including trabecular meshwork cells, human nonpigmented ciliary epithelial cells, retinal ganglion cells and optic nerve head astrocytes (Prasanna et al., 2003). ET-1 expression and its functions in LC cells however are not known. **The experiments in the specific aim 1 were designed to characterize the endothelin system in lamina cribrosa cells through which ET-1 may regulate the ECM of LC cells.**

Experimental Approach:

Immunocytochemistry for characterizing GFAP negative LC cells:

The GFAP negative LC cell lines that were used in the study were characterized by immunocytochemistry. Parafromaldehyde fixed LC cells were permeabilizeed with triton X-100. Following blocking of nonspecific binding with bovine serum albumin (BSA), Cells were incubated with primary monoclonal anti-GFAP antibody (Neomarkers, Fremont, CA). Following washes cells were incubated with secondary antibody, Alexa fluor 488 (Molecular probes, Eugene, OR). Cells were also incubated with DAPI to stain

the nuclei. Fluorescent images were captured using confocal microscopy (Carl Zeis, meditech; Germany).

Expression of ET_A, ET_B and prepro ET-1 by RT PCR and QPCR:

Total cellular RNA was isolated from LC cells using the Trizol B reagent (Life Technologies, Rockville, MD, USA). cDNA were synthesized from total RNA using random primers and AMV Reverse Transcriptase (Promega, Madison, WI, USA). Reactions without reverse transcriptase were also performed and used as negative controls for experiments. cDNA samples were amplified with specific primers for ppET-1, ET_A and ET_B using Taq polymerase in a DNA thermal cycler (Perkin-Elmer). For quantification of mRNA transcripts by QPCR, cDNA samples were amplified with specific primers for ET_A receptor, ET_B receptor and internal control β-actin using SYBR Green PCR core regents (PE Applied Biosystems, Foster City, CA, USA) in Cepheid Smart Cycler (Cepheid, Sunnyvale, CA, USA). The melting curves were generated to detect the melting temperatures of the specific products immediately after the PCR run. The relative mRNA levels were determined by the comparative C_T method (Pfaffl 2001).

Expression of ET_A & ET_B by Western blott:

Membrane fractions from LC cells were isolated by a method previously described (Dibas et al., 1996) and were separated by SDS –PAGE. The proteins were transferred to nitrocellulose membrane, probed with primary anti ET_A and anti ET_B antibodies (1:200 rabbit polyclonal; Almone labs) and secondary anti rabbit HRP conjugated secondary antibody (1:10000; Amersham Biosciences). The membranes were developed with ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Densitometric analysis of the

bands were performed using the image-analysis software. (Scion; National institutes of health, Bethesda, MD).

[Ca^{2+}] i measurement:

ET-1 mediated changes in [Ca^{2+}]i mobilization was studied by real-time fura-2 fluorescence microscopy. To determine the role of endothelin receptors ET_A and ET_B in ET-1 mediated [Ca^{2+}]i mobilization, ET_A and ET_B receptor antagonists BQ610 and BQ788 respectively were used in the study. The experiments were also carried out in the presence of calcium free and calcium containing buffer to determine the role of extra cellular calcium in ET-1 mediated [Ca^{2+}]i mobilization. Changes in Fura-2 fluorescence following treatment with ET-1 was monitored and recorded under a Nikon Diphot microscope using metaflour software. [Ca^{2+}]i in nM was determined by ratiometric analysis of fura -2 fluorescence [340:380 excitation] using the equation derived by Grynkiewicz et al., (Grynkiewicz G et al., 1985).

In-vitro Nitric oxide assay:

ET-1 induced nitric oxide (NO) releasae was measured using a Griess colorimetric assay. LC cells will be grown to confluence in 24 well plates. To determine the role of endothelin receptors ET_A and ET_B in ET-1 mediated NO release, ET_A and ET_B receptor antagonists BQ610 and BQ788 respectively were used in the study. The NO released in the media was measured as nitrite, the final oxidation breakdown product of nitric oxide (NO) using a Griess colorimetric NO synthase (NOS) assay kit (Calbiochem, San Diego, CA). A standard nitrite curve wass generated according to the manufacturer's protocol and Nitrite concentration in the culture medium was determined from the standard curve.

Statistical analysis:

Data was represented as Mean+/-SEM. Students t-test was applied for the analysis of paired groups and comparisons between multiple groups was analyzed by analysis of variance (ANOVA). Statistical analysis with values of $p<0.05$ was considered significant.

Specific Aim 2: To determine the effect of endothelin-1 on collagen regulation in LC cells:

d. To determine by QPCR the expression of collagens I and VI and endothelin-1 mediated changes in expression.
e. The changes in collagen I and VI in response to ET-1 will be determined by Western blot and immunocytochemistry.
f. The role of ET_A/ET_B receptor in ET-1 mediated collagen regulation will be determined by selective ET_A and ET_B receptor antagonist BQ610 and BQ788 respectively

Rationale:

Increases in extracellular matrix deposition including collagen type I, type IV, type VI, fibronectin, is observed in glaucomatous LC (Hernandez et al., 1987; Morrison et al., 1989; Miller and Quigley 1988; Hernandez 2000 Sawaguchi etal., 1999). Extensive ECM remodeling, is proposed to compromise the structural integrity of LC resulting in excavation of the optic disc and associated optic nerve degeneration and ganglion cell loss (Quigley et al., 1983; Sakugawa and Chihara 1985; Martin et al., 2003). The extra cellular matrix profile of LC cells describes as fibroblastoid like cells isolated from

Lamina cribrosa corresponds to the matrix profile observed *in-vivo* (Hernandez et al., 1988; Lambert et al., 2001). ET-1, a potent vaso active peptide, has been implicated in the pathophysiology of POAG (Yorio et al., 2003). ET-1 also recognized for its pro-fibrotic properties, plays an important role in initiating and maintaining fibrosis of various tissues and cell types (Tsukada et al., 2005; Phan SH 2002; Eddy AA 2000; Wakatsuki et al., 2004). The role of ET-1 as a profibrotic factor in glaucoma pathogenesis remains to be studied. **The experiments in specific aim 2 have been designed to address the hypothesis that ET-1 increases the expression of collagen I and VI in LC cells.**

Experimental design and approach:

ET-1 mediated regulation of collagen I, collagen VI, in LC cells by RTPCR:

Total cellular RNA from confluent LC cells was isolated using the Trizol B reagent (Life Technologies, Rockville, MD, USA) and cDNA will be synthesized from total RNA using random primers and AMV Reverse Transcriptase (Promega, Madison, WI, USA). Reactions without reverse transcriptase was also performed and used as negative controls for experiments. For quantification of mRNA transcripts by QPCR, cDNA samples were amplified with specific primers for COL1α1, COLVI α1 and internal control β-actin using SYBR Green PCR core regents (PE Applied Biosystems, Foster City, CA, USA) in Cepheid Smart Cycler (Cepheid, Sunnyvale, CA, USA). The melting curves were generated to detect the melting temperatures of the specific products immediately after the PCR run. The relative mRNA levels were determined by the comparative C_T method (Pfaffl 2001).

ET-1 mediated regulation of collagen I, collagen VI, in LC cells by Western Blott:

ET-1 mediated collagen synthesis was determined by analyzing both, the secreted and as well as deposited collagen type I and VI in LC cells. In order to determine the secreted collagen I and VI media of the treated cells were collected and concentrated 50-fold by centrifugation through a membrane (10-kDa cutoff; Amicon; Millipore, Bedford, MA), according to the manufacturer's instructions. In order to determine ET-1 mediated collagen type I and type VI synthesis and deposition total cell lystates of LC cells were prepared. To determine the role of endothlein receptors in ET-1 mediated collagen reglulation ET_A and ET_B receptor antagonists BQ610 and BQ788 were used in the study. Equal amounts of protein samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. Membranes were blocked with non-fat dry milk and incubated with 1:100 monoclonal anti collagen I (Calbiochem San Diego CA) and 1:100 polyclonal anti collagen VI (Chemcon Temecula, CA) . Following washes the membrane were incubated with anti mouse/anti rabbit HRP conjugated secondary antibodies (1:10000; Amersham Biosciences) and the blots were developed with Super Signal West Femto Maximum Sensitivity Substrate kit (Pierce Biotechnology, Inc, Rockford, IL). Densitometric analysis of the bands was performed using the image-analysis software (Scion; National institutes of health, Bethesda, MD).

ET-1 mediated regulation of collagen I, collagen VI, in LC cells bymmunocytochemistry:

Parafromaldehyde fixed LC cells were incubated with bovine serum albumin (BSA), to block the non specific binding. Following waskes Cells were incubated with with 1:100 monoclonal anti collagen I (Calbiochem San Diego CA) and 1:100 polyclonal anti collagen VI (Chemcon Temecula, CA) primary antibodies. Following washes cells were incubated with 1:400 dilution anti mouse/ anti rabbit secondary antibody, Alexa fluor 488 (Molecular probes, Eugene, OR). Cells were incubated with DAPI to stain the nuclei. Fluorescent images were captured using confocal microscopy (Carl Zeis, meditech; Germany).

Measurement of total collagen synthesis by [^{3}H] proline incorporation assay:

ET-1 mediated Collagen synthesis was assessed by measuring the uptake of [^{3}H] proline as previously described with slight modifications (Ku et al., 2006). The major biosynthetic destination of proline is collagen and therefore the amounts of radioactive [^{3}H] proline incorporated into insoluble and soluble protein fractions provides a reliable index of total collagen synthesis (Mukherjee and Sen 1990; Ku et al., 2006). In order to determine the receptors involved in ET-1 mediated increase in collagen synthesis, ET_A receptor antagonist, BQ610 and ET_B receptor antagonist BQ788 were used in the study. An equal portion of the solubilised proteins obtained form the cell layers or media were added to scintillation cocktail and [^{3}H] proline incorporation was measured using beta counter, Packard Tricarb 1600 TR liquid scintillation analyzer (Parkard, UK) while an equal portion of the solubilised proteins obtained form the cell layers or media were utilized to determine the total protein content using the bicinchonic (BCA) protein assay

(Pierce Biotechnology, Inc, Rockford, IL). The total radiocactivity counted in each sample was normalized to the respective total protein content.

Statistical analysis:

Data was represented as Mean+/-SEM. Students t-test was applied for the analysis of paired groups and comparisons between multiple groups was analyzed by analysis of variance (ANOVA). Statistical analysis with values of $p<0.05$ was considered significant.

Specific Aim 3: To determine the effect of endothelin-1 on collagen regulation in rat optic nerve head following intra-vitreal ET-1injection.

b. To determine endothelin-1 mediated increase in collagen I and VI in rat optic nerve head by immunohistochemistry following intra-vitreal ET-1injection.
b. To determine the role of ET_A/ET_B receptor in ET-1 mediated increase in collagen I and VI in rat optic nerve head following intra-vitreal ET-1injection.

Rationale:

Loss of retinal ganglion cells associated with extensive extra cellular matrix remodeling of the optic nerve head at the level of lamina cribrosa with increase in ECM molecules including collagen type I, type IV, type VI and tenascin a stress reactive protein, is a characteristic pathologic feature of POAG eyes (Hernandez et al., 1987; Morrison et al., 1989; Miller and Quigley 1988; Hernandez 2000 Sawaguchi et al., 1999). Extensive ECM remodeling, is proposed to compromise the structural integrity of LC resulting in excavation of the optic disc and associated optic nerve degeneration and ganglion cell loss (Quigley et al., 1983; Sakugawa and Chihara 1985; Martin et al., 2003). ET-1 a

potent vasoactive peptide has been implicated in POAG (Yorio et al., 2003). Elevated aqueous humor levels and plasma levels of ET-1 have been reported in glaucomatous subjects (Sugiyama et al., 1995; Noske et al., 1997). Elevated levels of ET-1 are also observed in elevated IOP model of glaucoma in rats (Prasanna et al., 2005; Kallberg et al., 2002). Injections of ET-1 (2nmole) into rat eyes have shown to cause loss of retinal ganglion cells with an axon block and activation of optic nerve head astrocytes (Stokely et al., 2002; Chauhan et al., 2004; Lau et al., 2006). ET-1, as a profibrotic factor, is also involved in various types of tissue fibrosis resulting in increases in collagen type I and VI synthesis and accumulation (Sukada et al., 2005; Phan SH 2002; Eddy AA 2000; Wakatsuki et al., 2004). ET-1 could alter collagen synthesis and render the LC tissue less compliant to stress. **The experiments in this specific aim are designed to study the hypothesis that intra-vitreal injection of ET-1 increases the levels of collagens I, & VI in rat eyes.**

Experimetnal design and approach:

ET-1 mediated regulation of collagen I, collagen VI, in rat optic nerve head by Immunohistochemistry following intra-vitral injection of ET-1:

Adult Wistar- Kyoto rats were injected with ET-1 intra –vitreally. In order to determine thre role of ET_A and ET_B receptors in ET-1 mediated collagen regulation, ET_B deficient transgenic adult Wistar-Kyoto rats (sl/sl / ET_B KO) were used in the study. Following ET-1 treatment, the rats were sacrificed and eyes enucleated. The eyes were fixed in 4% paraformaldehyde. Following fixation the eyes were dehydrated with gradient dilutions of ethanol and paraffin embedded. 5μm thick sections of paraffin embedded eyes were

obtained with a microtome and placed one glass slides (Platinum microslides, Germany). Sections were de-paraffinised in xylene, re-hydrated with a series of dilutions of ethanol and PBS. Sections were then blocked with BSA. The sections were washed in 1X PBS. Following washes the sections were incubated with 1:100 primary goat polyclonal anti collagen type I antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and 1:100 dilution of anti collagen type VI antibody (Chemicon Temecula,CA). Following washes, sections were incubated with 1:400 dilution of secondary anti goat antibody, Alexa fluor 633 or 1:400 dilution of secondary anti rabbit antibody, Alexa fluor 488 (Molecular probes, Eugene, OR). and fluorescent images of mounted sections were taken using confocal microscopy (Carl Zeiss Meditec, Inc., Thornwood, NY).

Statistical Analysis:

Fluorescent intensities of collagen types I and VI were quantified using the Image J software. Data obtained by Image J were analyzed with one-way analysis of variance (ANOVA) for multiple comparisons and represented as Mean ± SEM.

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CHAPTER II

Endothelin-1, Endothelin A and B Receptor Expression and their Pharmacological properties in GFAP Negative Human Lamina Cribrosa Cells.

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Abstract

Primary open angle glaucoma (POAG) is a progressive optic neuropathy, characterized, in part by extensive extra cellular matrix remodeling and collapse of the lamina cribrosa (LC). Endothelin-1 (ET-1), a potent vasoactive peptide and its receptors, endothelin receptor A (ET_A) and endothelin receptor B (ET_B), have been implicated in glaucomatous optic neuropathy. In this study we examined the expression of ET-1 and its receptors in GFAP negative LC cells. RTPCR analysis revealed that LC cells express both ET_A, ET_B receptors and prepro- ET-1, the primary gene transcript of ET-1. A dose-dependent increase in intra-cellular calcium concentrations was observed in the presence of 1, 10 & 100nM ET-1. Increased intracellular calcium concentrations were blocked by the ET_A selective antagonist BQ610 but not by the ET_B specific antagonist BQ788. Desensitization to ET_A - mediated increase in intracellular calcium was observed in LC cells following pre-treatment with ET-1 for 24 hrs. Western blot analysis of LC cells treated with ET-1 for 24 hrs revealed a decreased expression of ET_A receptor protein at 1, 10 & 100nM concentrations, while a dose dependent increase in the ET_B receptor was observed with a significant increase at 100nM. Quantitative PCR showed a dose-dependent decrease in ET_A receptor mRNA levels and an increase in the mRNA levels of ET_B receptors. A Griess colorimetric assay was used to measure the NO released from LC cells and ET-1 induced a dose-dependent increase in NO release which was significant at 100nM concentration. ET-1 induced NO release was significantly blocked by BQ788, an ET_B selective antagonist, and as well as BQ610, an ET_A selective antagonist. These results suggested that human lamina cribrosa cells expressed functional ET_A and ET_B

receptors and their expression and function was altered in response to prolong exposure to ET-1. This may have an implication in the normal physiology of LC cells and in POAG subjects where elevated levels of ET-1 could impact LC function.

Keywords. Endothelin, Lamina Cribrosa, Glaucoma

Introduction

Glaucoma, an optic neuropathy, affects more than 60 million people worldwide resulting in irreversible blindness (Thylefors and Negrel 1994; Quigley and Broman 2006). Primary open angle glaucoma (POAG) is the most prevalent form commonly associated with elevated intraocular pressure (IOP) and results in a progressive loss of vision due to the loss of retinal ganglion cells (RGC) (Quigley 2005). Various mechanisms including elevated IOP, ischemia and glutamate mediated excitotoxicity have been implicated in retinal ganglion cell death (Kuehn et al., 2005). The lamina-cribrosa (LC) is a distinct region of the ONH composed of perforated connective tissue through which the RGC axons exit the eye (Anderson 1969; Birch et al., 1997). LC provides metabolic and mechanical support to the exiting nerve fibers against a pressure gradient established by the high IOP and the surrounding low intra cranial pressure (Burgoyne et al., 2005). Marked disruption in the architecture of the LC is observed in POAG subjects which includes, backward displacement, distortion, collapse and extra cellular matrix reorganization (Miller and Quigley 1988). These changes in LC have been associated with blockade of axonal transport, resulting in optic nerve degeneration and loss of RGCs by apoptosis (Quigley et al., 1983; Sakugawa and Chihara 1985; Martin et al., 2003). LC cells and ONH astrocytes (ONA) are the two types of cells identified in the Lamina cribrosa. LC cells located in cribriform plates do not express GFAP. This marker is only found in ONA (Hernandez et al., 1988; Lambert et al., 2001). Both ONA and LC cells have been implicated in glaucoma pathology (Hernandez 2000).

Endothelin-1 (ET-1) and its isoforms, ET-2 and ET-3, belong to a family of 21-amino acid vasoactive peptides that mediate their effects through seven trans-membrane G-protein coupled receptors, endothelin receptor A (ET_A) and endothelin receptor B (ET_B), (Rubanyi and Polokoff 1994). Initially discovered for its potent vaso-constrictive activity, ET-1 has been implicated in normal and pathophysiological conditions of several systems, including the cardiovascular, renal, pulmonary and central nervous system (Kedzierski and Yanagisawa 2001). ET-1 levels were found to be 2-3 fold higher in human aqueous humor than in plasma (Lepple-Wienhues et al., 1992). Furthermore, POAG subjects had significantly higher levels of ET-1 in plasma and aqueous humor compared to their age matched controls (Sugiyama et al., 1995; Noske et al., 1997). Elevated ET-1 levels have also been observed in animal models of glaucoma (Prasanna et al., 2005; Kallberg et al., 2002). Intravitreal ET-1, when administered at similar doses into various animal models resulted in loss of retinal ganglion cells by apoptosis (Lau et al., 2006), caused activation of glia (Lau et al., 2006) and blockade of axonal transport (Stokely et al., 2002). The effects of ET-1 on LC cells and the role of the endothelin system in LC are not known. The objective of the present study was to determine if ET-1 and its receptors are present and are coupled to signaling pathways that may influence LC cell function.

Materials and methods

Cell culture: Lamina cribrosa (LC) cells were derived from a 66-year-old donor with glaucoma and from a 90-year-old normal with no evidence of glaucoma. Optic nerve head astrocytes (ONA) used in the study were derived from 66-year old donor. These cell lines used in the study were a generous gift from Dr Robert Wordinger (UNTHSC, Fort worth, TX) and Dr Abott Clark (Alcon labs, Fort worth, TX) (Lambert et al., 2004). LC and ONA cell lines used were previously characterized by immunofluorescent staining for various markers including glial fibrillary acidic protein (GFAP), NCAM, α-smooth muscle actin and a variety of extra cellular matrix proteins, including collagen types I, III, and IV, elastin, laminin, and fibronectin (Lambert et al., 2001). Both LC cells and ONA used in this study were from passages 4 to 7 and were similar to that used by Lambert et al., 2001. The cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified eagle medium (DMEM; Invitrogen-Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and penicillin/streptomycin/glutamine (Invitrogen-Gibco, Grand Island, NY).

Immunocytochemistry: LC and ONA cells cultured on glass coverslips, were fixed with 4% paraformaldehyde, permeablized with 0.2% Triton X-100 and nonspecific binding blocked with 5% Bovine serum albumin (BSA). Cells were then incubated with 1:200 primary monoclonal anti-GFAP antibody (Neomarkers, Fremont, CA) diluted in 1% BSA. Cells incubated with 1% BSA alone served as a negative control. Following washes cells were incubated with 1:400 dilution secondary antibody, Alexa fluor 488 (Molecular probes, Eugene, OR). Cells were incubated with 300nM DAPI to stain the nuclei. The

coverslips were mounted on Fluorsave reagent (Calbiochem, San Diego, CA) and fluorescent images were taken using confocal microscopy (Carl Zeiss Meditec, Inc., Thornwood, NY).

RT-PCR: LC cells were grown to confluence in 100mm dishes. Total cellular RNA was isolated using the Trizol B reagent (Life Technologies, Rockville, MD, USA). cDNA was synthesized from 5 μg of total RNA using random primers and AMV Reverse Transcriptase (Promega, Madison, WI, USA). Reactions without reverse transcriptase were also performed and used as negative controls for experiments. cDNA samples were amplified with specific primers for ppET-1, ET_A and ET_B using Taq polymerase in a DNA thermal cycler (Perkin-Elmer) [40 cycles: denaturation 94°C, 1min; annealing 60°C, 1min; extension 72°C, 2min]. Control RT-PCR reactions without reverse transcriptase or cDNA served as negative controls and did not result in amplification products suggesting that the reactions were not contaminated with genomic DNA. PCR primers for ppET-1, ET_A, ET_B and β-actin used in this study have been previously published (Zhang et al., 2003; Prasanna et al., 2002). In brief, PCR primers were designed from their respective cDNA sequence using Gene Jockey II program (BioSOFT, Ferguson, MO, USA) or Primer 3 program (provided in the public domain at http://www.basic.nwu.edu/biotools/Primer3.html by the Massachusetts Institute of Technology, Cambridge, MA). The PCR primers and their expected amplified product size are listed in Table 1.

QPCR: For quantification of mRNA transcripts by QPCR, amplification was performed as previously described with modifications (Zhang et al., 2003). Briefly 2.5 μl cDNA

samples were amplified with specific primers for ET_A receptor, ET_B receptor and β-actin was used as internal control. For QPCR, SYBR Green PCR core regents (PE Applied Biosystems, Foster City, CA, USA) was used. QPCR amplifications were performed for 50 cycles of denaturation at 95°C for 60sec, annealing 60°C for 60 sec, extension 72°C for 120 sec (for ET_A and β-actin) or 58°C annealing for 60sec and extension at 72°c for 30 sec (for ET_B) in Cepheid Smart Cycler (Cepheid, Sunnyvale, CA, USA). The melting curves were generated to detect the melting temperatures of the specific products immediately after the PCR run. The relative mRNA levels were determined by the comparative C_T method (as described in PE Biosystems User Bulletin #2: http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). The relative mRNA levels in treated versus control are represented as mean percentage ± SEM of three individual experiments. Amplified PCR products were run on 1% or 2% agarose gel stained with ethidium bromide in parallel with 100-bp DNA markers. PCR primers for ppET-1, ET_A, ET_B and β-actin used in QPCR were similar to the primers used in RTPCR and their expected amplified product size are listed in Table 1.

$[Ca^{2+}]i$ measurement: LC cells were incubated with 3μM Fura -2 for 20min (Molecular probes, Eugene, OR) in Hank's Balanced Salt Solution (HBSS) buffer, with or without $CaCl_2$ [in mmoles: 137 Nacl, 2.5 $CaCl_2$, 5 Kcl, 1.47 $MgSO_4$, 2.8 $NaHCO_3$, 5 glucose, 1.47 KH_2PO_4, 1.2 $Mgcl_2$, 1.47 Na_2HPO_4 & 20 HEPES pH 7.4]. In some experiments cells were pre–incubated for 30min with either 1μM BQ610 an ET_A selective antagonist or 1μM BQ788 an ET_B receptor selective antagonist. Cells were treated with 1nM, 10nM and 100nM concentrations of ET-1. Changes in Fura-2 fluorescence following treatment

with ET-1 was monitored and recorded under a Nikon Diaphot microscope using Metafluor software. $[Ca^{2+}]i$ in nM was determined by ratio metric analysis of Fura -2 fluorescence [340:380 excitation] using the equation derived by Grynkiewicz et al., (1985).

Western Blott: LC cells were grown to confluency in 100mm dishes and treated with ET-1 at concentrations of 1, 10 and 100nM in serum free media for 24 hrs. Membrane fractions from LC cells were isolated by a method previously described (Dibas et al., 1996). Briefly LC cells were harvested in PBS, centrifuged and resuspended in homogenizing buffer [in mmoles: 3.2 Tris Hcl, 16 Sodium pyrophosphate, 1.6 EDTA, 0.5 EGTA, 0.6 Ammonium molybdate 3.2 DTT & protease inhibitors]. The resuspended cells were subjected to sonication and clarified lysates were subjected to centrifugation at 100,000g to obtain membrane fractions. Resuspended proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with primary anti ET_A and anti ET_B antibodies (1:200 rabbit polyclonal; Almone labs). Following incubation with anti rabbit HRP conjugated secondary antibody (1:10000; Amersham Biosciences), the blots were developed with ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Densitometric analysis of the bands was performed using the image-analysis software (Scion; National institutes of health, Bethesda, MD). The relative band intensities in treated versus control is represented as mean percentage ± SEM of three individual experiments.

Nitrite Assay: LC cells were grown to confluence in 24 well plates. The cells, in some experiments were pre–incubated for 30min with either 1μM BQ610 or 1μM BQ788, ET_A

and ET_B receptor antagonists, respectively. Following treatment with 1nM, 10nM and 100nM ET-1, 100nM ET-1 +BQ788, 100nM ET-1 +BQ610, BQ788 alone and BQ610 alone in serum free media for 24 hrs, the culture media was collected and analyzed for NO. The NO released in the media was measured as nitrite, the final oxidation breakdown product of nitric oxide (NO), using a Griess colorimetric NO synthase (NOS) assay kit (Calbiochem, San Diego, CA). A standard nitrite curve was generated according to the manufacturer's protocol and Nitrite concentration in the culture medium was determined from the standard curve.

Results

Expression of ET_A, ET_B receptors and pre-pro ET-1 in lamina cribrosa cells.

LC cell lines from the 66 and 90-year-old donors showed no immunoreactivity to GFAP (Fig. 1(A), & Fig. 1(B) respectively) while an intense staining for GFAP was observed in optic nerve head astrocytes (Fig. 1(C)). Cells incubated with secondary antibody in the absence of primary antibody showed no immunoreactivity (Fig. 1(D)). RT-PCR analysis of total RNA isolated from these two LC cell lines showed the expression of both ET_A and ET_B receptors (Fig. (2)). These two LC cell lines also express pre-proET-1, the primary gene transcript and precursor of endothelin-1 (Fig. (2)).

ET-1 mediated intra cellular calcium [Ca^{2+}] i mobilization in LC cells.

The basal levels of [Ca^{2+}]i in LC cells incubated in buffer with or with out calcium was about 90 nM. LC cells treated with 1nM, 10nM and100nM concentrations of ET-1 in calcium containing buffer, resulted in a dose-dependent, increase in [Ca^{2+}]i with

concentrations of $[Ca^{2+}]i$ rising above 1μM (Fig. (3) A). The increase in intracellular calcium levels were transient and were followed by a sustained increase in $[Ca^{2+}]i$ with levels above the base line. LC cells treated with ET-1 in calcium free media also showed a dose-dependent, transient increase in $[Ca^{2+}]i$, however the sustained increase above the base line was not observed (Fig. (3)B). Preincubation of LC cells with BQ610, a selective ET_A receptor antagonist, completely blocked the ET-1 mediated increase in intracellular calcium levels (Fig. 3(C)). BQ788, a selective ET_B receptor antagonist, had no effect on ET-1 mediated increases in intra cellular calcium levels (Fig. 3(C)). Interestingly, LC cells pre-treated with ET-1 for 24 hrs were unable to respond to subsequent ET-1 treatment in terms of its effect on intra cellular calcium (Fig. 3(C)). A summary of the results on calcium mobilization is presented in Table 2. There was no apparent differences in calcium responses to ET-1 in cell lines obtained from the POAG and normal patient.

ET-1 mediated regulation of ET_A and ET_B receptor expression in LC cells.

Western blot analysis of LC cells treated with 1nM, 10nM and 100nM, ET-1 for 24hrs, revealed that ET-1 at all doses significantly reduced the ET_A receptor expression (Figures 4A, 4B). The most significant down regulation was observed at 1nM concentration. Probing the same blot for expression of ET_B receptors showed a dose-dependent increase in the expression of ET_B receptors (Fig. 4(A) and Fig. 4(C)). QPCR analysis indicated that ET-1 decreased ET_A receptor mRNA expression in a dose dependent manner (Fig. 4(D) and Fig. 4(E)), while a dose-dependent increase in ET_B receptor mRNA expression was observed (Fig. 4(D) and Fig. 4(F)). These results suggested that ET-1 regulated the

expression of its receptors both at the level of protein and mRNA. The decrease in ET_A receptors, following treatment with ET-1, could probably explain the loss of ET-1mediated increase in calcium mobilization experiments.

ET-1–mediated NO release from LC cells.

In order to determine if the ET_B receptor changes were linked to a cellular response, we determined ET-1 mediated release of NO from LC cells using a Griess colorimetric assay. Treating LC cells with ET-1 for 24 hrs resulted in, an increase in nitrite accumulation in the culture medium (Fig. 5). There was a dose-dependent trend and a statistically significant increase in nitrite levels at the 100nM concentration of ET-1. The ET-1 mediated increase in nitrite accumulation was significantly blocked with BQ788, an ET_B receptor antagonist, and partially with BQ610 an ET_A receptor antagonist, suggesting that, both receptors contributed to NO production (Fig. 5).

Discussion

The mechanisms contributing to the loss retinal ganglion cells in glaucoma are not completely understood, however, a primary site of injury appears to be at the level of the lamina cribrosa (Quigley, 2005; Hernandez, 2000). Recent studies have shown that LC cells respond to mechanical stretch upregulating the expression of various extracellular protein molecules similar to that observed in glaucomatous subjects and animal models (Kirwan et al., 2005). *In vitro* cultures of LC cells appear to express neurotrophin receptors and to secrete neurotrophins and thus LC cells may serve as neurotophic support to the RGC axons (Lambert et al., 2004). Endothelin, a potent vasoactive peptide

has been proposed to play an important role in the pathophysiology of glaucoma (Yorio et al., 2002). What role ET has in LC cell function is not known. Presently, we have identified pre-proET-1 and ET-1 receptors, ET_A and ET_B, in LC cells and determined the linkage between ET receptors with down stream LC signaling pathways.

ET-1, ET-3 and both ET_A & ET_B receptors are widely distributed in the eye and their physiological functions in normal and pathological states, such as glaucoma are well characterized (Yorio et al., 2002; Prasanna, et al., 2003). The increase in endothelin-1 synthesis and secretion can be triggered by various cytokines including tumor necrosis factor-α (TNFα), Transforming growth factor- β (TGF-β) and thrombin (Kurihara et al., 1989; Emori, et al., 1992; Prasanna et al., 1998; Woods et al., 1999; Narayan et al., 2004). Mechanical stress also can induce the expression and secretion of ET-1 in endothelial cells, epithelial cells and astrocytes (Chen et al., 2001; Tschumperlin et al., 2003; Ostrow and Sachs 2005). Optic nerve head astrocytes express pre-pro-ET-1 and are capable of synthesizing ET-1 (Desai et al., 2004). Recently endothelin converting enzymes have been characterized in the human and bovine optic nerve head (Dibas et al., 2005). The expression of pre-pro ET-1 in LC cells suggests that LC cells could also contribute to the endogenous secretion of ET-1 under normal and pathological conditions like glaucoma. As an autocrine action, ET-1 could influence LC function. Our findings that ET-1 can increase intracellular calcium in LC cells suggests that, ET-1 could affect the contractile state of this cell type, which contains smooth muscle actin (Lambert et al., 2001)

Biphasic increases in intracellular free calcium, characterized by an initial transient peak followed by a sustained increase, is a key event in ET-1 mediated receptor activation in several other cell types including smooth muscle cells, endothelial cells, epithelial cells and astrocytes (Pollock et al., 1995). ET-1 mediated activation of the Gq coupled ET_A receptor, results in the classic phospholipase C activation, IP3 generation and mobilization of intracellular calcium stores. Such actions result in a rise in free cytosolic calcium and correspond to the initial transient peak observed following ET-1 stimulation. The sustained release of intracellular calcium, however has been attributed to the influx of extracellular calcium via the activation of L-type calcium channels, the store operated calcium channels or the non selective cation ion channels located in the plasma membrane (Kawanabe and Nauli 2005; Miwa et al., 2005). ET-1 mediates calcium mobilization in various cell types of the eye including trabecular meshwork cells, ciliary smooth muscle cells, retinal pigment epithelial cells and optic nerve head astrocytes (Prasanna et al., 2003). Calcium mobilization in LC cells was also consistent with that seen in other ocular cells types, characterized by a similar biphasic response which appeared to be mediated by the ET_A receptor. The sustained increase in intracellular calcium by ET-1, was completely abolished in calcium free external media, while the transient peak was not affected suggesting the involvement of extracellular calcium influx in the sustained phase of the ET-1 mediated increase in intracellular calcium response. Brief exposure or intermittent pulses of ET-1, results in rapid desensitization of ET-1 mediated calcium mobilization in various cell types (Gandhi et al., 1990; Cyr and Kris 1993; Cyr et al., 1993; Stojilkovic and Catt 1996). The rapid prolonged homologus

desensitization is unique to ET-1 induced signaling while other seven trans-membrane agonists, like histamine, do not result in rapid prolonged desensitization (Oles et al., 1997). The recovery of calcium signaling following desensitization is varied in different cell types lasting for more than 20 hrs (Oles et al., 1997). In LC cells treated with ET-1 for 24 hrs there was no response to additional ET-1 treatment on $[Ca^{2+}]i$ mobilization, suggesting a desensitization mechanism. Several mechanisms could explain such an action, including receptor endocytosis, defective G-protein coupling, reduced surface expression of ET_A receptors or receptor phoshporylation (Pollock et al., 1995). In LC cells treated with ET-1 for 24 hrs we also observed a decrease in membrane receptor protein, as well as a decrease in expression of ET_A receptors at the level of transcription. The decrease in ET_A receptor protein levels at the membrane may result as a consequence of prolonged exposure to agonist as it has been shown for other G-protein coupled receptor proteins like β-adrenergic agonists (Grady et al., 1997; Wallukat 2002). The ET-1 mediated decrease in ET_A receptor levels appeared to be initiated, inpart, from transcriptional regulation. Such an activity seems to be appropriate in the presence of continuous ET-1. It was interesting to observe that the receptor protein expression following 24 hr treatment with ET-1 was significantly decreased at 1nM ET-1 with a similar decrease in mRNA expression. However with increasing doses of ET-1 the mRNA expression continued to decrease while the protein expression appears to recover although still less than control. Since membrane receptor protein was used for the measurement, we think the recovery was a result of recycling of the ET_A receptor following internalization. Such recycling of the ET_A receptor has been shown previously

(Bremnes et al., 2000). It was also observed that ET_B receptor expression was elevated in the continuous presence of ET-1. This too is expected as, the ET_B receptor serves as a clearance receptor and prolonged exposure with ET-1 does result in increase in ET_B receptor expression in other cell types to help clear the additional ET-1 present (Rogers et al., 1997; Iwasa et al. 1999; D'Orleans-Juste et al., 2002; Schinelli 2002).

Several pathological conditions including cardiovascular and CNS injury, associated with elevated ET-1 levels, also show an upregulated ET_B receptor expression (Rogers et al., 1997; Iwasa et al. 1999; D'Orleans-Juste et al., 2002; Schinelli 2002). Increased ET_B receptor expression was also observed at the level of the optic nerve head in animal models of glaucoma and human glaucomatous eyes (Prasanna et al., 2005; Wang et al., 2006). ET_B receptors serve as clearance receptors for ET-1 and therefore play an important role in maintaining normal levels of the potent peptide ET-1 (Fukuroda et al., 1994; Dupuis et al., 1996). However, the shift in expression, towards the ET_B receptor subtype could also result in altered signal transduction because, ET_A and ET_B receptors are coupled to different G proteins and down stream targets (Takagi et al., 1995). It has been observed that mechanical deformation and ET-1 results in ET_B receptor upregulation and apoptosis of smooth muscle cells isolated from rabbit blood vessels (Cattaruzza et al., 2002). The reactive phenotype of astrocytes, including enhanced GFAP expression and proliferation appears to be mediated by the ET_B receptor subtype and inhibition of ET_B receptors results in a protective effect following CNS injury (Baba 1998; Koyama et al., 1999). Chronic exposure to ET-1 in cultured fibroblasts also

results in a switch of receptor subtype from ET_A to ET_B, resulting in an ET_B mediated increase in collagen synthesis (Horstmeyer et al., 2005). Similarly a switch in receptor expression in LC cells, a similar phenotype to fibroblasts, could account for some of the extracellular matrix changes that often accompany glaucoma pathology. The role of the ET_B receptor and nitric oxide (NO) in maintaining the vascular tone is well established in the vasculature (D'Orleans-Juste et al., 2002). Excessive NO release and its metabolite, peroxynitrates, however, are damaging to neurons and have been implicated in various CNS disorders and glaucoma (Neufeld 1999). NO is synthesized from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS). NOS exists in different isoforms including constitutive forms (eNOS and nNOS) and the inducible NOS (iNOS) found in activated tissue (Alderton et al., 2001). The constitutive forms of NOS are calcium sensitive enzymes and require higher concentration of calcium for activation in comparison to iNOS (Salerno et al., 1997). ET-1 enhances the activity of all the isoforms of nitric oxide synthase (eNOS, nNOS & iNOS) through the activation of ET_B receptors resulting in increased NO production in several cell types including astrocytes and endothelial cells (Hirata Y et al., 1993; Warner et al., 1989; Oda et al.,1997 Prasanna et al., 2000; Zhang et al., 2003). We have demonstrated that ET-1 also mediates NO release in LC cells. ET-1 mediated NO release was blocked by both the ET_B receptor antagonist, BQ788, and the ET_A receptor antagonist, BQ610, suggesting that both ET_B and ET_A receptors are involved in ET-1 mediated NO release by LC cells. To what extent NO production by LC cells participates in LC function or pathology is not yet clear. It could be produced as a result of the response to ET_A mediated calcium mobilization as a

relaxant or as a consequence of ET_B activation. The finding that both the ET_A and ET_B antagonists reduced NO production suggests that, this may involve more than one receptor's signaling. Certainly the significant increase in intracellular calcium through ET_A receptors could play a role in NO production through some cellular feedback mechanism, balancing the calcium/contractile actions with NO/relaxant effects as part of cellular homeostatic mechanism or the higher calcium levels could trigger constitutive NOS to produce NO.

In conclusion, we have characterized and extended the expression of the endothelin and its receptors to include LC cells. To support such an action we have identified pre-pro endothelin-1, endothelin receptors and their effects on GFAP negative primary LC cells. Since LC cells appear to be important in the pathogenesis of glaucoma and ET-1 has been implicated in this disease, ET-1's contribution to the pathogenesis of glaucoma could involve actions on LC cells. However, the pathways by which ET could potentially promote pathological changes in LC, is an area that needs further investigation.

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Fig.1. Immunofluroscence staining for GFAP. LC cells (A) showed no GFAP staining. Nuclei are DAPI stained (blue). DIC Image of LC cells (D). Intense staining for GFAP (green) was seen throughout the cytoplasm in ONH astrocytes from a 66 year old donor (C). DIC image of ONH astrocytes (D). Negative control, treated with secondary antibody showed no GFAP staining (E) and its corresponding DIC image (F). Scale bar in L=50μm.

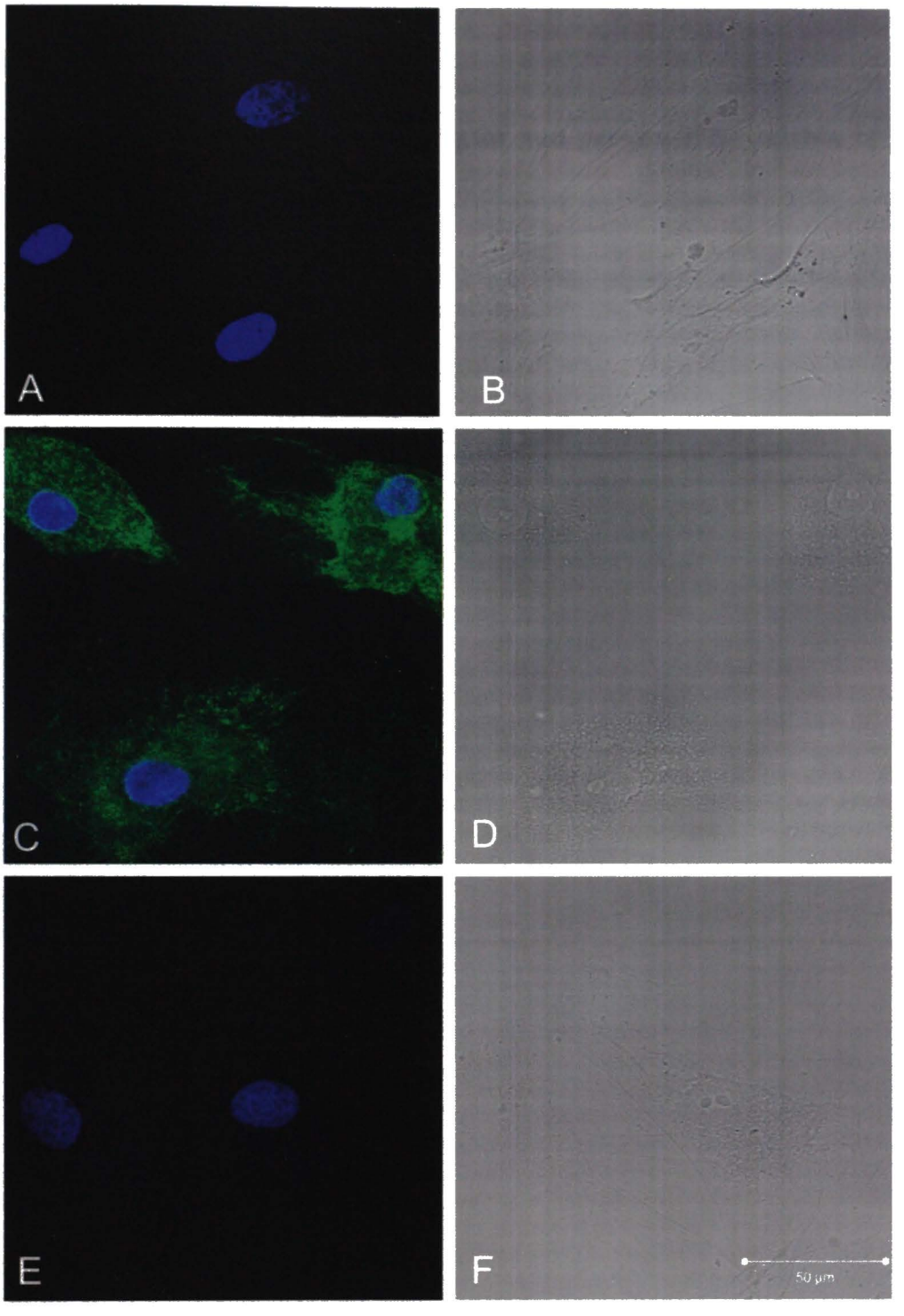

A
B
C
D
E
F
50 µm

Fig.2. Expression of ET_A, ET_B receptor and pre-pro ET-1 mRNA in LC cells. RT-PCR products of ET_A receptor, ET_B receptor and pre-pro ET-1 from a 66-year old, normal donor (A) and from a 90-year old, glaucomatous donor (B). RT-PCR control with no reverse transcriptase (C).

Table 1. PCR primer sequences and expected product sizes

Gene	Primer	Product size
ETA	(S) ACTCATCAACCCACTAATTTGGT (A) TTSTGCTGTTCSCCCCTATATTC	561
ETB	(S) TCACTGTGCTGAGTCTATGTGC (A) AGCAGCTTCGCAGCTAACTTCC	206
ppET-1	(S) TATCAGCAGTTAGTGAGAGG (A) CGAAGGTCTGTCACCAATGTGC	180
β-Actin	(S) TGTGATGGTGGGAATGGGTCAG (A) TTTGATGTCACTCACGATTTCC	514

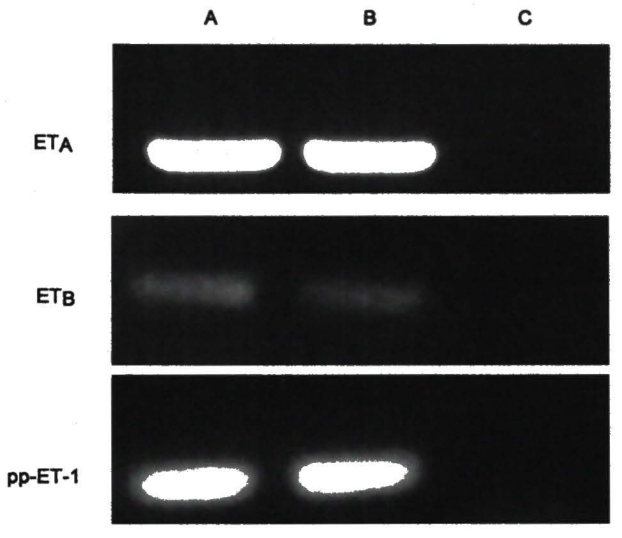
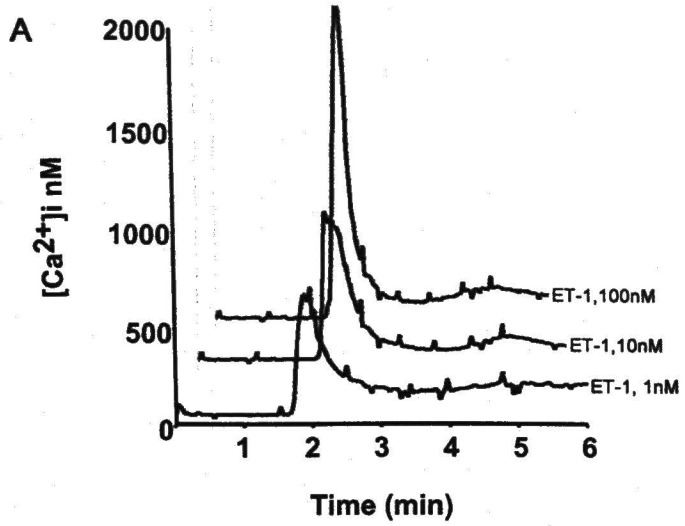
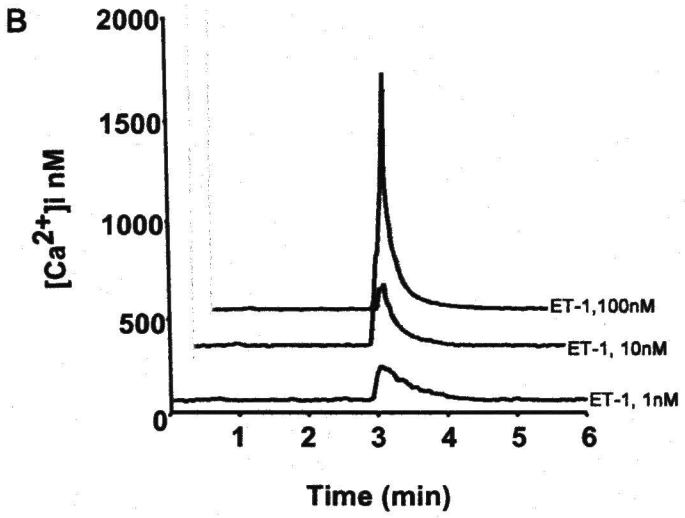

A
B
C
ETA
ETB
pp-ET-1

Fig.3. ET-1 mediated $[Ca^{2+}]i$ mobilization in LC cells determined by Fura -2 calcium imaging. Representative curves illustrating the dose dependent increase in $[Ca^{2+}]i$ with 1,10 and 100nM ET-1, characterized by an initial transient spike followed by a sustained increase with levels significantly above base line (A). Dose-dependent increase in $[Ca^{2+}]i$ with 1, 10 and 100nM ET-1 in calcium free media, characterized by a initial transient peak only, which is not followed by a sustained increase, suggesting a role of extracellular Ca^{2+} in ET-1 response (B). Furthermore pretreatment with ET-1 100nM for 24 hrs prevents the increase in $[Ca^{2+}]i$ by acute ET-1 administration (as indicated by arrow (C)). Blockade of the ET_A receptor by BQ610 prevents ET-1 mediated $[Ca^{2+}]i$ mobilization, while BQ788 an ET_B selective antagonist has no effect on ET-1 induced $[Ca^{2+}]i$ mobilization (C).


A
2000
1500
1000
500
0
[Ca2+]i nM
ET-1,100nM
ET-1,10nM
ET-1, 1nM
1
2
3
4
5
6
Time (min)


B
2000
1500
1000
500
0
[Ca2+]i nM
ET-1,100nM
ET-1, 10nM
ET-1, 1nM
1
2
3
4
5
6
Time (min)

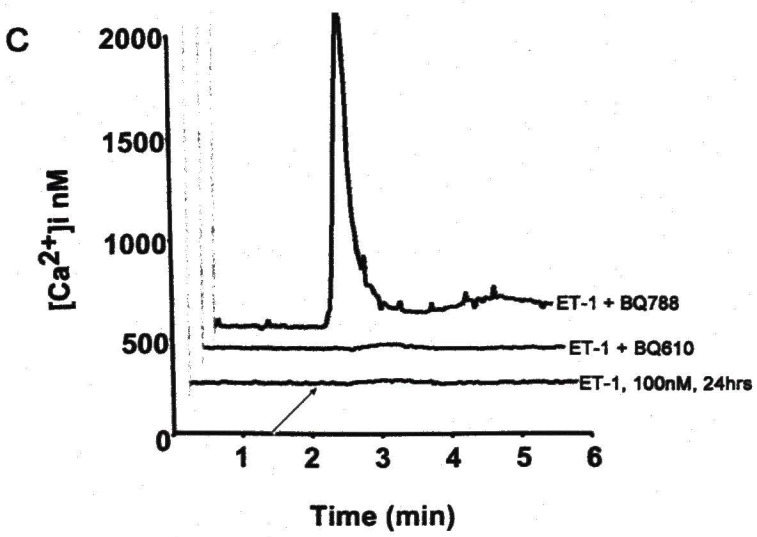

C
2000
1500
1000
500
0
[Ca2+]i nM
ET-1 + BQ788
ET-1 + BQ610
ET-1, 100nM, 24hrs
1
2
3
4
5
6
Time (min)

Table 2. Summary of ET-1 mediated $[Ca^{2+}]i$ mobilization in LC cells determined by Fura-2 Imaging

Treatment	$[Ca^{2+}]I$ nM	Cells (n)
ET-1 Dose Response		
Baseline before treatment	56±10	15
ET-1, 1nM	429±100*	15
Baseline After treatment	91±18**	15
Baseline before treatment	181± 25	15
ET-1, 10nM	730± 67*	15
Baseline After treatment	169±14**	15
Baseline before treatment	82±15	22
ET-1, 100nM	1533±601*	22
Baseline After treatment	345±55**	22
ET-1 Dose Response in Ca^{2+} free media		
Baseline before treatment	51±10	15
ET-1, 1nM	137±23*	15
Baseline After treatment	63±12	15
Baseline before treatment	74±9	15
ET-1, 10nM	341± 30*	15
Baseline After treatment	96±17	15
Baseline before treatment	112±12	22
ET-1, 100nM	1100±300*	22
Baseline After treatment	122±10	22
Regulation of ET-1 mediated [Ca2+]i		
Baseline before treatment	75±10	10
ET-1 (100nM), 24hrs	75±10	10
Baseline before treatment	82±15	10
ET-1 (100nM) + BQ610 (1μM)	82±15	10
Baseline before treatment	181± 25	25
ET-1 (100nM) + BQ788 (1μM)	1630±650*	25
Baseline After treatment	325±30**	25

Statistical significance between *baseline, peak and 1 minute post peak mean values, **baseline after and before treatment by One-way ANOVA and Student-Newman-Keuls multiple comparison Test at $p < 0.05$. Date are expressed in mean nM ± SEM.

Fig. 4. Effects of ET-1 on ET_A and ET_B receptor expression in LC cells determined by western blot and QPCR analysis. Western blot analysis of ET_A and ET_B receptor protein expression, following the treatment with ET-1, (1, 10 & 100nM) for 24 hrs (A). The quantification of band intensities for ET_A and ET_B receptors are represented as mean percentage ± SEM compared with the corresponding control band (B). A significant decrease in ET_A receptor protein expression was observed with all doses of ET-1, with a greatest decrease at 1nM. In the same blot a dose-dependent increase in ET_B receptor protein expression was seen, with a significant increase at 100nM. QPCR products separated on ethidium bromide stained 1.1% agarose gel of ET_A receptor, ET_B receptor and the internal control β-actin, following the treatment with ET-1 (1,10 & 100nM) for 24 hrs (C). QPCR data presented as the mean percentage ± SEM of mRNA levels of ET_A and ET_B receptor expression compared with the respective control (D). A significant decrease in ET_A receptor mRNA expression was observed while a dose-dependent increase in ET_B receptor mRNA was observed following the treatment with ET-1. *Statistical significance of ET-1 treatment versus control as determined by one-way ANOVA and student-Newman-Keuls multiple comparison test ($p<0.05$). Experiments were repeated 3 times in LC cells from a 66-year old donor.

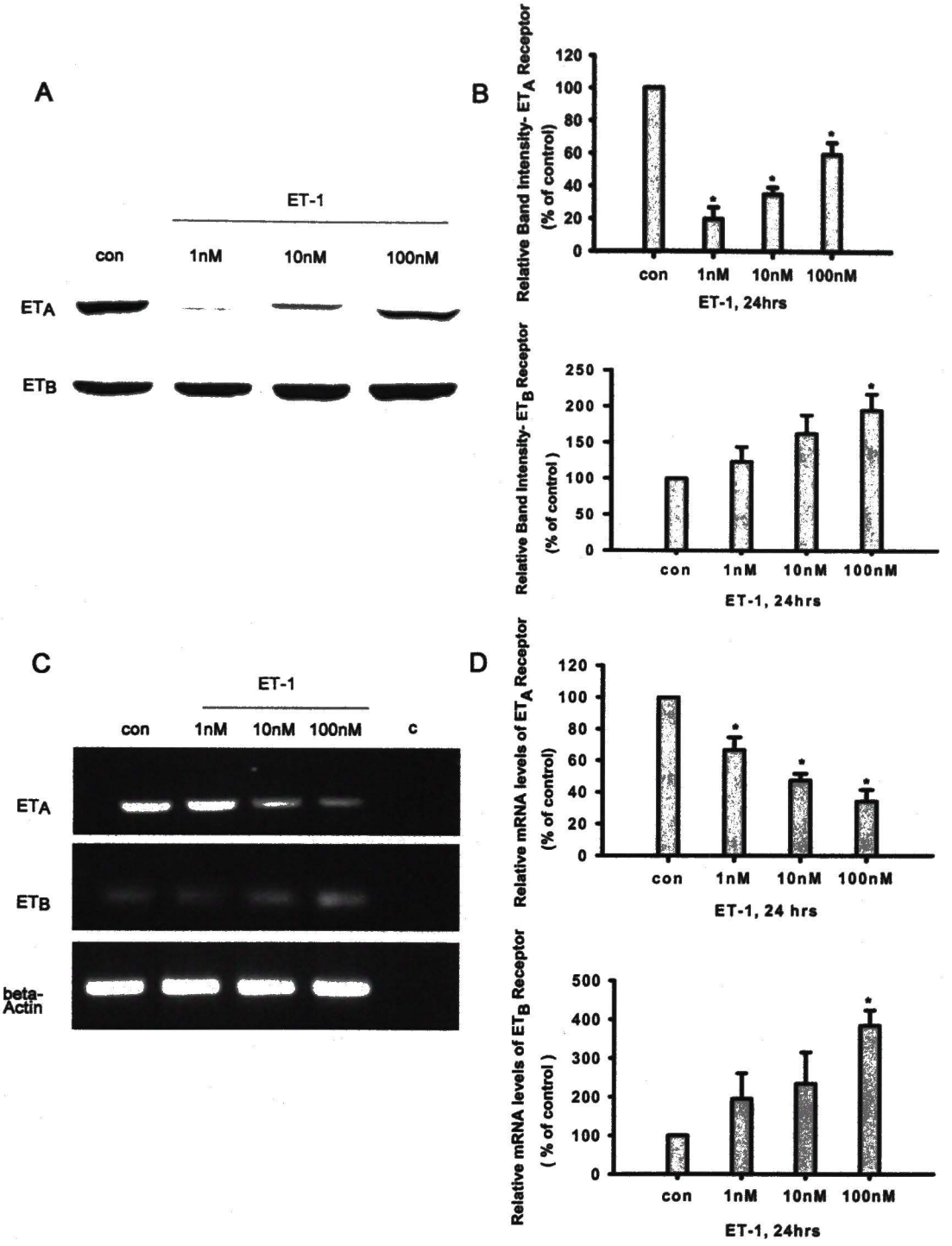
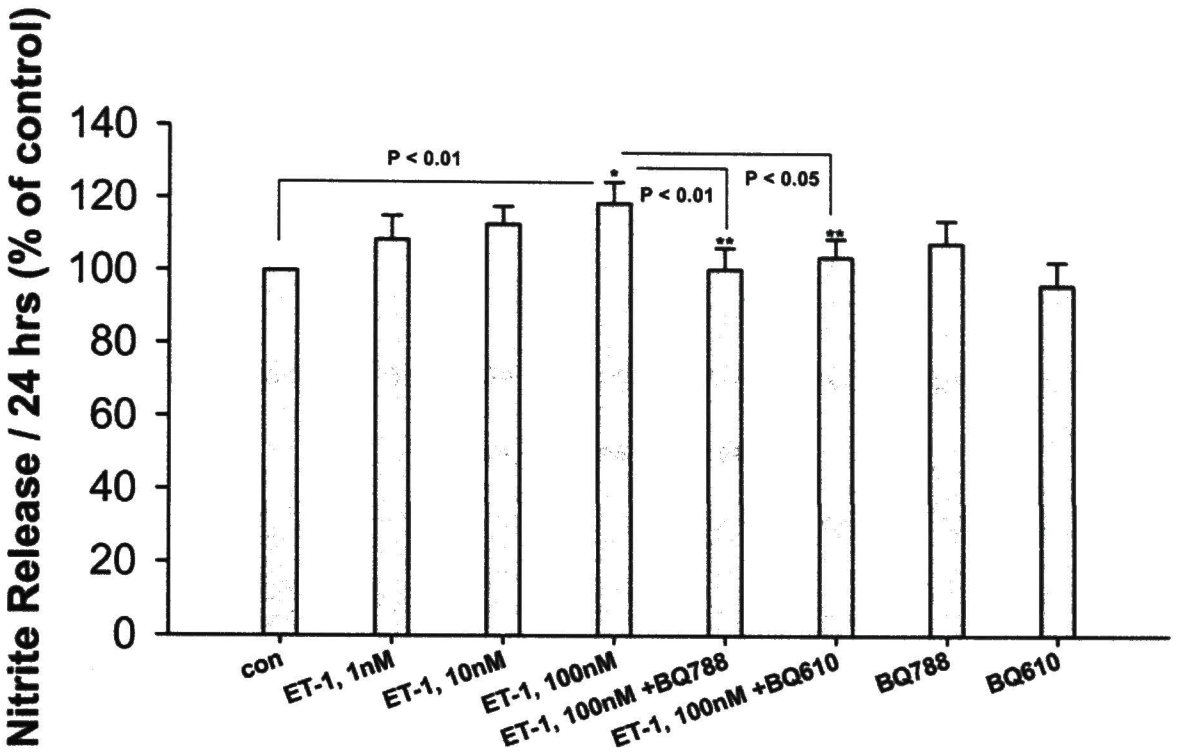

A
ET-1
con
1nM
10nM
100nM
ETA
ETB
B
Relative Band Intensity- ETA Receptor
(% of control)
120
100
80
60
40
20
0
con
1nM
10nM
100nM
ET-1, 24hrs
Relative Band Intensity- ETB Receptor
(% of control)
250
200
150
100
50
0
con
1nM
10nM
100nM
ET-1, 24hrs
C
ET-1
con
1nM
10nM
100nM
c
ETA
ETB
beta-
Actin
D
Relative mRNA levels of ETA Receptor
(% of control)
120
100
80
60
40
20
0
con
1nM
10nM
100nM
ET-1, 24 hrs
Relative mRNA levels of ETB Receptor
(% of control)
500
400
300
200
100
0
con
1nM
10nM
100nM
ET-1, 24hrs

Fig. 5. Effects of ET-1 on nitric oxide (NO) release form LC cells. NO released into the culture media from LC cells following various treatment conditions for 24 hrs including, control with no treatment, ET-1 (1, 10, &100 nM), cells pre-incubated with ET_B receptor antagonist (BQ788 -1μM) or ET_A receptor antagonist (BQ610 -1μM) for 30 min and subsequently treated with ET-1 (100nM) in the presence of respective antagonists for 24 hrs. The antagonist BQ610 and BQ788 were also tested without ET-1 addition. NO released was converted to nitrites and measured using the Griess colorimetric assay. Data are expressed as the mean percentage ± SEM of nitrite released in the culture medium. A dose dependent increase in nitrite release was observed with ET-1 and the ET-1 mediated release of NO was significantly inhibited by the ET_B receptor antagonist, BQ788 and as well as the ET_A receptor antagonist, BQ610. The antagonists, BQ788 and BQ610 alone did not significantly affect NO release. * statistical significance of ET-1 versus control; **statistical significance of ET-1 +BQ7788 versus control; **statistical significance of ET-1 +BQ610 versus ET-1 alone, as determined by one-way ANOVA and student-Newman-Keuls multiple–comparison test ($p<0.05$). Experiments were repeated four times on LC cells obtained from 66-year old donor.


Nitrite Release / 24 hrs (% of control)
140
120
100
80
60
40
20
0
P < 0.01
P < 0.01
P < 0.05
*
**
**
con
ET-1, 1nM
ET-1, 10nM
ET-1, 100nM
ET-1, 100nM +BQ788
ET-1, 100nM +BQ610
BQ788
BQ610

CHAPTER III

Endothelin-1 Mediated Regulation of Extracellular Matrix Collagens in Cells of Human Lamina Cribrosa.

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Experimental Eye Research

(In Revision)

Abstract

Primary Open Angle Glaucoma (POAG) is a progressive optic neuropathy characterized by loss of retinal ganglion cells, optic nerve degeneration and characteristic excavation of the optic disc. Extensive ECM remodeling with increase in collagen type I, IV, VI, and elastin degeneration has been associated with a compromise in structural integrity of the LC resulting in excavation of the optic disc and ganglion cell loss. Endothelin-1(ET-1), a potent vaso-active peptide, also mediates extracellular matrix regulation resulting in an increase in collagen deposition in various cell types and tissues and has been proposed to play a key role in glaucoma pathology. The role of ET-1 in the regulation of extracellular matrix collagens at the level of optic nerve head is not known. In this study we have examined the role of ET-1 in extracellular matrix collagen regulation in primary cultures of human lamina cribrosa cells. Our hypothesis is that ET-1 increases remodeling of the ECM of cells of the lamina cribrosa. Such actions could contribute to the development of optic neuropathy. QPCR analysis revealed that ET-1 mediated an increase in mRNA levels of collagen type I alpha 1 and collagen type VI alpha 1 chains at all doses of ET-1 with a significant increase at 1nM and 10nM concentration in LC cells. A dose dependent increase in collagen type I and type VI protein deposition and secretion was also observed by Western blot in response to ET-1 and was significant at 10nM and 100nM concentrations of ET-1. ET-1 increased the [^{3}H] proline uptake in LC cells suggesting that ET-1 contributed to an increase in total collagen synthesis in LC cells. ET-1 - mediated increase in collagen type I, type VI and total collagen synthesis was significantly blocked by the ET_A receptor antagonist, BQ610, as well as with the ET_B

receptor antagonist, BQ788, suggesting the involvement of both receptor subtypes in ET-1 mediated collagen synthesis in LC cells. These results suggest that ET-1 regulates extra cellular matrix–collagen synthesis in LC cells and may contribute to ECM remodeling at the level of LC of POAG subjects who have elevated plasma and aqueous humor levels of endothelin-1.

Keywords. Endothelin, Lamina Cribrosa, Extacellular matrix, Collagens, Glaucoma

Introduction

Primary Open Angle Glaucoma (POAG), a leading cause of blindness worldwide, is a progressive optic neuropathy characterized by loss of retinal ganglion cells, optic nerve degeneration with characteristic excavation of the optic disc (Quigley 2005; Quigley and Broman 2006;). Elevated intraocular pressure (IOP) and age are important risk factors (Leibowitz et al., 1980; Klein et al., 1992). Various mechanisms including elevated IOP, ischemia and glutamate mediated excitotoxicity have been implicated in retinal ganglion cell death and the primary site of injury appears to be at level of the lamina cribrosa (Hernandez, 2000; Kuehn et al., 2005; Quigley, 2005). The lamina-cribrosa (LC) is a distinct region of the ONH composed of perforated connective tissue plates through which the RGC axons exit the eye (Anderson 1969 and Birch et al., 1997). LC provides mechanical and metabolic support to the exiting nerve fibers against a pressure gradient established by the high IOP and the surrounding low intracranial pressure (Burgoyne et al., 2005). Marked disruption in the architecture of the LC is observed in POAG subjects and includes, backward displacement, distortion and collapse of the LC and a corresponding extracellular matrix (ECM) reorganization (Miller and Quigley 1988). These changes in LC have been associated with blockade of axonal transport, resulting in optic nerve degeneration and loss of RGCs by apoptosis (Quigley et al., 1983; Sakugawa and Chihara 1985; Martin et al., 2003). Increase in ECM components including collagen type I, type IV, type VI, and elastin degeneration is observed in LC of POAG subjects and animal models of glaucoma (Hernandez et al., 1987; Quigley 1988; Morrison et al., 1989; Miller and Sawaguchi et. al, 1999; Hernandez et al., 2000). Excess accumulation of

collagens, the principal components of ECM, results in fibrosis leading to loss in normal structure and function of the tissue (Varga et al., 2005). The changes in collagens observed in POAG could therefore alter the biomechanical properties of LC and result in the loss of structural integrity (Tengroth and Ammitzboll 1984; Rehnberg et al., 1987). Pathophysiological changes in the optic nerve head astrocytes (ONA) and lamina cribrosa cells (LC) including, hypertrophy, migration. ECM regulation and proliferation have been attributed to the changes observed in the lamina cribrosa of POAG subjects (Hernandez 2000; Prasanna et al., 2002; Kirwan et al., 2005; Morrison et al., 2005). LC cells constitute a major cell type in the lamina cribrosa region and are characterized by the lack of glial fibrillary acidic protein (GFAP) (Hernandez et al., 1988; Lambert et al., 2001; Kirwan et al., 2005). *In vitro* cultures of LC cells appear to express neurotrophin receptors and secrete neurotrophins and thus LC cells may serve as neurotophic support to the RGC axons (Lambert et al., 2004). LC cells share biochemical similarities to trabecular meshwork cells *in vitro* and *in vivo*, indicating that perhaps POAG could involve both the trabecular meshwork and lamina cribrosa (Tengroth and Ammitzboll 1984 and Steely et al., 2000). Recent studies have shown that LC cells respond to mechanical stretch upregulating the expression of various extracellular protein molecules including collagen I, IV, VI, fibronectin, and elastin similar to that observed in glaucomatous subjects and animal models (Kirwan, et al., 2005). LC therefore represents an important pro-fibrotic tissue prone to tissue fibrosis resulting in loss of structural integrity and collapse of LC.

Endothelin-1 (ET-1) and its isoforms, ET-2 and ET-3, belong to a family of 21-amino acid vasoactive peptides and play a regulatory role in vascular homeostasis mediating their effects through seven trans-membrane G-protein coupled receptors, endothelin receptor A (ET_A) and endothelin receptor B (ET_B). Apart from its known vasoactive properties ET-1 also influences a number of cellular events including proliferation, differentiation and apoptosis (Rubanyi and Polokoff; 1994; Kedzierski and Yanagisawa 2001). POAG subjects have significantly higher levels of ET-1 in plasma and aqueous humor compared to their age matched controls (Sugiyama et al., 1995 and Noske et al., 1997). Elevated IOP animal models of glaucoma also demonstrate significant increase in ET-1 levels (Kallberg et al., 2002; Prasanna et al., 2005). Intravitreal administration of ET-1 in various animal models results in the loss of retinal ganglion cells by apoptosis, a blockade of axonal transport and activation of optic nerve head astrocytes that results in optic neuropathy similar to that observed in glaucoma (Stokely et al., 2002; Chauhan et al., 2004; Lau et al., 2006). ET-1 therefore may play a key role in glaucoma pathology (Yorio et al., 2002). ET-1 also plays an important role as a pro- fibrotic factor in initiating and maintaining fibrosis of various tissues resulting in increases in collagen synthesis and ECM accumulation in several cell types including cardiac myocytes, fibroblasts and smooth muscle cells (Eng and Friedman 2000; Eddy 2000; Wakatsuki et al., 2004; Clozel and Salloukh 2005; Tsukada et al., 2006). ET-1 is also capable of modulating the effects of other pro-fibrotic factors like, transforming growth factor-beta and connective tissue growth factor. Inhibiting ET-1 activity using a dual ET_A and ET_B receptor antagonist prevents ET-1 mediated tissue fibrosis (Rodriguez-Vita et al., 2005; Clozel

and Salloukh 2005). The role of ET-1 in increasing ECM components has also been demonstrated in diabetic retinopathy (Khan et al., 2006). However the role of ET-1 in the regulation of ECM at the level of optic nerve head remains to be studied. In the present study we examined ET-1 mediated extra cellular matrix collagen changes in lamina cribrosa cells.

Materials and methods

Cell culture: Primary cultures of human Lamina cribrosa (LC) cells derived from four normal donors (ages 58, 84, 85, 87 yrs) were characterized previously as an LC population and were a generous gift from Dr Robert Wordinger (UNTHSC, Fort Worth, TX) and Dr Abe Clark (Alcon labs, Fort Worth, TX) (Lambert et al., 2004; 2001). The cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified eagle medium (DMEM; Invitrogen-Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and penicillin/streptomycin/glutamine (Invitrogen-Gibco, Grand Island, NY).

QPCR: LC cells were grown to confluence in 100mm dishes. Total cellular RNA was isolated using the Trizol B reagent (Life Technologies, Rockville, MD, USA). cDNA was synthesized from 5 μg of total RNA using random primers and AMV Reverse Transcriptase (Promega, Madison, WI, USA). Reactions without reverse transcriptase were also performed and used as negative controls for experiments. For quantification of mRNA transcripts by QPCR, amplification was performed as previously described with modifications (Zhang et al., 2003). Briefly 2.5 μl cDNA samples were amplified with specific primers for COL I alpha 1, COL VI alpha 1 and β-actin was used as internal

control were amplified using SYBR Green PCR core regents (PE Applied Biosystems, Foster City, CA, USA). QPCR amplifications were performed for 50 cycles of denaturation at 95°C for 60sec, annealing 60°C for 60 sec, extension 72°C for 120 sec (for ET_A and β-actin) or 58°C annealing for 60sec and extension at 72°c for 30 sec (for ET_B) in Cepheid Smart Cycler (Cepheid, Sunnyvale, CA, USA). The melting curves were generated to detect the melting temperatures of the specific products immediately after the PCR run. The relative mRNA levels were determined by the comparative C_T method (as described in PE Biosystems User Bulletin #2: http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). The relative mRNA levels in treated versus control are represented as mean percentage ± SEM of four individual experiments. Amplified PCR products were run on 0.75% agarose gel stained with ethidium bromide in parallel with 100-bp DNA markers. Control RT-PCR reactions without reverse transcriptase or cDNA served as negative controls and did not result in amplification products suggesting that the reactions were not contaminated with genomic DNA. PCR primers for COL I alpha 1, COL VI alpha 1 and β-actin used in this study were designed from their respective cDNA sequence using Gene Jockey II program (BioSOFT, Ferguson, MO, USA) or Primer 3 program (provided in the public domain at http://www.basic.nwu.edu/biotools/Primer3.html by the Massachusetts Institute of Technology, Cambridge, MA). The authenticity of QPCR products was confirmed by DNA sequencing and a BLAST search of the sequence through National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The PCR primers and their expected amplified product size are listed in Table 1.

Immunocytochemistry: LC cultured on glass coverslips were treated with or with out ET-1 (100nM) in serum free medium for 48 hrs. Following treatment the cells were fixed with 4% paraformaldehyde. Nonspecific binding was blocked with 5% Bovine serum albumin (BSA). Cells were then incubated with 1:200 primary mouse monoclonal anti-collagen type I antibody (Calbiochem, Fremont, CA) or 1:200 primary rabbit polyclonal anti-collagen type VI antibody diluted in 1% BSA. Cells incubated with 1% BSA alone served as a negative control. Following washes cells were incubated with 1:400 dilution secondary anti mouse antibody, Alexa fluor 488 or 1:400 dilution secondary anti rabbit antibody, Alexa fluor 633 (Molecular probes, Eugene, OR). Following washes cells were incubated with 300nM DAPI to stain the nuclei. The coverslips were mounted on Fluorsave reagent (Calbiochem, San Diego, CA) and fluorescent images were taken using confocal microscopy (Carl Zeiss Meditec, Inc., Thornwood, NY).

Western Blot: LC cells were grown to confluency in 60mm dishes. Following various treatments the media was collected and concentrated by Microcon centrifugal filter device (10-kDa cutoff; Amicon; Millipore, Bedford, MA). Cell lysates were obtained by directly lysing the cells in RIPA lysis buffer [1% NP-40, 0.5% sodium deooxycholate, 0.1% sodium dodecyl sulfate in 1X PBS]. Protein content was determined by bicinchonic acid (BCA) protein assay (Pierce Biotechnology, Inc, Rockford, IL). Equal amounts of proteins were supplemented with SDS sample buffer, separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were probed with primary anti collagen type I antibody [1:200 mouse monoclonal (Calbiochem, San Diego, CA) or 1:200 goat polyclonal (Santa Cruz Biotechnology, Inc, Santa Cruz, CA)] or primary anti

collagen type VI antibody [1:200 rabbit polyclonal (Chemicon Temecula, CA)]. Following incubation with anti mouse/ rabbit HRP conjugated secondary antibody (1:10000; GE Health Care, Piscataway, NJ) or anti goat HRP conjugated secondary antibody (1; 5000; Santa Cruz Biotechnology, Inc, Santa Cruz, CA) the blots were developed with Super Signal West Femto Maximum Sensitivity Substrate kit (Pierce Biotechnology, Inc, Rockford, IL). The blots were stripped and reprobed with primary anti beta-tubulin antibody [1:200 rabbit polyclonal (Santa Cruz Biotechnology, Inc, Santa Cruz, CA)] followed by anti rabbit HRP conjugated secondary antibody (1:10000; GE Health Care, Piscataway, NJ) for normalizing the protein loading. Densitometric analysis of the bands was performed using the image-analysis software (Scion image; National institutes of health, Bethesda, MD). The relative band intensities in treated versus control is represented as mean percentage ± SEM of four individual experiments.

Measurement of total collagen synthesis by [^{3}H] proline incorporation assay:

Collagen synthesis was assessed by measuring the uptake of [^{3}H] proline as previously described with slight modifications (Ku et al., 2006). Briefly cells were seeded into 24-well plates. Confluent wells were serum starved over night and subjected to various treatment conditions and included control with no treatment, ET-1 100nM, cells pre-incubated with the ET_A receptor antagonist (BQ788 -1μM) or the ET_B receptor antagonist (BQ610 -1μM) for 30 min and subsequently treated with ET-1 (100nM) in the presence of respective antagonists, and the antagonists BQ610 or BQ788 alone. [^{3}H] Proline (Perkin Elmer Waltham, Massachusetts) was added to each well at a final concentration of 1 μCi/ml, and remained in the medium for the rest of the incubation period along with

various treatments. After 48 hrs of treatment the media was removed from the wells. Proteins in the media were precipitated by adding trichloroacetic acid (TCA) to give a final concentration of 10%, and left on ice for one hour. Precipitated protein was collected by centrifugation at 14000 g for 30 min, washed with 4 ml ice-cold 10% TCA to remove any unincorporated labeled proline and centrifuged again. The supernatant was carefully removed and the pellet suspended in 0.3 ml of 0.3 M NaOH-0.1% SDS and warmed to 37 °C for 1hr. The cell layer was washed twice with PBS and precipitated with 1 ml of ice-cold 10% TCA for 30 min at 4^0C. Following washes with 10 % ice cold TCA, the proteins were solubilised by incubating with 0.3 ml of 0.3 M NaOH-0.1% SDS at 37°C for 1 hr. An equal portion (0.2 mL) of the solubilised proteins obtained form the cell layers or media were added to 3ml scintillation cocktail and utilized to count the radioactivity using beta counter, Packard Tricarb 1600 TR liquid scintillation analyzer (Parkard, UK) while an equal portion of the solubilised proteins obtained form the cell layers or media (0.1 mL) were utilized to determine the total protein content using the bicinchonic (BCA) protein assay (Pierce Biotechnology, Inc, Rockford, IL). The total radiocactivity counted in each sample were normalized to the respective total protein content. Data are expressed as the mean percentage ± SEM of [^{3}H] proline incorporated in cells or proteins in media of 8 individual wells of similar treatment groups.

Results

ET-1 mediated regulation of COL I α1 and COL VI α1 mRNA expression in LC cells.

QPCR analysis of total RNA isolated from LC cell lines treated with 1nM, 10nM and 100nM, ET-1 for 24hrs was performed. ET-1 at all doses increased the COL I α1 mRNA levels (Fig. 1(A) and Fig. 1(B)). The most significant increase was observed at 1nM concentration followed by ET-1 10nM concentration. A similar trend was observed for the expression COL VI α1 mRNA levels where in, ET-1 increased the COL VI α1 mRNA levels at all doses with a most significant increase at 1nM concentration followed by 10nM concentration (Fig. 1(C) and Fig. 1(D)). These results suggested that ET-1 regulated the expression of COL I α1 and COL VI α1 possibly at the level of transcription and increased the steady state levels of COL I α1 and COL VI α1 mRNA.

ET-1 mediated regulation of collagen type I expression in LC cells.

An increase in immunoreactivity for collagen type I protein was observed following ET-1 100nM treatment for 48 hrs in LC cell lines, suggesting that ET-1 mediated an increase in collagen type I deposition (Fig. 2(A)). Cells incubated with secondary antibody alone in the absence of primary antibody showed no immunoreactivity and served as control (Fig. 2(A)). Western blot analysis of LC cell lysates treated with 1nM, 10nM and 100nM, ET-1 for 48hrs, revealed that ET-1 increased the collagen type I expression in a dose-dependent manner with a significant increase at 10nM and 100nM concentrations (Fig. 2(B) and Fig. 2(C)). Western blot analysis of LC cell culture media also revealed a dose dependent increase in collagen type I secretion (Fig. 2(D) and Fig. 2(E)). These results

suggested that ET-1 increased both the deposition and secretion of collagen type I in a dose-dependent manner in LC cells.

ET-1 mediated regulation of collagen type I expression in LC cells.

An increase in immunoreactivity for collagen type VI protein was observed following 100nM ET-1 treatment for 48 hrs in LC cell lines, suggesting that ET-1 also mediated an increase in collagen type VI deposition (Fig. 3(A)). Cells incubated with secondary antibody alone in the absence of primary antibody showed no immunoreactivity and served as control (Fig. 3(A c)). A dose-dependent increase in collagen type VI expression was observed as determined by Western blot analysis of LC cell lysates treated with 1nM, 10nM and 100nM, ET-1 for 48hrs, with a modest but statistically significant increase at 10nM and 100nM concentrations (Fig. 3(B) and Fig. 3(C)). Western blot analysis of LC cell culture media also revealed a dose dependent increase in collagen type VI secretion with a significant increase at 10nM and 100nM concentrations of ET-1 (Fig. 3(D) and Fig. 3(E)).

ET-1 mediated regulation of collagen type I & type VI expression in LC cells and Role of endothelin receptors ET_A and ET_B.

In order to determine the role of ET-1 receptors in ET-1 mediated collagen regulation, the cells were preincubated with either a specific ET_A receptor antagonist, BQ610, or a specific ET_B receptor antagonist, BQ788, and subsequently treated with ET-1 for 48hrs. Western blot analysis of LC cell lysates revealed that 100nM ET-1 significantly increased the expression of collagen type I protein and the increase was blocked partially with the ET_A receptor antagonist BQ610 but a further significant decrease was seen with the ET_B

receptor antagonist BQ788. The antagonists BQ610 and BQ788 alone did not affect collagen type 1 expression significantly (Fig. 4(A) and Fig. 4(B)). Similarly, 100nM ET-1 significantly increased the expression of collagen type VI protein and this increase was also differentially regulated where by, the increase was blocked partially with ET_B receptor antagonist BQ788 but a further significant decrease was seen with the ET_A receptor antagonist, BQ610. The antagonists BQ610 and BQ788 alone did not affect collagen type VI expression significantly (Fig. 4(C) and Fig. 4(D)). These results suggested that both ET_A and ET_B receptors are involved in ET-1 mediated upregulation of both collagen type I and type VI.

ET-1 mediated regulation of total collagen synthesis in LC cells.

Based on the previous data that ET-1 appears to increase both collagen type I and type VI we decided to measure increases in collagen synthesis. The major biosynthetic destination of proline is collagen and therefore the amounts of radioactive [^{3}H] proline incorporated into insoluble and soluble protein fractions provides a reliable index of total collagen synthesis (Mukherjee and Sen 1990; Ku et al., 2006). In order to determine the receptors involved in ET-1 mediated increase in collagen synthesis, the cells were preincubated with either an ET_A receptor antagonist, BQ610 or an ET_B receptor antagonist, BQ788 and subsequently treated with ET-1 for 48hrs in the presence of [^{3}H] proline. ET-1 at 100nM significantly increased the [^{3}H] proline incorporation into cell protein as well as that of the cultured media. ET-1 mediated increase in [^{3}H] proline incorporation was blocked partially with the ET_A receptor antagonist BQ610. A further significant inhibition was observed with the ET_B receptor antagonist, BQ788. The

antagonists BQ610 and BQ788 alone did not have any effect on [^{3}H] proline incorporation (Fig. 5(A) and Fig. 5(C)).

Discussion

The lamina cribrosa is a distinct region of the optic nerve head (ONH) composed of perforated connective tissue plates through which the axons of retinal ganglion cells (RGC) exit the eye (Anderson 1969; Birch et al., 1997). The connective tissue of LC comprises various extra cellular matrix molecules including collagens, elastin, fibronectin and proteoglycans (Rehnberg et al., 1986; Goldbaum et al., 1989; Morrison et al., 1989). The composition of ECM renders resiliency and compliance to LC and therefore its ability to sustain changes in intraocular pressure (IOP) without the loss of structural integrity (Burgoyne et al., 2005; Morrison et al., 2005). A number of studies have indicated LC as the primary site of injury in glaucoma pathology (Hernandez 2000; Quigley 2005). Extensive extracellular matrix remodeling at the level of LC in glaucomatous subjects and animal models of glaucoma has been suggested (Hernandez 2000). Moreover, the ability of LC cells to respond to profibrotic triggers mediating enhanced ECM synthesis implicates LC as an important pro-fibrotic tissue that could lead to loss of structural integrity and collapse of LC in gluacoma (Hernandez 2000 and Kirwan et al., 2005). An increase in collagen deposition including collagen type I, IV, VI and elastin degeneration has been observed in glaucomatous LC (Hernandez et al., 1987; Miller and Quigley 1988; Morrison et al., 1989; Sawaguchi et al., 1999; Hernandez 2000). The increase in collagen deposition could alter the biomechanical properties of

LC, rendering LC less compliant to stress such as increase in intra ocular pressure and contribute to its collapse, resulting in excavation of the optic nerve head, blockade of axonal transport and associated RGC loss observed in glaucoma (Tengroth and Ammitzboll 1984; Rehnberg et al., 1987). Excessive ECM deposition could also render a non permissive environment for the surviving RGCs to regenerate their axons (Hernandez 2000). The increase in extracellular matrix synthesis has been attributed to the mechanical effects of elevated IOP and or due to the activity of various cytokines including transforming growth factor- β (TGF-β) (Kirwan et al., 2005; Morrison et al., 2005;). Activated optic nerve head astrocytes and LC cells in response to raised IOP or cytokines have been suggested to contribute to enhanced synthesis of ECM in the optic nerve head (Hernandez 2000; Kirwan et al; 2005). Endothelin-1, a potent vasoactive peptide has been proposed to play an important role in the pathophysiology of glaucoma (Yorio et al., 2002). Endothelin-1 has been increasingly recognized for its role as a pro-fibrotic factor resulting in enhanced ECM synthesis and has been widely implicated in the pathology of various connective tissue disorders (Eng and Friedman 2000; Eddy 2000; Wakatsuki et al., 2004; Clozel and Salloukh 2005; Tsukada et al., 2006). The role of ET-1 in ECM regulation in glaucoma pathogenesis is not known. Our lab recently demonstrated that ET-1 is capable of increasing fibronectin deposition by optic nerve head astrocytes (He et al., 2007). In the present study we have demonstrated that ET-1 is also able to increasing extracellular matrix collagen synthesis, deposition, and secretion in human primary LC cells.

Increase in collagen type I has been associated with marked reduction of compliance of several tissues resulting in fibrosis and loss of normal structure and function of the tissue (Varga et al., 2005). Profibrotic agents like transforming growth factor-β (TGF-β), endothelin-1(ET-1) or mechanical stress has been implicated in excess collagen type I synthesis and deposition in various tissues (Eddy 2000; Wakatsuki et al., 2004; Tsukada et al., 2006). Associated with fibrotic tissues is the increase in collagen type VI and is considered as an early marker for tissue fibrosis (Specks et.al, 1995; Hatamochi et.al., 1996; Gerling et.al, 1997; Groma 1998; Zeichen et.al., 1999). The exact role of collagen VI is not clear however, collagen VI is proposed to interact with cell receptors of the integrin type and serve to associate ECM to cells (Van der Rest and Garrone 1991). Increase in collagen VI has also been proposed to increase the rate of collagen type I fibril formation (Harumiya et al., 2002; Minamitani et al., 2004;), result in loss of elastic properties of skin in cutis laxa (Hatamochi et al., 1996), prevent apoptosis of serum starved fibroblasts (Ruhl et al., 1999) and contribute to migration of glioblastoma cells (Han and Daniel 1995; Han et al., 1995). Indeed, cyclic albumin drug carriers that selectively interact with collagen type VI, have been developed for targeting antifibrotic drugs to fibrotic tissues (Beljaars et al., 2000; Schuppan and Porov 2002). Increase in collagen type I and collagen type VI is a characteristic feature of ECM remodeling in LC of glaucomatous subjects and animal models of glaucoma (Hernandez 2000; Morrison et al., 2005). Elevated IOP models of glaucoma have demonstrated the deposition of collagen I and VI at the optic nerve head to be an early event and are correlated linearly to degree of IOP- induced injury (Johnson et al., 2000; Guo et al., 2005; Morrison et al.,

2005; Johnson et al., 2007). We were therefore interested in studying the role ET-1 played in regulation of collagens type I and VI in LC cells. The dose and time point selected for ET-1 in the present study are consistent with previous studies (Shi-wen et al., 2001; Hafizi et al., 2004; Horstmeyer et al., 2005; He et al., 2007). Collagen type I molecule consists of three polypeptide chains, two identical α1 and a distinct α2 forming a triple helix. The genes of identical α1 (1) chain and the distinct α2 (1) chain of collagen type I are located in 17 and 7 chromosome respectively and are regulated coordinatively in the ratio of 2:1 (Van der Rest and Garrone 1991). Collagen VI is a heterotrimeric molecule made of three polypeptide chains, αI (VI) α2 (VI) and α3 (VI) with the genes for α1 (VI), α2 (VI) chains located in chromosome 22 and α3 (VI) chain located in chromosome 2 are also regulated in coordinative manner (Van der Rest and Garrone 1991). ET-1 treatment in LC cells resulted in an increase in mRNA levels of both collagen type I α I and collagen type VI αI chains at all doses of ET-1, suggesting a transcriptional regulation of collagen genes by ET-1. Western blot analysis following ET-1 treatment also demonstrated an increase in deposition and secretion of both collagen type I and type VI proteins in LC cells. The increase in protein expression of collagen type I and VI was dose-dependent with a significant increase at 10 and 100nM concentrations of ET-1 in contrary to the increase in mRNA levels which were significantly increased at 1nM and 10nM concentrations of ET-1. Besides increase in transcription the overall increase in collagen synthesis has been attributed to increase in half life and mRNA stability, or decreased collagen degradation (Stefanovic et al., 1997; Stefanovic et al., 1999; Friedman et al. 2000; Bedossa and Paradis 2003). Endothelin-1

not only enhances collagen synthesis in various tissues and cell types but also limits degradation by increasing the activity of tissue inhibitors of matrix metalloproteases (TIMPS), key enzymes that inhibit matrixmetallo proteases (MMP's) responsible for collagen degradation (Thirunavukkarasu et al., 2004; Koyama et al., 2007;) . Our lab has recently shown that ET-1 at 100nM significantly increases the levels of TIMP1 and TIMP2 in optic nerve head astrocytes (He et al., 2007). ET-1 mediated increases in collagen levels in LC cells could therefore involve both an increase in expression and decrease in collagen degradation. ET-1 mediated increase in collagen type I is well characterized in various tissues, however to the best of our knowledge, current study presents for the first time that ET-1 can directly regulate collagen type VI expression.

Endothelin-1 (ET-1) mediates its effects through seven transmembrane G-protein coupled receptors, endothelin receptor A (ET_A) and endothelin B (ET_B) that are coupled to different G proteins and down stream targets (Yanagisawa 1994; Takagi et al., 1995). ET-1 mediated activation of the Gq coupled ET_A receptor, results in the classic phospholipase C activation, IP3 generation, mobilization of intracellular calcium stores while activation of ET_B receptor results in nitric oxide (NO) release via a cGMP pathway. (D'Orleans-Juste et al., 2002). In cardiovascular and pulmonary tissues ET-1 mediated collagen synthesis and ECM deposition have been primarily attributed to stimulation of ET_A receptors (Hafizi et al., 2004; Rodriguez-Vita et al., 2005). In dermal and hepatic tissues however, ET_B receptors, contribute to ET-1 mediated collagen synthesis and ECM deposition (Gandhi et al., 2000; Shi-wen et al., 2001). ET_A receptor serve as regulators of collagen homeostasis by inducing both its synthesis and degradation while it has been

shown that ET_B receptor mediates only collagen synthesis (Guarda et al., 1993; Shi-wen et al., 2001 Tostes et al., 2002). Chronic exposure of ET-1 in cultured fibroblasts also resulted in a switch of receptor subtype from ET_A to ET_B, resulting in an ET_B mediated increase in collagen synthesis (Shi-wen et al., 2001; Horstmeyer et al., 2005;). An upregulation of ET_B receptors results in autoinduction of ET-1 synthesis and further enhances ET-1 effects through ET_B receptors (Iwasaki et al., 1995). Prevention of ET_B upregulation indeed inhibits hepatic stellate cell activation and collagen synthesis (Xuedong et al., 2003). An upregulation of ET_B receptors has also been observed in human glaucomatous optic nerve head as well as elevated IOP models of glaucoma (Prasanna et al., 2005 and Wang et al., 2006;). In our previous study, we have shown that ET-1 treatment in LC cells resulted in an upregulation of ET_B receptors that was significant at 100 nM concentrations of ET-1 (Rao et al., 2007). The shift in receptor subtype from ET_A to ET_B could in part explain the increase in collagen deposition that is observed at 100nM concentrations of ET-1 in the present study. Both BQ610 an ET_A receptor antagonist and BQ788 an ET_B receptor antagonist were able to each partially inhibit collagen type I and collagen type VI deposition suggesting the involvement of both receptor subtypes in ET-1 mediated collagen deposition in LC cells. Similar results have been reported previously were dual ET_A and ET_B receptor anatagonists effectively inhibit ET-1 mediated collagen synthesis (Shi-wen et al., 2001; Morgera et al., 2003; Clozel and Salloukh 2005).

The primary structure of collagen chains is made for the most part of repeating Gly-Xaa-Yaa triplets with a high content of Pro in the Xaa position and OH-Pro in the Yaa

position (Van der Rest and Garrone 1991). ET-1 mediated enhanced [^{3}H] proline incorporation has been used for determining the effects of ET-1 on total collagen synthesis in several tissues and cell types (Guo et al., 2004; Hafizi et al., 2004). ET-1 also mediated enhanced [^{3}H] proline incorporation in cell protein and cultured media of LC cells. ET-1 mediated [^{3}H] proline incorporation was blocked by both the ET_B receptor antagonist, BQ788, and the ET_A receptor antagonist, BQ610. These results suggest that ET-1 increases total collagen synthesis in LC cells and is mediated by both ET_B and ET_A receptors.

In conclusion, we have demonstrated that ET-1 contributes to enhanced collagen synthesis and secretion in LC cells. Elevated ET-1 concentrations have been observed in POAG subjects and in elevated IOP models of glaucoma (Sugiyama et al., 1995; Noske et al., 1997; Kallberg et al., 2002; Prasanna et al., 2005). It is well established that exogenous administration ET-1, in various animal models, results in loss of retinal ganglion cells by apoptosis, blocks axonal transport, activates optic nerve head astrocytes and results in optic neuropathy similar to that observed in glaucoma (Stokely et al., 2002; Chauhan et al., 2004; Lau et al., 2006). ET-1 mediated increase in collagen synthesis by LC cells could therefore contribute to ECM remodeling observed at the level of LC in glaucomatous subjects and further contribute to the pathology of primary open angle glaucoma.

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Fig.1. Effects of ET-1 on COL I α1 and COL VI α1 mRNA determined QPCR analysis.

QPCR products separated on ethidium bromide stained 0.75 % agarose gel of COL I α1, COL VI α1 and internal control β-actin, following the treatment with ET-1 (1, 10 & 100nM) for 24 hrs (A, C). QPCR data is presented as the mean percentage ± SEM of mRNA levels of COL I α1, COL VI α1 expression compared with the respective control (B, D). ET-1 increased the mRNA expression of COL I α1, COL VI α1 message at all doses of ET-1. A significant increase was observed at 10nM and 100nM concentrations of ET-1. *Statistical significance of ET-1 treatment versus control as determined by one-way ANOVA and student-Newman-Keuls multiple comparison test ($p<0.05$). Experiments were repeated 4 times, two times each in LC cell lines from two different donors.

Table 1. PCR primer sequences and expected product sizes

Gene	Primer Sequence	Product size (bp)
COLI alpha1	(S) GATGGACTCAACGGTCTCC (A) CCTTGGGGTTCTTGCTGATG	458
COL VI alpha1	(S) CTGGGCGTCAAAGTCTTCTC (A) ATTCGAAGGAGCAGCACACT	211
Beta-Actin	(S) TGTGATGGTGGGAATGGGTCAG (A) TTTGATGTCACTCACGATTTCC	514

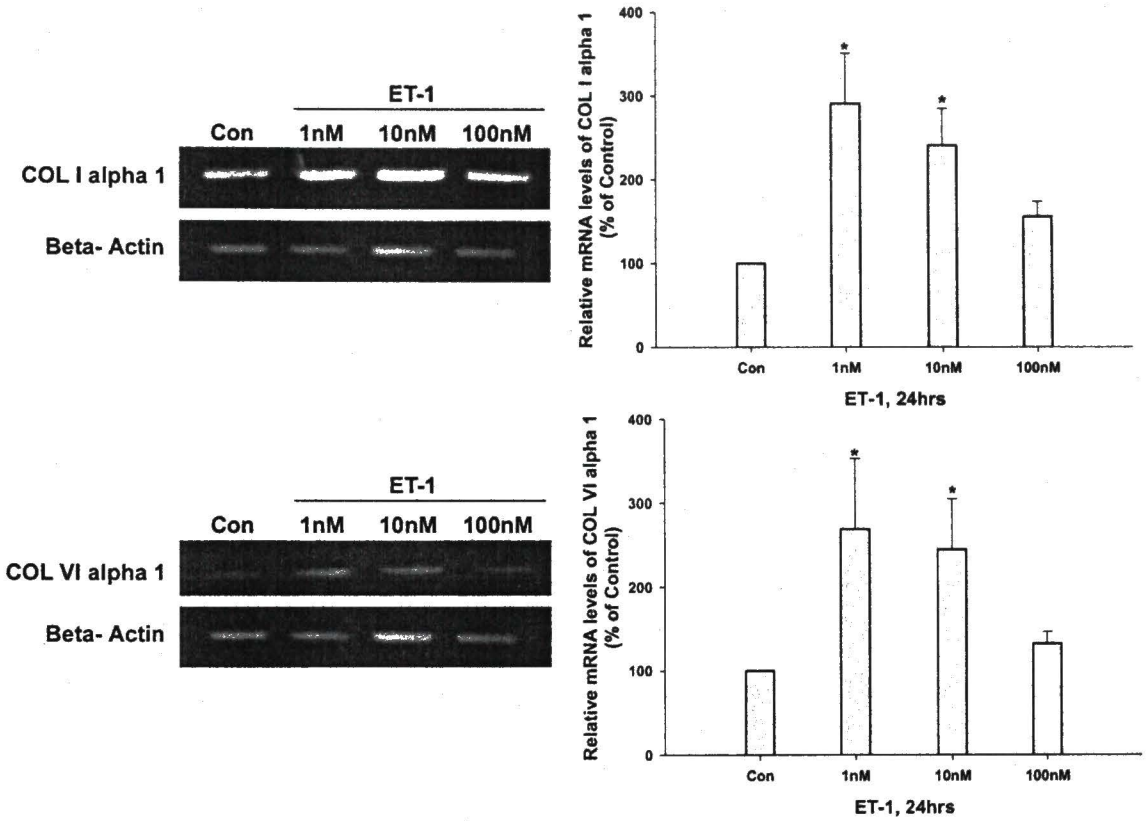

ET-1
Con
1nM
10nM
100nM
COL I alpha 1
Beta- Actin
Relative mRNA levels of COL I alpha 1 (% of Control)
400
300
200
100
0
Con
1nM
10nM
100nM
ET-1, 24hrs
ET-1
Con
1nM
10nM
100nM
COL VI alpha 1
Beta- Actin
Relative mRNA levels of COL VI alpha 1 (% of Control)
400
300
200
100
0
Con
1nM
10nM
100nM
ET-1, 24hrs

Fig.2. Effect of ET-1 on collagen type I.

ET-1 increased the deposition of collagen type I in LC cells as observed by Immunostaining. Immunofluroscent staining for collagen type I in LC cells not treated (control) or treated with ET-1 (100nM) for 48hrs (A). Negative control, treated with secondary antibody alone showed no staining for collagen type I (A). Nuclei are DAPI stained (blue). Scale bar in L=50μm. Representative Western blot of collagen type I and internal control beta-tubulin protein expression in LC cell lysates, following the treatment with ET-1, (1, 10 & 100nM) for 48 hrs (B). The quantification of band intensities of collagen type I is represented as mean percentage ± SEM compared with the corresponding control band (C). A dose-dependent increase in collagen type I following treatment with ET-1 was observed and was significant at ET-1 10nM and 100nM concentrations. Representative western blot of collagen type I in LC cell culture media, following treatment with ET-1, (1, 10 & 100nM) for 48 hrs (D). Comassie stained gel suggested a uniform loading. The quantification of band intensities collagen type I is represented as mean percentage ± SEM compared with the corresponding control band (E). A dose-dependent increase in collagen type I in culture media following treatment with ET-1 was observed. *Statistical significance of ET-1 treatment versus control as determined by one-way ANOVA and student-Newman-Keuls multiple comparison test ($p<0.05$). Experiments were repeated 4 times, two times each in LC cell lines from two different donors.

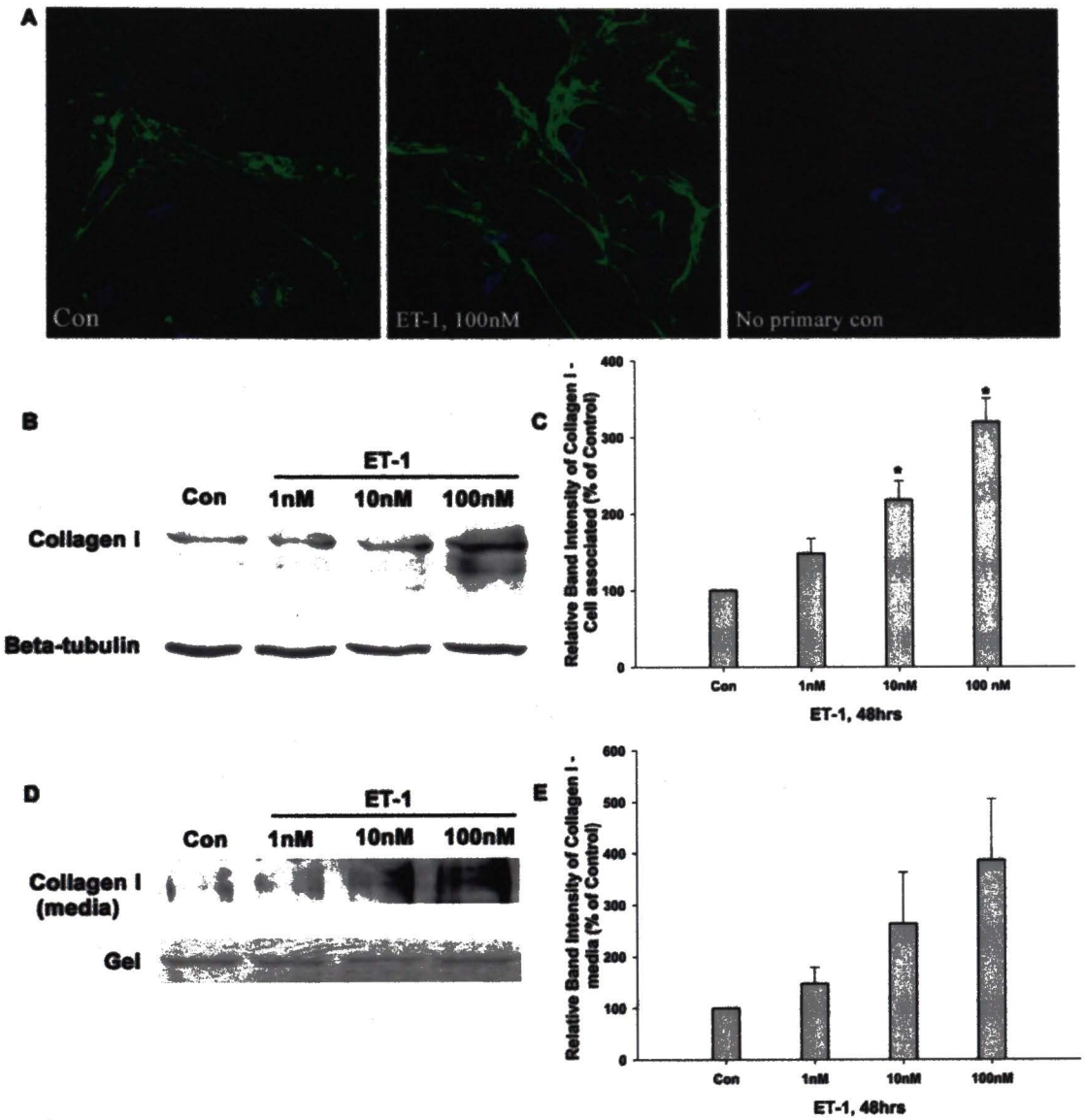

A
Con
ET-1, 100nM
No primary con
B
ET-1
Con
1nM
10nM
100nM
Collagen I
Beta-tubulin
C
Relative Band Intensity of Collagen I - Cell associated (% of Control)
400
300
200
100
0
*
*
Con
1nM
10nM
100 nM
ET-1, 48hrs
D
ET-1
Con
1nM
10nM
100nM
Collagen I (media)
Gel
E
Relative Band Intensity of Collagen I - media (% of Control)
600
500
400
300
200
100
0
Con
1nM
10nM
100nM
ET-1, 48hrs

Fig.3. Effect of ET-1 on collagen type VI.

ET-1 increased the deposition of collagen type VI in LC cells as observed by Immunostaining. Immunofluroscent staining for collagen type VI in LC cells not treated (control) or treated with ET-1 (100nM) for 48hrs (A). Negative control, treated with secondary antibody alone showed no staining for collagen type VI (A). Nuclei are DAPI stained (blue). Scale bar in L=50μm. Representative western blot of collagen type VI and internal control beta-tubulin protein expression in LC cell lysates, following the treatment with ET-1 (1, 10 & 100nM) for 48 hrs (B). The quantification of band intensities collagen type VI is represented as mean percentage ± SEM compared with the corresponding control band (C). A dose-dependent increase in collagen type VI following treatment with ET-1 was observed and was significant at ET-1 10nM and 100nM concentrations. Representative western blot of collagen type VI in LC cell culture media, following the treatment with ET-1 (1, 10 & 100nM) for 48 hrs (D). Comassie stained gel suggested a uniform loading. The quantification of band intensities of collagen type VI is represented as mean percentage ± SEM compared with the corresponding control band (E). A dose-dependent increase in collagen type VI in culture media following treatment with ET-1 was observed and the increase was significant at 10nM and 100nM concentration of ET-1. *Statistical significance of ET-1 treatment versus control as determined by one-way ANOVA and student-Newman-Keuls multiple comparison test ($p<0.05$). Experiments were repeated 4 times, two times each in LC cell lines from two different donors.

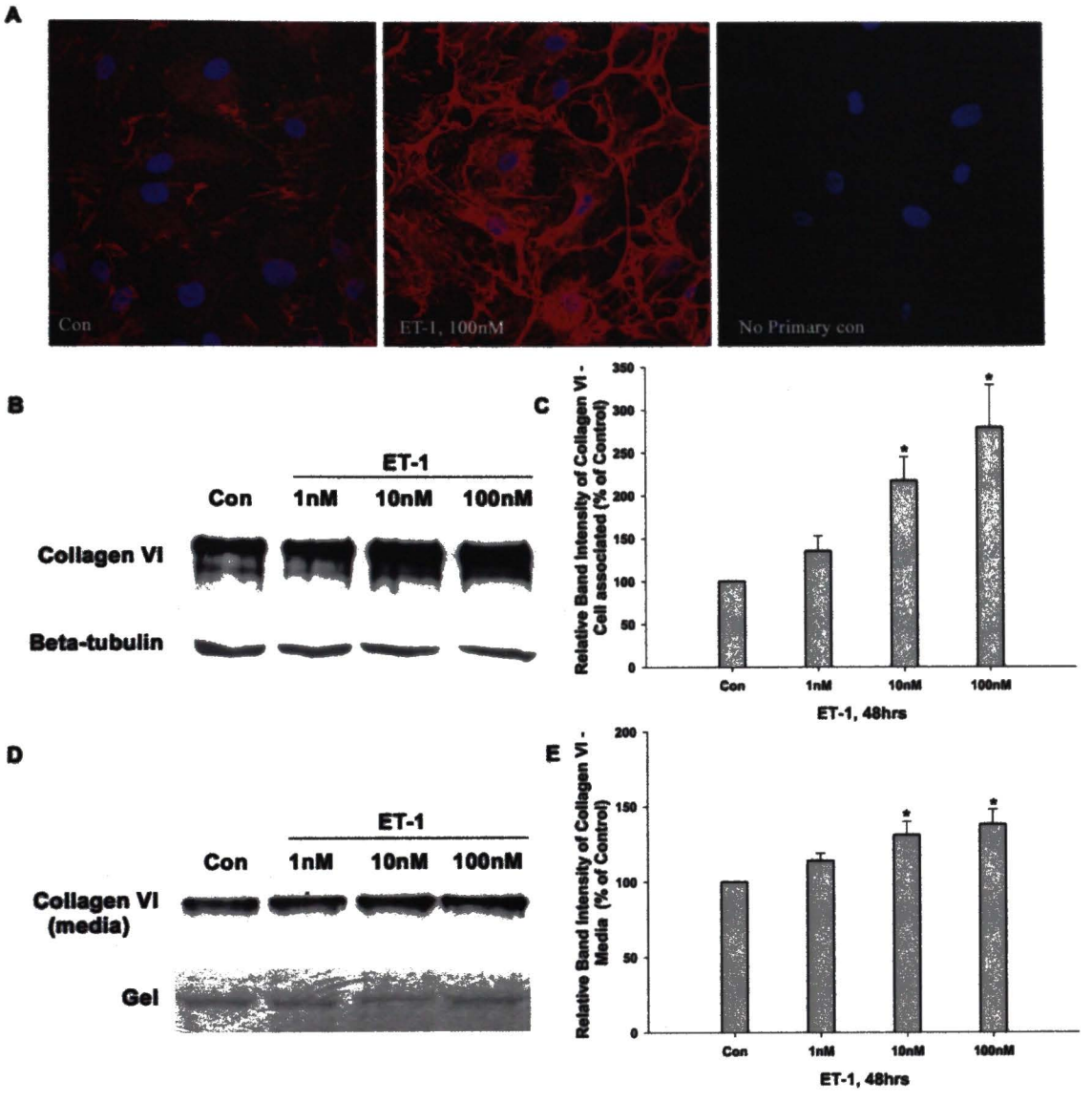

A
Con
ET-1, 100nM
No Primary con
B
ET-1
Con
1nM
10nM
100nM
Collagen VI
Beta-tubulin
C
Relative Band Intensity of Collagen VI - Cell associated (% of Control)
350
300
250
200
150
100
50
0
Con
1nM
10nM
100nM
ET-1, 48hrs
D
ET-1
Con
1nM
10nM
100nM
Collagen VI (media)
Gel
E
Relative Band Intensity of Collagen VI - Media (% of Control)
200
150
100
50
0
Con
1nM
10nM
100nM
ET-1, 48hrs

Fig.4. Effects of ET-1 on collagen type I and type VI is mediated by ET_A and ET_B receptors.

Representative western blot of collagen type I and internal control beta-tubulin protein expression in LC cell lysates, with or without the treatment with ET-1, 100nM, ET-1 100nM treated in the presence of ET_A antagonist BQ610 or ET_B antagonist BQ788 or BQ610/ BQ788 alone for 48 hrs (A). The quantification of band intensities of collagen type I is represented as mean percentage ± SEM compared with the corresponding control band (B). An increase in collagen type I following treatment with ET-1 100nM was partially and significantly blocked with either ET_A antagonist BQ610 and also with the ET_B antagonist BQ788. BQ610 and BQ788 alone had no significant effects on collagen type I regulation. Representative western blot of collagen type VI and internal control beta-tubulin protein expression in LC cell lysates, with or without the treatment with ET-1, 100nM, ET-1 100nM treated in the presence of ET_A antagonist BQ610 or ET_B antagonist BQ788 or BQ610/ BQ788 alone for 48 hrs (C). The quantification of band intensities collagen type VI represented as mean percentage ± SEM compared with the corresponding control band (D). An increase in collagen type VI following treatment with ET-1 100nM was partially and significantly blocked with the ET_A antagonist, BQ610 and also blocked with ET_B antagonist, BQ788. BQ610 and BQ788 alone had no significant effects on collagen type VI regulation. * Statistical significance of ET-1 versus control; **statistical significance of ET-1 +BQ610 versus ET-1 alone; **statistical significance of ET-1 +BQ788 versus ET-1 alone, as determined by one-way ANOVA

and student-Newman-Keuls multiple–comparison test ($p<0.05$). Experiments were repeated four times, two times each on LC cell lines obtained from two different donors.

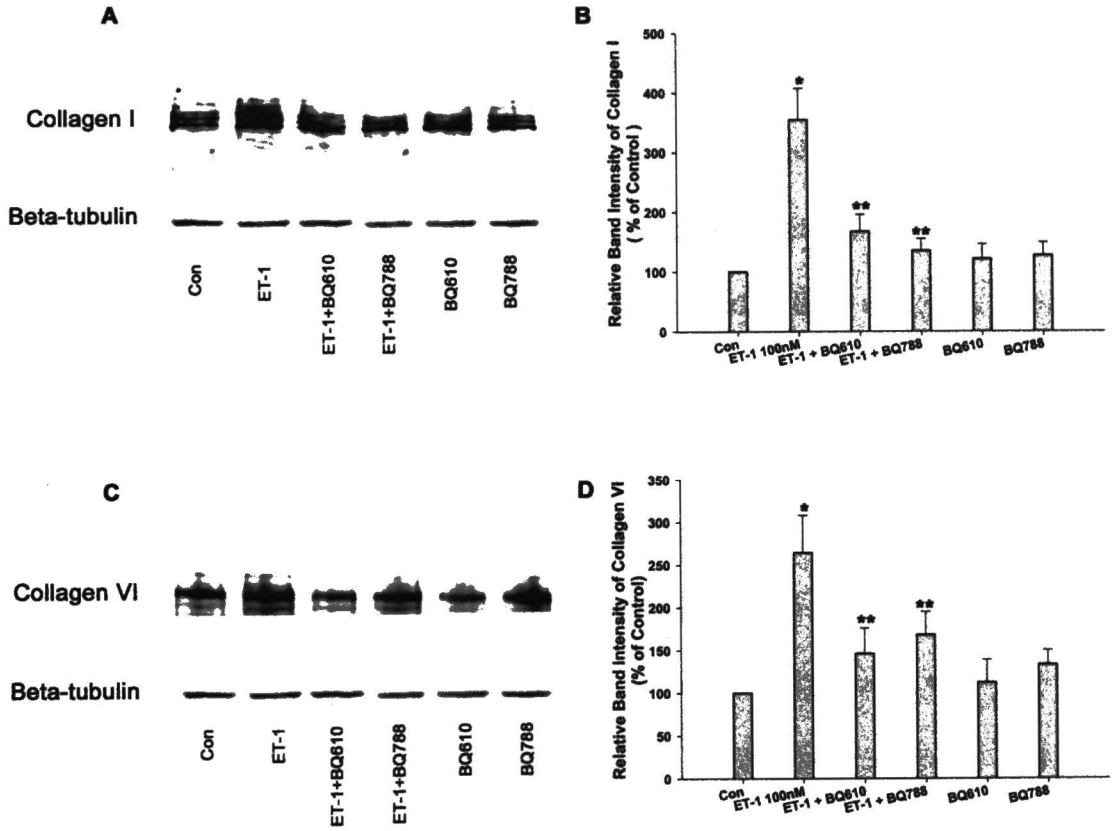
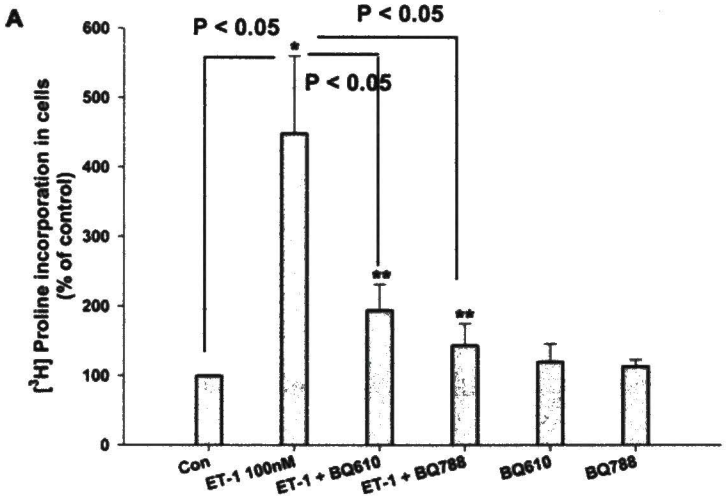
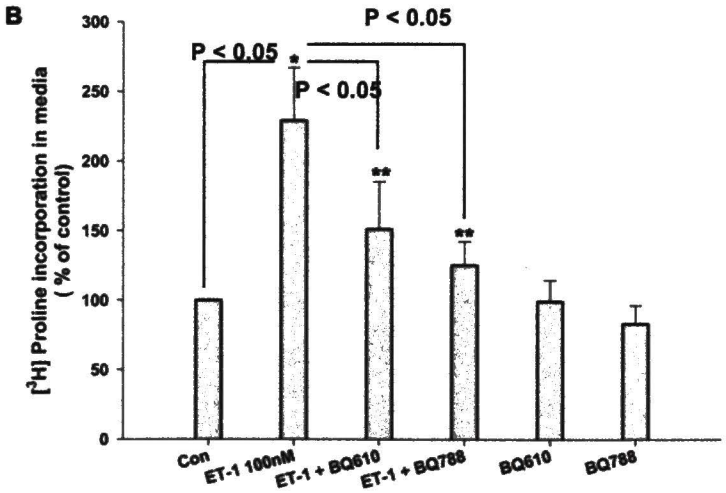


Fig.5. Effect of ET-1 on total collagen synthesis.

Total collagen synthesis was assessed by determining [^{3}H] proline uptake into cellular proteins and proteins secreted into the media. [^{3}H] proline uptake into cell protein (A) and proteins secreted into the media (B) following various treatment conditions for 48 hrs including, control with no treatment, ET-1 100nM, cells pre-incubated with ET_A receptor antagonist (BQ788 -1μM) or ET_B receptor antagonist (BQ610 -1μM) for 30 min and subsequently treated with ET-1 (100nM) in the presence of respective antagonists, and the antagonists BQ610 or BQ788 alone. ET-1 significantly increased [^{3}H] proline uptake into cell protein as well as proteins secreted into the media. ET-1 mediated increase in [^{3}H] proline uptake was partially and significantly blocked with the ET_A antagonist BQ610 and also significantly blocked with the ET_B antagonist BQ788. BQ610 and BQ788 alone had no significant effects on [^{3}H] proline uptake. Data are expressed as the mean percentage ± SEM of [^{3}H] proline incorporated cells or proteins in media. * Statistical significance of ET-1 versus control; **statistical significance of ET-1 +BQ610 versus ET-1 alone; **statistical significance of ET-1 +BQ788 versus ET-1 alone, as determined by one-way ANOVA and student-Newman-Keuls multiple–comparison test ($p<0.05$). Experiments were repeated eight times, four times each on LC cell lines obtained from two different donors.


A
P < 0.05
P < 0.05
P < 0.05
[3H] Proline incorporation in cells (% of control)
600
500
400
300
200
100
0
*
**
**
Con
ET-1 100nM
ET-1 + BQ610
ET-1 + BQ788
BQ610
BQ788


B
P < 0.05
P < 0.05
P < 0.05
[3H] Proline incorporation in media (% of control)
300
250
200
150
100
50
0
*
**
**
Con
ET-1 100nM
ET-1 + BQ610
ET-1 + BQ788
BQ610
BQ788

CHAPTER IV

Endothelin-1 Mediated Regulation of Extracellular Matrix Collagens in rat optic nerve head

Vidhya R. Rao, Raghu R. Krishnamoorthy and Thomas Yorio

(To be submitted)

Abstract

Primary Open Angle Glaucoma (POAG) is a progressive optic neuropathy characterized by loss of retinal ganglion cells, optic nerve degeneration and characteristic excavation of the optic disc. Extensive ECM remodeling with increase in collagen type I, IV and VI, has been associated with structural changes in the optic nerve head and retinal ganglion cell loss. Endothelin-1(ET-1), a potent vaso-active peptide, influences extracellular matrix regulation resulting in an increase in collagen deposition in various cell types and tissues and ET-1 has been proposed to play a key role in glaucoma pathology. The role of ET-1 in the regulation of extracellular matrix collagens at the level of optic nerve head however is not known. Our hypothesis is that ET-1 increases collagen synthesis resulting in ECM remodeling at the optic nerve head and contributes to the development of optic neuropathy. In this study we have examined the role of ET-1 in extracellular matrix collagen regulation in the rat optic nerve heads. Immunohistochemical analysis of optic nerve head sections revealed an increase in collagen type I and type VI deposition following intravitreal injection of ET-1. ET-1 mediated collagen VI deposition was less in ET_B deficient transgenic rats indicating the involvement of ET_B receptors in the regulation of collagen VI synthesis. These results suggest that ET-1 regulates extracellular matrix collagen synthesis in rat optic nerve head and contributes to ECM remodeling

Keywords. Endothelin, Optic nerve head , Extacellular matrix, Collagens, Glaucoma

Introduction

Primary open angle glaucoma (POAG) is a leading cause of blindness world wide (Quigley 2005). Loss of retinal ganglion cells is associated with extensive extracellular matrix remodeling at the level of lamina cribrosa and is considered a characteristic pathologic feature of the disease (Quigley, 2005; Hernandez, 2000). An increase in various ECM molecules including collagen types I, IV, VI and tenascin, a stress reactive protein, has been observed in glaucomatous optic nerve head (Hernandez et al., 1987; Morrison et al., 1989; Miller and Quigley 1988; Hernandez 2000 Sawaguchi et al., 1999). Extensive ECM remodeling is proposed to compromise the structural integrity of LC resulting in excavation of the optic disc and associated axonal transport block, optic nerve degeneration and ganglion cell loss (Quigley et al., 1983; Sakugawa and Chihara 1985; Martin et al., 2003). Elevated intra ocular pressure is an important risk factor for the disease and reducing IOP is the only therapy used to delay the progression of the disease (Marquis and Whitson 2005). Elevated animal models of glaucoma including primates, rodents and various other species express characteristic extracellular matrix remodeling with increase in collagen deposition and loss of retinal ganglion cells (Hernandez et al., 1987; Quigley 1988; Morrison et al., 1989; Miller and Sawaguchi et. al, 1999; Hernandez et al., 2000). The increase in collagen deposition is linearly correlated with the loss of retinal ganglion cells in various animal models of glaucoma with elevated IOP (Johnson et al., 2000; Guo et al., 2005; Morrison et al., 2005; Johnson et al., 2007). The mechanisms contributing to ECM remodeling have not been elucidated. Excessive ECM deposition, including collagen types I and VI with the loss of structural integrity, is

consistent with the pathology of fibrotic diseases (Varga et al., 2005; Kirwan et al., 2005). Important pro-fibrotic factors including mechanical stress such as elevated IOP, endothelin-1 (ET-1) and transforming growth factor –beta (TGF-β) have been implicated in POAG (Yorio et al., 2002; Kirwan et al., 2005). ET-1 appears to play a key role in pathophysiology of POAG (Yorio et al., 2002). Elevated aqueous humor levels and plasma levels of ET-1 have been reported in glaucomatous subjects (Sugiyama et al., 1995; Noske et al., 1997). Elevated levels of ET-1 are also observed in elevated IOP models of glaucoma (Prasanna et al., 2005; Kallberg et al., 2002). Injections of ET-1 (2nmole) into rat eyes have shown to cause loss of retinal ganglion cells, blockade of axonal transport and activation of optic nerve head astrocytes (Stokely et al., 2002; Lau et al., 2006). ET-1 as a profibrotic factor, is also involved in various types of tissue fibrosis resulting in increases in collagen type I and VI synthesis and accumulation (Tsukada et al., 2005; Phan SH 2002; Eddy AA 2000; Wakatsuki et al., 2004). We have previously reported that ET-1 extends its pro-fibrotic effects on lamina cribrosa cells *in vitro* by increasing collagen type I and type VI synthesis and deposition. In the present study we wished to determine if ET-1 also mediated its pro-fibrotic effects in rat eyes.

Materials and Methods

ET_B deficient transgenic Wistar- Kyoto rats were a kind gift form Dr. Yanagisawa (UT Southwestern Medical Center, Dallas). ET_B-deficient homozygous (*sl/sl / ET_B KO*) and wild-type (+/+) Wistar- Kyoto rats were used in the study. Genotyping using, polymerase chain reaction was performed on DNA isolated from tail biopsy specimens, as described (Gariepy et al., 1998). All animals were allowed free access to standard laboratory rat

chow and tap water and were housed under controlled humidity, temperature and a 12-h light/dark cycle. All studies were conducted in accordance with NIH guidelines, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the University of North Texas Health Science Center Committee on Animal Welfare.

Intravitreal injections of ET-1:

Adult Wistar- Kyoto rats were divided into following treatment groups: Group -1; wild type control rats (+/+; n=2), Group 2; wild type ET-1 injected (+/ +, ET-1; n=2) and Group 3; ET-1 injected, ET_B deficient (sl/sl, ET-1; n=2). Rats were anaesthetized with the standard rat cocktail anesthesia. Once the animal was unconscious and corneal blink response lost, the rat was carefully positioned under the dissecting scope and eyelids spread apart just to see the limbus. Vehicle, with or without ET-1 (final dose of 2 nmol) was injected into the vitreous of the left eye in increments of 1ul every 30 seconds with a $30^1/_2$-gauge needle attached to a syringe (microliter 710, 22s gauge; Hamilton Co., Reno, NV) by polyethylene tubing (PE-20, Clay Adams Brand; BD Biosciences, Sparks, MD). The dose of ET-1 chosen was based on dose-response studies in our laboratory concerning the effects on axonal transport of ET-1 (Stokely et al., 2002).

Immunohistochemistry:

Following 48 hrs of treatment, the rats were sacrificed and eyes enucleated. Small incision with a #11 scappel blade was made at the limbus of the eyes and fixed in 4% paraformaldehyde for 4 hrs. Following fixation the eyes were dehydrated with gradient dilutions of ethanol and paraffin embedded. 5μm thick sections of paraffin embedded

eyes were obtained with a microtome and placed one glass slides (Platinum microslides, Germany). Sections were de-paraffinised in xylene, re-hydrated with a series of dilutions of ethanol and PBS. Sections were then blocked with 5% BSA for an hour. The sections were washed in 1X PBS. Following washes the sections were incubated overnight at 4^0C with 1:100 primary goat polyclonal anti collagen type I antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and 1:100 dilution of anti collagen type VI antibody (Chemicon Temecula,CA) diluted in 1% BSA. Sections incubated with 1% BSA alone served as negative control. Following washes, sections were incubated with 1:400 dilution of secondary anti goat antibody, Alexa fluor 633 or 1:400 dilution of secondary anti rabbit antibody, Alexa fluor 488 (Molecular probes, Eugene, OR). Following washes the sections were mounted on Fluorsave reagent (Calbiochem, San Diego, CA) and fluorescent images were taken using confocal microscopy (Carl Zeiss Meditec, Inc., Thornwood, NY).

Statistical Analysis:

Fluorescent intensities of collagen types I and VI from eight sections of each treatment group were quantified using the Image J software. Data obtained by Image J were analyzed with one-way analysis of variance (ANOVA) for multiple comparisons and represented as Mean ± SEM.

Results

ET-1 mediated regulation of collagen type I and type VI expression in rat optic nerve head.

Immunohistochemical analysis of rat optic nerve head sections revealed the expression of collagen types I and VI in the pre-laminar, laminar and as well as retro-laminar regions of the optic nerve head. The expression of collagen types I and VI were similar to the total collagen distribution as observed by the Gomori's stain in the optic nerve head (Fig. 1 (A)). An increase in collagen types I and VI deposition following ET-1 injection was observed, compared to the vehicle treated control eyes (Fig. 1 (B) & (C)). The Increase in collagens type I and VI expression following ET-1 injection was observed in the pre-laminar, laminar and as well as retro-laminar regions of the optic nerve head sections (Fig. 1 (B) & (C)).

Role of endothelin receptors ET_A and ET_B in ET-1 mediated regulation of collagen type I expression in optic nerve head.

In order to determine the involvement of ET_A and ET_B receptors in ET-1 mediated collagen regulation, ET-1 was administered intravitreally in both the wild type (+/+) and as well as ET_B deficient transgenic rats (sl/s / ET_B KO). Immunohistochemical analysis of collagen type I in optic nerve head sections following ET-1 injection revealed an increase in collagen type I deposition compared to vehicle treated controls (Fig. 2 (A) & (B)). No significant decrease in collagen type I deposition was observed in ET_B deficient (sl/sl) transgenic rats in comparison to the wildtype injected rats suggesting that ET_A receptors

appeared to be primarily involved in ET-1 mediated enhanced deposition of collagen type I.

Role of endothelin receptors ET_A and ET_B in ET-1 mediated regulation of collagen type VI expression in optic nerve head.

Immunohistochemical analysis of collagen type VI in optic nerve head sections following ET-1 injection revealed an increase in collagen type VI deposition compared to vehicle treated controls (Fig. 3 (A) & (B)). Collagen type VI deposition was markedly decreased in ET_B deficient (sl/sl / ET_B KO) transgenic rats in comparison to the wildtype injected rats suggesting that ET_B receptors were primarily involved in ET-1 mediated enhanced deposition of collagen type VI.

Discussion

Endothelin-1 (ET-1) is a 21-amino acid vasoactive peptide which plays a regulatory role in vascular homeostasis. Besides its role in the maintenance of vascular homeostasis, ET-1 also influences a number of cellular events including proliferation, differentiation and apoptosis (Rubanyi and Polokoff; 1994; Kedzierski and Yanagisawa 2001). ET-1 plays an important role as a pro- fibrotic factor in initiating and maintaining fibrosis of various tissues resulting in increases in collagen synthesis and ECM accumulation in several cell types including cardiac myocytes, fibroblasts and smooth muscle cells (Eng and Friedman 2000; Eddy 2000; Wakatsuki et al., 2004; Clozel and Salloukh 2005; Tsukada et al., 2006). ET-1 has therefore been implicated in fibrotic diseases affecting various systems including respiratory, cardiac, renal and liver. ET-1 appears to play a key role in

glaucoma pathology (Yorio et al., 2002). POAG subjects have significantly higher levels of ET-1 in plasma and aqueous humor compared to their age matched controls (Sugiyama et al., 1995; Noske et al., 1997). Elevated ET-1 levels in aqueous humor have also been observed in elevated IOP animal models of glaucoma (Prasanna et al., 2005; Kallberg et al., 2002). Various cell types in the eye could serve as a source for ET-1 including the ciliary epithelial cells in the anterior segment of the eye (Wollensak et al., 1998), optic nerve head astrocytes (Deasi et al., 2004; Prasanna et al., 2003) , Lamina cribrosa cells (Rao et al., 2007) and endothelial cells (Chen et al., 2001; Tschumperlin et al., 2003; Ostrow and Sachs 2005). Pathophysiological concentrations of ET-1 in the optic nerve head or the retina, the primary site of pathology, however is not yet known. Retro bulbar or Intra-vitreal ET-1 administration into various animal models results in dose dependent loss of retinal ganglion cells by apoptosis and activates optic nerve head astrocytes. (Chauhan et al., 2004; Lau et al., 2006). Previously our lab has characterized ET-1 mediated axonal transport blockade following a single intravitreal dose of 2nmole ET-1 in SD rats. Since extensive extracellular matrix deposition including collagen types I and VI is a characteristic feature of PAOG and is linearly correlated with the loss of retinal ganglion cells we wished to see if ET-1 also mediated an increase in ECM deposition in rat optic nerve head following intravitreal administration.

In the present study we have demonstrated by immunohistochemistry that intra-vitreal administration of a single dose of 2 nmole ET-1 resulted in enhanced deposition of collagen type I and type VI in rat optic nerve head. The expression of collagens type I

and VI was similar to previous reports (Morrison et al., 1995). ET -1 mediated enhanced deposition of collagen type1 and type VI was observed around the blood vessels in the pre laminar regions. ET-1 mediated increase in collagen deposition was also observed in the laminar and as well as the retrolaminar regions similar to that previously reported of enhanced deposition of ECM in elevated IOP models of glaucoma (Morrison et al., 2000). Our in vitro studies have also demonstrated that ET-1 increased collagen synthesis, deposition and secretion of collagens type I and VI in Lamina cribrosa cells. Besides lamina cribrosa cells, optic nerve head astrocytes have also been implicated glaucoma pathology and could contribute to enhanced collagen deposition in response to glaucomatous stimuli including TGF-β and ET-1 (He et al., 2007; Fuchshofer et al., 2005). The enhanced deposition of collagens throughout the optic nerve head including pre laminar, laminar and post laminar regions following ET-1 injections suggests that perhaps other cells types including optic nerve head astrocytes and as well as cells of the vasculature could contribute to ET-1 mediated collagen deposition.

Endothelin-1 (ET-1) and its isoforms, ET-2 and ET-3, mediate their effects through seven transmembrane G-protein coupled receptors, endothelin receptor A (ET_A) and endothelin B (ET_B) with specificities of ET-1 > ET-2 > ET-3 and ET-1 = ET-3, respectively (Yanagisawa 1994; Takagi et al., 1995). ET-1 mediated activation of the Gq coupled ET_A receptor, results in the classic phospholipase C activation, IP3 generation, mobilization of intracellular calcium stores, while activation of ET_B receptor results in nitric oxide (NO) release via a cGMP pathway. (D'Orleans-Juste et al., 2002). ET_B receptor also serves as a clearance receptor for ET-1 and helps regulate the

concentrations of this potent peptide in tissues and circulation (Rogers et al., 1997; Iwasa et al., 1999; D'Orleans-Juste et al., 2002; Schinelli 2002). Pathological conditions including cardiovascular and CNS injury, associated with elevated ET-1 levels, show an upregulated ET_B receptor expression (Rogers et al., 1997; I wasa et al., 1999; D'Orleans-Juste et al., 2002; Schinelli 2002). While ET_B receptor serves to clear the excess ET-1, increases in ET_B receptors also results in detrimental effects. For instance, it was observed that mechanical deformation and release of ET-1 results in ET_B receptor upregulation resulting in apoptosis of smooth muscle cells via ET_B receptors (Cattaruzza et al., 2002). Similarly, the reactive phenotype of astrocytes, including enhanced GFAP expression and proliferation, appeared to be mediated by the ET_B receptor subtype and inhibition of ET_B receptors resulted in a protective effect following CNS injury (Baba 1998; Koyama et al., 1999). Increase in ET_B receptor expression and ET_B receptor mediated enhanced collagen synthesis has been associated with various fibrotic conditions affecting the skin and the liver (Gandhi et al., 2000; Shi-wen et al., 2001). An increase in ET_B receptor expression has been reported in optic nerve heads of human glaucomatous eyes (Wang et al., 2006). Increases in ET-1 and ET_B receptor expression was also observed in the optic nerve heads of animal models of glaucoma with elevated IOP (Prasanna et al., 2006). In studies that demonstrated ET-1 mediated inhibition of axonal transport in rat optic nerves, similar effects were observed following ET-3 administration, which selectively activates ET_B receptors, indicating that changes in axonal transport were largely ET_B-receptor–mediated (Stokley et al., 2002). ET-1 also results in retinal ganglion cell apoptosis both *in vitro* and *in vivo* and appears to be

mediated through ET_B receptors (Krishnamoorthy et al., 2007). In our studies using ET_B deficient transgenic rats we observed a marked decrease in collagen VI deposition in optic nerve head sections following ET-1 injection compared to collagen deposition in ET-1 injected wild type rats (Fig.4.). An apparent decrease in ET-1 mediated collagen VI deposition in ET_B deficient transgenic rats suggests that the ET_B receptor, in part, contributes to ET-1 mediated enhanced collagen deposition.

In conclusion, we have demonstrated that ET-1 contributes to enhanced collagen deposition in rat optic nerve heads. ET-1 mediated deposition of collagen VI was less in ET_B deficient transgenic rats in comparison to wild type ET-1 injected rats, suggesting the involvement of ET_B receptors in enhanced collagen VI deposition. ET-1, in various animal models, results in loss of retinal ganglion cells by apoptosis, blocks axonal transport, activates optic nerve head astrocytes and results in optic neuropathy similar to that observed in glaucoma (Stokely et al., 2002; Chauhan et al., 2004; Lau et al., 2006). Our studies suggest that ET-1 could also contribute to increase in collagen synthesis and promote a fibrotic condition that further enhances the progression of the POAG.

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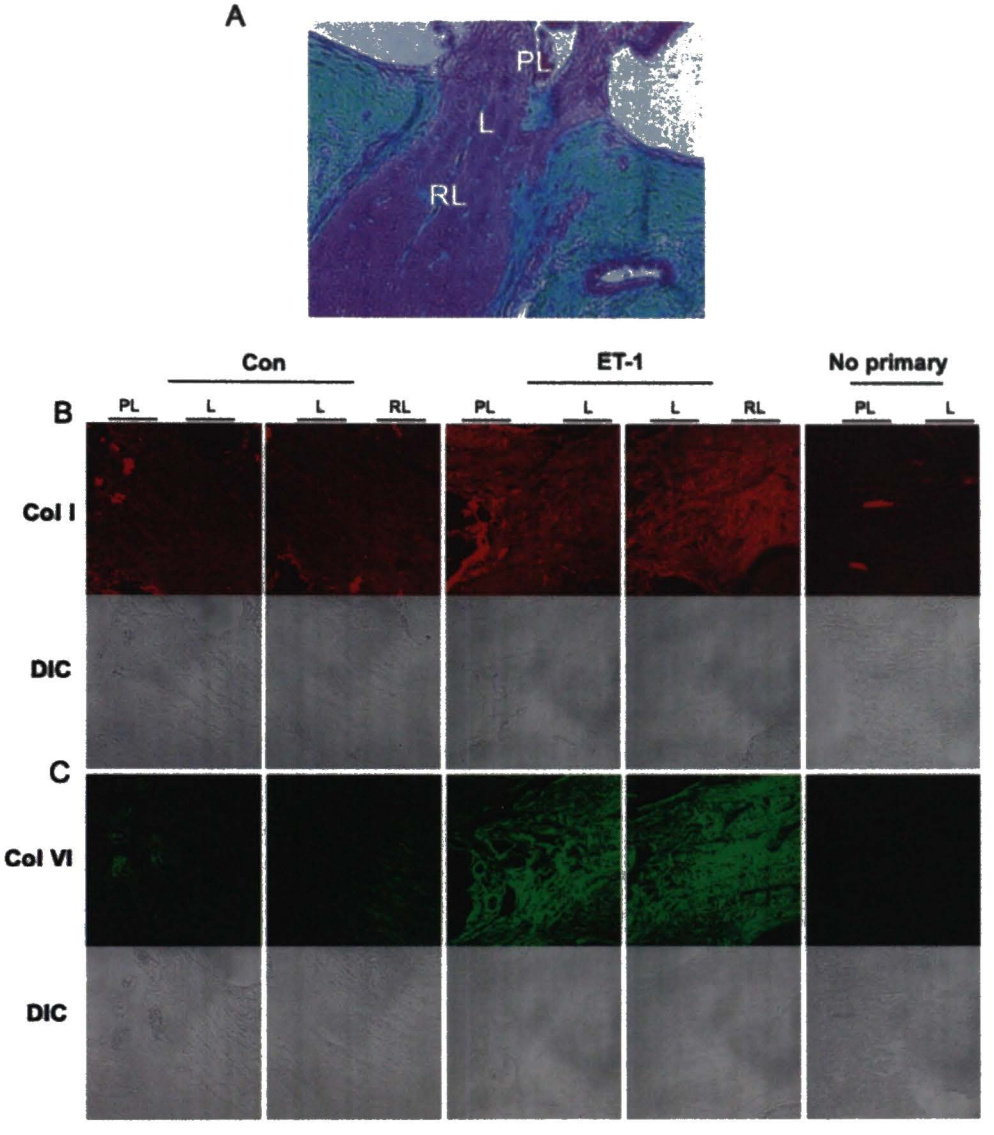
Fig.1. Collagen expression and ET-1 mediated regulation of collagen type I and type VI expression in rat optic nerve head

Gomori's Trichrome stain (green) for total collagen in the pre-laminar (PL), Laminar (L) and retro-laminar (RL) regions in a sagittal section through the optic nerve head (A). Representative immunohisto sections of wild type (+/+) rats showing the expression of Collagen type I in pre- laminar (PL) , laminar (L), retro-laminar (RL) regions of optic nerve head, in control and ET-1 injected eyes (B). Representative immunohisto sections of wild type (+/+) rats showing the expression of Collagen type VI in pre- laminar (PL), laminar (L), retro-laminar (RL) regions of optic nerve head, in control and ET-1 injected eyes (C).


A
PL
L
RL
Con
ET-1
No primary
B
PL
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L
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PL
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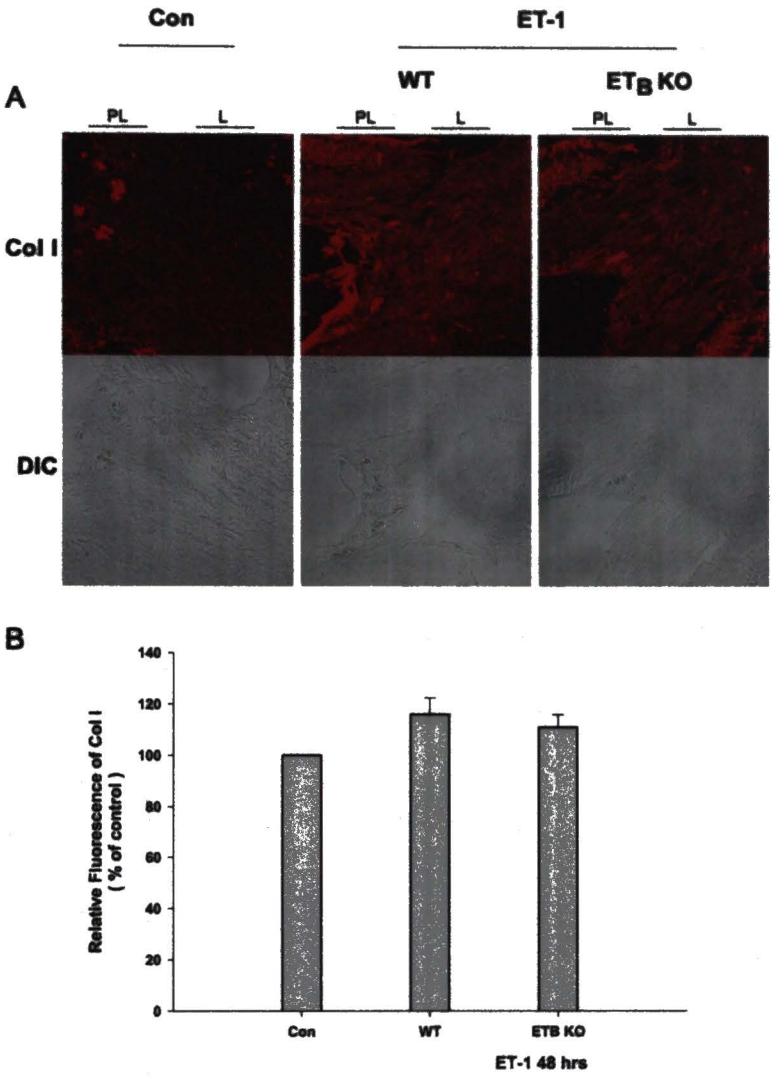
Col I
DIC
C
Col VI
DIC

Fig.2. Role of endothelin receptors ET_A and ET_B in ET-1 mediated regulation of collagen type I expression in optic nerve head.

Representative immunohisto sections of pre-laminar (PL) and laminar (L) regions of optic nerve head, showing the expression of Collagen type I, in control, ET-1 injected wild type (+/+) rats and ET -1 injected ET_B deficient (sl/sl / ET_B KO) rats (Figure 2 (A)). Relative fluorescence intensity for collagen type I staining in treatment groups including, control, ET-1 injected wild type (+/+) and ET-1 injected ET_B deficient (sl/sl / ET_B KO) rats as analyzed by Image J software and subsequently subjected to one-way analysis of variance (ANOVA) for multiple comparisons (Fig. 2(B)). Data are represented as the mean ± SEM (n=2; 8 consecutive optic nerve head sections per n).


Con
ET-1
WT
ETB KO
A
PL
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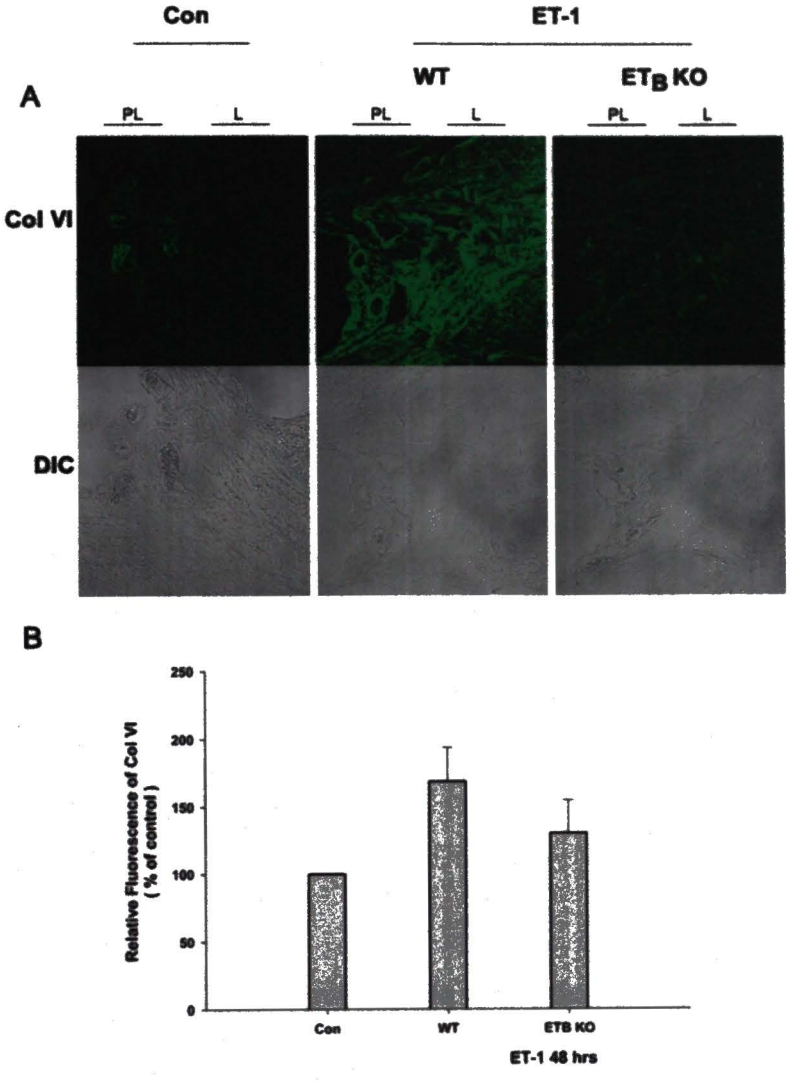
PL
L
PL
L
Col I
DIC
B
Relative Fluorescence of Col I
(% of control)
140
120
100
80
60
40
20
0
Con
WT
ETB KO
ET-1 48 hrs

Fig.3. Role of endothelin receptors ET_A and ET_B in ET-1 mediated regulation of collagen type VI expression in optic nerve head.

Representative immunohisto sections of pre-laminar (PL) and laminar (L) regions of optic nerve head, showing the expression of Collagen type VI, in control, ET-1 injected wild type (+/+) rats and ET -1 injected ET_B deficient (sl/sl / ET_B KO) rats (Figure 3 (A)). Relative fluorescence intensity for collagen type VI staining in treatment groups including, control, ET-1 injected wild type (+/+) and ET-1 injected ET_B deficient (sl/sl / ET_B KO) rats as analyzed by Image J software and subsequently subjected to one-way analysis of variance (ANOVA) for multiple comparisons (Fig. 3(B)). Data are represented as the mean ± SEM (n=2; 8 consecutive optic nerve head sections per n).


Con
ET-1
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Col VI
DIC
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Relative Fluorescence of Col VI
(% of control)
250
200
150
100
50
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Con
WT
ETB KO
ET-1 48 hrs

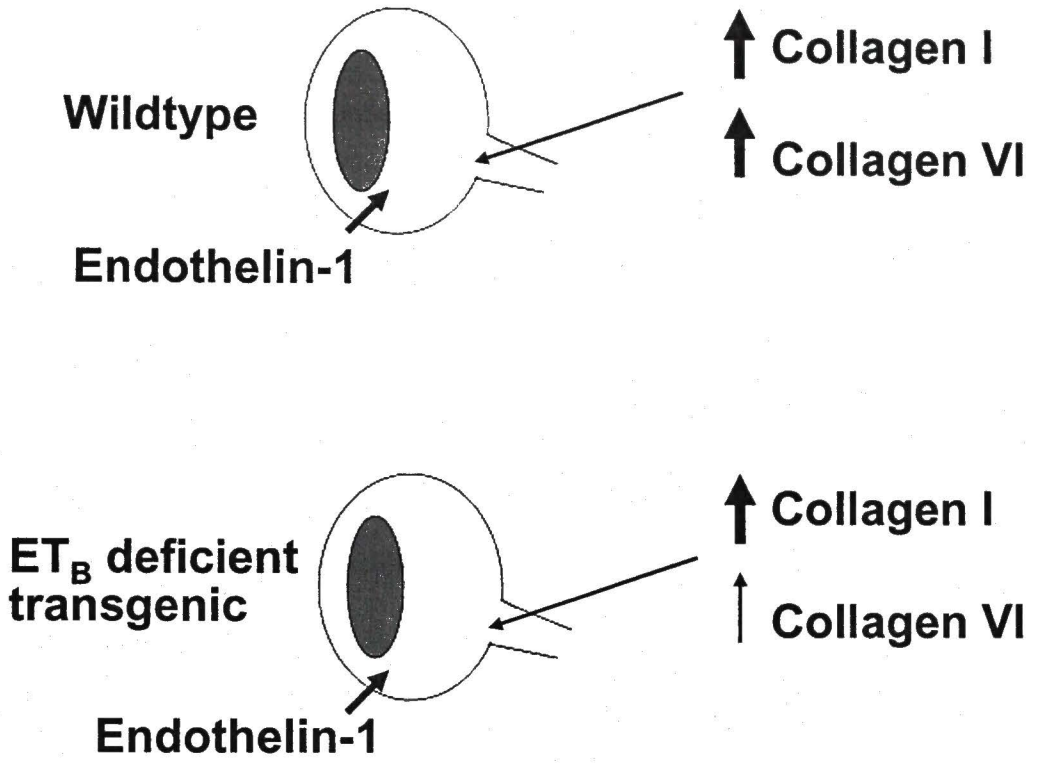

Fig. 4. ET-1 mediated collagen regulation in rat optic nerve head

Schematic representation of ET-1 mediated collagen deposition in rat optic nerve head following intra-vitreal injection as observed by immunohistochemistry. ET-1 increased collagen type I and VI depostition in optic nerve head of wildtype rats. ET-1 mediated collagen I deposition was not affected in ET_B deficient transgenic rats however there was a marked decrease in ET-1 mediated collagen VI deposition in ET_B deficient transgenic rats in comparison to wild type rats. These results suggest that ET-1 increases collagen type I and type VI *in vivo* at the level of optic nerve head and ET-1 mediated increase collagen VI deposition involves ET_B receptors.

Chapter V

Conclusions

Primary open angle glaucoma (POAG) is a leading cause of irreversible blindness worldwide. Loss of retinal ganglion cells by apoptosis underlies the loss of vision associated with POAG. Extensive extracellular matrix remodeling at the level of optic nerve head, with enhanced deposition of collagen types I, VI, and structural changes of LC is a characteristic pathologic feature of the disease. Enhanced deposition of collagens in the optic nerve head appears to occur very early in the disease process and linearly correlates to the loss of retinal ganglion cells suggesting that ECM remodeling at the level of optic nerve head may have an important role in the disease progression. Extensive ECM remodeling with enhanced collagen deposition resulting in loss of structural integrity observed in POAG is consistent with the pathology of fibrosis. Mechanical stress and profibrotic factors including transforming growth factor beta (TGFβ) and endothelin-1(ET-1), implicated in fibrotic diseases, are also implicated in POAG suggesting that POAG may involve a fibrotic mechanism. ET-1 a potent vasoactive peptide plays a key role in glaucoma pathology. Mechanisms by which ET-1 contributes to POAG appear to include vascular effects of ET-1 resulting from ischemia as well as non vascular effects, including activation of optic nerve head astrocytes, blockade of retinal ganglion cell axonal transport and apoptosis of RGCs. The present

study focused on the hypothesis that ET-1 enhances collagen deposition in the optic nerve head and contributes to a fibrotic mechanism in POAG.

ET-1 primarily acts as an autocrine and paracrine factor mediating its effects through G protein coupled seven transmembrane ET_A and ET_B receptors. ET-1 mediates biphasic increases in intracellular calcium levels via Gq coupled ET_A receptors, and ET_B receptor mediated NO release are well characterized signaling mechanisms of ET-1 in various cell types. ET_B receptor also serves as a clearance receptor for ET-1 and several pathological conditions associated with elevated ET-1 levels including POAG demonstrate an upregulation of ET_B receptors. While ET_B receptor upregulation serves to clear the excess ET-1, an upregulation of ET_B receptor results in signaling cascades that are linked to detrimental effects including apoptosis, hypertrophy, and enhanced collagen synthesis. Lamina cribrosa cells are a major cell type of the optic nerve head and respond to glaucomatous stimuli upregulating the expression of various extracellular protein molecules including collagen I and VI, suggesting that LC cells represent an important pro-fibrotic cell type prone to fibrosis. In specific aim 1 of the proposed study we have characterized the expression ET-1 and its receptors in LC cells. LC cells express both ET_A and ET_B receptors. LC cells express prepro-endothelin-1 the primary gene transcript for ET-1 suggesting that LC cells could contribute to ET-1 secretion at the level of LC. ET-1 mediates intracellular calcium mobilization in LC cells primarily via ET_A receptors. ET-1 also mediates NO release in LC cells and involves both ET_A and ET_B receptors. We observed a down regulation of ET_A receptors and ET_A receptor mediated intracellular

calcium mobilization in response to chronic ET-1. Consistent with what has been seen in glaucoma patholologgy we also observed an ET-1 mediated upregulation of ET_B receptors in LC cells (Prasanna et al., 2005; Wang et al., 2006). These findings suggested that ET-1's contribution to the pathogenesis of the optic nerve could also involve actions on LC cells.

Extensive ECM remodeling at the level of LC is thought to result in a compromise of the structural integrity of LC resulting in excavation of the optic disc and associated optic nerve degeneration and ganglion cell loss. Increases in extracellular matrix deposition including collagen type I and type VI was observed in glaucomatous LC (Hernandez et al., 1987; Morrison et al., 1989; Miller and Quigley 1988; Hernandez 2000 Sawaguchi et al., 1999). ET-1 plays an important role in initiating and maintaining fibrosis of various cell types and has been implicated in fibrotic disease (Sukada et al., 2005; Phan SH 2002; Eddy AA 2000; Wakatsuki et al., 2004).

In specific aim 2 we have demonstrated that ET-1 mediates its profibrotic effects in LC cells. ET-1 increased the mRNA levels of both collagen type I α I and collagen type VI αI chains at all doses of ET-1, suggesting a transcriptional regulation of collagen genes by ET-1. ET-1 increased the deposition and secretion of both collagen type I and type VI in LC cells. The increase in protein expression of collagen type I and VI was dose-dependent with a significant increase at 10 and 100nM concentrations of ET-1 in contrary to the increase in mRNA levels which were significantly increased at 1nM and 10nM

concentrations. The difference in mRNA and protein levels of the different collagen types could occur as a result of other mechanisms including enhanced mRNA stability or decreased collagen degradation. Chronic exposure of ET-1 results in a switch of receptor subtype from ET_A to ET_B, resulting in an ET_B -mediated increase in collagen synthesis in various cell types (Gandhi et al., 2000; Shi-wen et al., 2001). A similar switch in receptor subtype was observed in LC cells, where ET_B receptors were significantly upregulated at 100nM concentration of ET-1 (Fig.1.) and could in part explain the increase in collagen deposition that is observed at higher concentrations of ET-1. ET-1 mediated increase in collagen synthesis and deposition however appeared to be mediated by both ET_A and ET_B receptors as both ET_A and ET_B receptor antagonists were capable of inhibiting ET-1 mediated collagen synthesis suggesting that ET_B receptor upregulation in response to ET-1 could be downstream of ET_A receptor mediated signaling. The studies suggested that, ET-1 increases collagen synthesis and deposition by LC cells and could therefore contribute to ECM remodeling observed at the level of LC in glaucomatous subjects and further contribute to the pathology of primary open angle glaucoma.

LC in human eyes is very well developed consisting of ten laminar connective tissue whereas, the LC in eyes of rats, is less developed with only 1-2 layers of sparse connective tissue. Extensive ECM remodeling of the optic nerve head with increase in collagen type I and type VI deposition, the characteristic pathologic feature of POAG however is retained in the rodent models of glaucoma (Johnson et al., 2000; Guo et al., 2005; Morrison et al., 2005; Johnson et al., 2007). Intra-vitreal ET-1 administration into

various animal models results in loss of retinal ganglion cells by apoptosis, activation optic nerve head astrocytes and blockade of axonal transport (Chauhan et al., 2004; Lau et al., 2006; Stokley et al., 2002). An increase in ET-1 and ET_B receptor expression is observed in optic nerve heads of human glaucomatous eyes and animal models of glaucoma. ET-1 mediated detrimental effects including blockade of axonal transport and RGC apoptosis appear to be mediated through ET_B receptors (Stokely et al., 2002; Krishnamoorthy et al., 2007). In specific aim 3 we have demonstrated that ET-1 results in enhanced deposition of collagen type I and type VI in rat optic nerve head. ET-1 mediated collagen VI deposition was decreased in ET_B deficient transgenic rats in comparison to the wild type controls. An apparent decrease in ET-1 mediated collagen VI deposition in ET_B deficient transgenic rats suggests that, ET_B receptor in part contributes to ET-1 mediated enhanced collagen VI deposition. ET-1 mediated increase in collagen deposition was also observed in the pre-laminar regions around the blood vessels, in the lamina regions and as well as the retro laminar regions similar to that observed in IOP models of glaucoma (Johnson et al., 2000; Guo et al., 2005; Morrison et al., 2005; Johnson et al., 2007). The enhanced deposition of collagens throughout the optic nerve head including prelaminar, laminar and retrolaminar regions following ET-1 injections suggests that perhaps other cells types including optic nerve head astrocytes and as well as cells of vasculature could contribute to ET-1 mediated collagen deposition (Fig.3.). These studies suggest that ET-1 could contribute to increase in collagen synthesis *in vivo*

In summary the studies addressed in the specific aims have demonstrated that Endothlein-1 increases collagen synthesis and deposition both *in vitro* in LC cells and as well as *in vivo* at the level of optic nerve head (Fig.2.). It is well established that ET-1, in various animal models, results in loss of retinal ganglion cells by apoptosis, blocks axonal transport, activates optic nerve head astrocytes and contributes to pathogenesis of POAG (Chauhan et al., 2004; Lau et al., 2006; Stokley et al., 2002). ET-1 mediated increase in collagen synthesis at the level of optic nerve head could render a fibrotic mechanism that further enhances the progression of POAG.

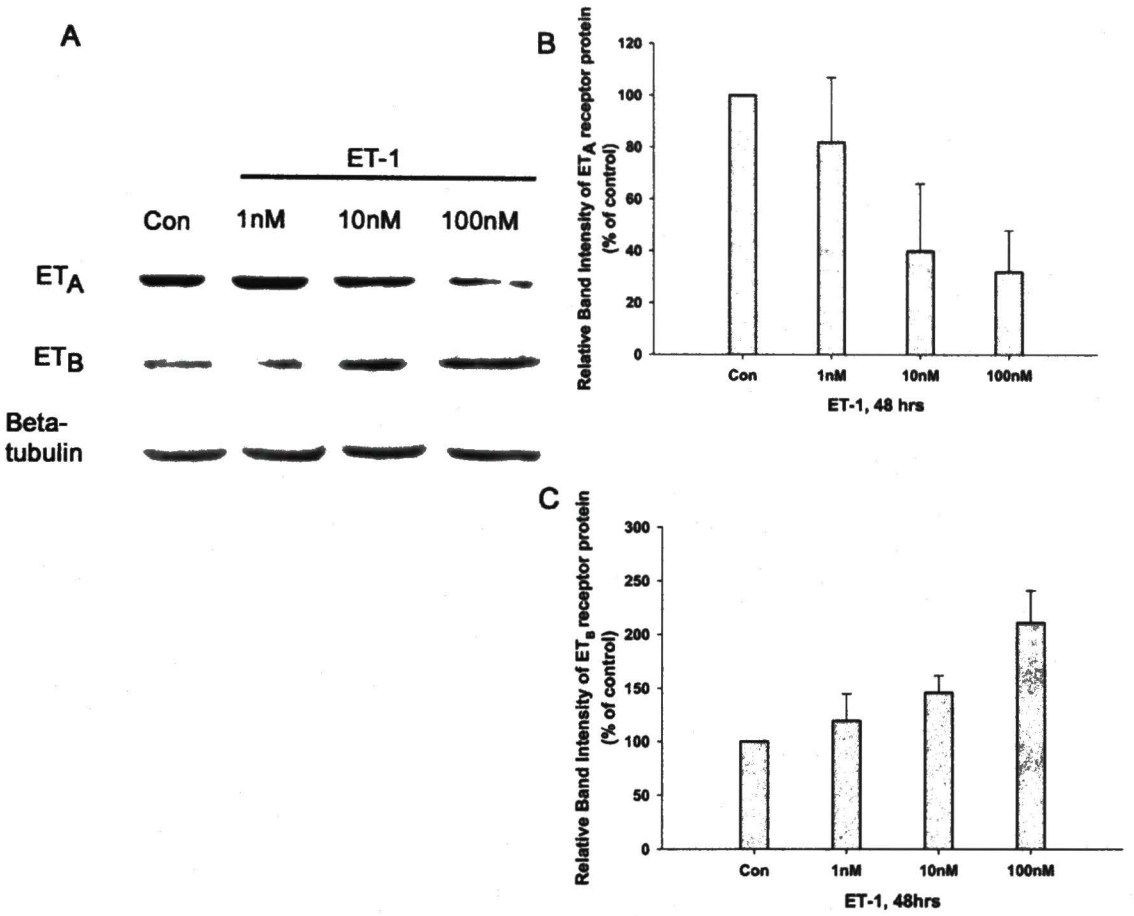

Fig.1. Effects of ET-1 on ET_A and ET_B receptor expression in LC cells determined by western blot. Western blot analysis of ET_A and ET_B receptor protein expression, following the treatment with ET-1, (1, 10 & 100nM) for 48 hrs (A). The quantification of band intensities for ET_A and ET_B receptors are represented as mean percentage ± SEM compared with the corresponding control band (B, C).

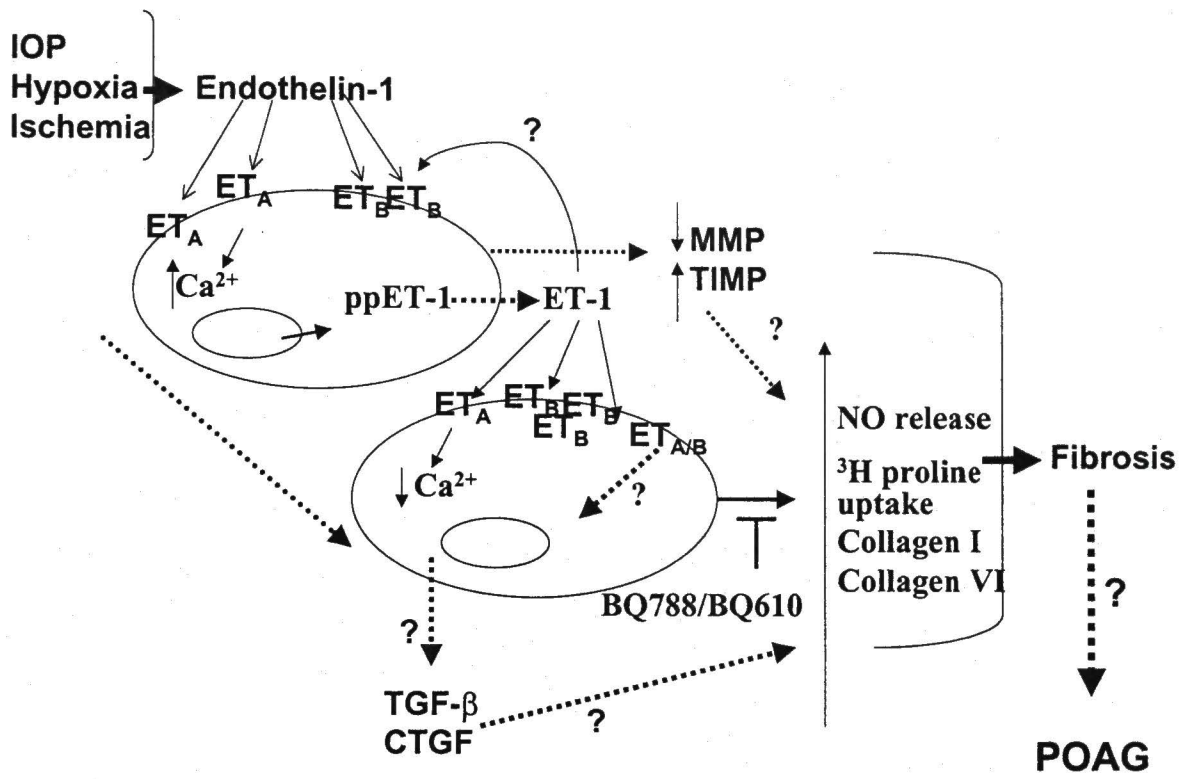

Fig. 2. ET-1 mediated mechanisms contributing to enhanced collagen deposition

Schematic representation of conclusions and future directions for research presented in the dissertation. ET-1 mediates collagen synthesis resulting in enhanced collagen I and collagen VI deposition *in vitro* in LC cells and as well as *in vivo* in rat optic nerve head. ET-1 mediated increase in collagen deposition is inhibited by both ET_A and ET_B receptor antagonists BQ610 and BQ788 respectively. ET_B receptor upregulation and ET_A receptor downregulation results in a switch in ET_A: ET_B ratio in LC cells following chronic exposure to ET-1. The switch in ET_A: ET_B receptor ratio may result in intracellular

signaling mechanisms that contribute to ET-1 mediated enhaced collagen synthesis. ET-1 mediated collagen synthesis could be down stream of ET-1 signialing via $ET_{A/B}$ heterodimers. Alternatively ET-1 could be mediate its profibrotic effects by inhibiting collagen degradation by upregulating TIMP's, downregulating MMP's or upregulating other profibrotic factors including CTGF and TGF-β.

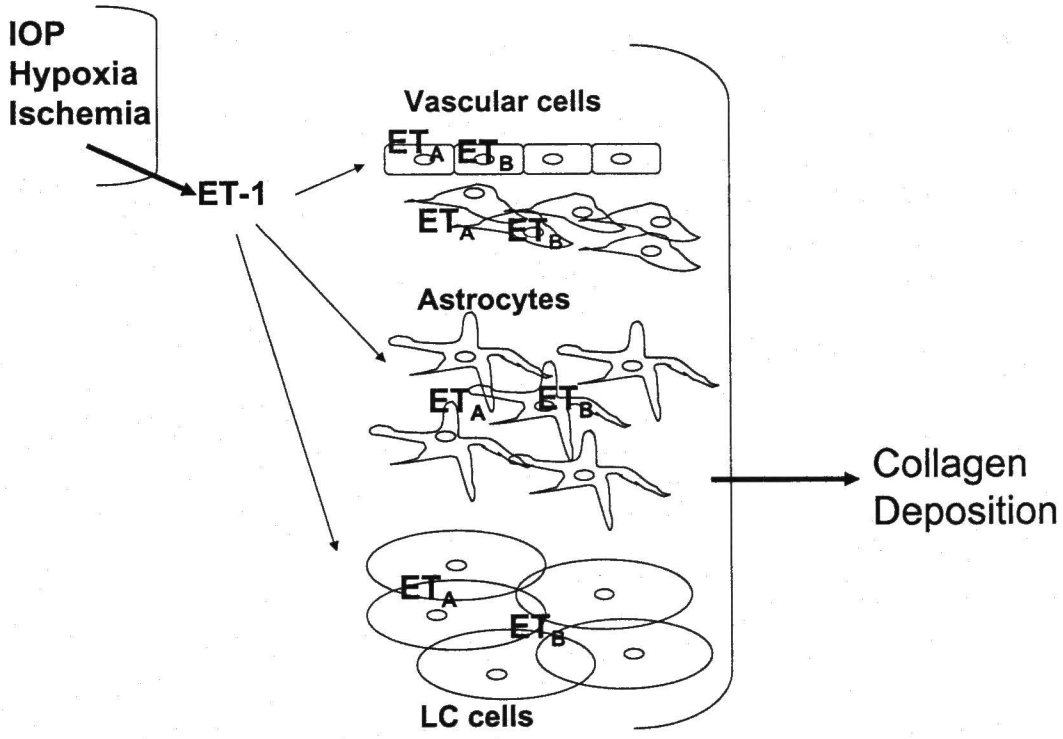

Fig.3. Possible role of other cell types in ET-1 mediated collagen deposition

The enhanced deposition of collagens throughout the optic nerve head including prelaminar, laminar and retrolaminar regions following ET-1 injections suggests that s other cells types including optic nerve head astrocytes and as well as cells of vasculature

(endothelial cells, smooth muscle cells and pericytes) could contribute to ET-1 mediated collagen deposition.

Future Directions

The main objective of this dissertation was to determine if ET-1, a key player in glaucoma pathology, confers a fibrotic mechanism by enhancing collagen type I and type VI deposition at the level of optic nerve head as observed in POAG. The specific aims addressed in the proposal have effectively demonstrated that ET-1 does indeed confer a fibrotic mechanism by enhancing collagen type I and type VI deposition both *in vitro* in LC cells and as well as *in vivo* in rat optic nerve head. Future studies would be directed towards understanding the mechanisms by which ET-1 regulates collagen synthesis and as well as the contribution of ET-1 mediated collagen regulation in the pathogenesis of POAG. The studies would be performed as outlined below.

1) To deliniate the role of Endothlein receptors ET_A and ET_B in ET-1mediated collagen synthesis: The role of ET-1 in POAG is well established. The contribution of ET receptors in ET-1 actions however remains to be studied and would be essential in order to design effective treatment strategies. In POAG an upregulation of ET_B receptors is observed at the level of optic nerve head and appears to contribute to the detrimental effects observed in POAG including RGC apoptosis, ONA activation and axonal transport blockade. In our studies with LC cells we observed an ET_B receptor

upregulation consistent with glaucoma pathology and a down regulation in ET_A receptor protein. The switch in receptor subtype form ET_A towards ET_B has been linked to enhanced collagen synthesis in various cell types. In LC cells a similar switch in receptor subtypes could contribute to the enhanced collagen synthesis. However, ET-1 mediated collagen synthesis appears to be blocked by both ET_A receptor antagonist and ET_B receptor antagonist individually. In order to determine if ET-1 mediated collagen synthesis involves both ET_A and ET_B receptors or if it were an ET_B dependent phenomenon, future studies would be done in the presence of ET_B selective agonist such as ET-3 and as well as in the presence of siRNA targeted to ET_B receptors. Apparent decrease in collagen VI deposition in our *in vivo* experiments in ET_B deficient transgenic rats suggests that ET-1 mediated collagen VI synthesis involves ET_B receptors. Future *in vivo* studies in the presence of ET_B receptor selective agonist ET-3 would further establish the significance of ET_B receptor in collagen VI synthesis. In our experiments with LC cells we observed a down regulation of ET_A receptor protein and its down stream signaling, intra cellular calcium mobilization. While it is very well known that ET_B receptors are upregulated in POAG, it is not known if POAG involves a down regulation in ET_A receptors. Future studies would be aimed at studying the ET_A receptor levels and the significance of ET_A receptor down regulation in POAG models. The pathology of POAG is contributed to elevated levels of ET-1. ET-1 unlike its isofroms is capable of binding to heterodimers of ET_A and ET_B receptors. ET-1 mediated collagen synthesis could be down stream of ET-1 signaling through hetrodimers of ET_A and ET_B receptors since both receptor antagonists are capable of inhibiting ET-1 mediated

collagen synthesis (Fig.2.). Significance of such a receptor signaling remains to be explored in glaucoma pathology and would be essential in determining if dual ET_A/ET_B receptor antagonist would be more effective in treating POAG than selective antagonists to ET_A or ET_B receptors.

2) To determine the molecular mechanisms contributing to ET-1 mediated enhanced collagen deposition: Extra cellular matrix collagen deposition is not only attributed to enhanced synthesis but also to its limited degradation. In our experiments in LC cells we observed a dose-dependent increase in collagen protein deposition, however the mRNA for collagens decreased at higher concentrations of ET-1 suggesting that other mechanisms besides increase in synthesis could be involved in ET-1 mediated collagen deposition. Future studies would be addressed to determine if ET-1 limited the degradation of collagen at higher concentrations. In order to address the above hypothesis, levels of Matrix metalloproteases (MMPs) the key enzymes responsible for collagen degradation and the levels of tissue inhibitors of matrix metalloproteases (TIMPS) the key enzymes that inhibit MMPs would be determined in response to ET-1 treatment (Figure 2). ET-1 exerts its profibrotic effects on tissues by also regulating other profibrotic factors including transforming growth factor beta (TGF β) and connective tissue growth factor (CTGF). TGF β is also implicated in POAG. Future studies would address if ET-1 alters the levels of TGFβ and CTGF *in vitro* and *in vivo* and there by further enhancing collagen deposition (Fig.2.).

3) To determine the contribution of ET-1 mediated collagen regulation in the pathogenesis of POAG: Extensive extracellular matrix remodeling of the optic nerve head with enhanced collagen type I and type VI deposition is a characteristic pathological feature of POAG. The underlying cause of blindness however is attributed to the loss of retinal ganglion cells. Studies with elevated IOP models of glaucoma have demonstrated that deposition of collagens type I and type VI is an early event in the disease process and correlates with the loss of retinal ganglion cells. In our *in vivo* studies we have demonstrated that ET-1 enhanced the deposition of collagen type I and VI very similar to that observed in elevated IOP models of glaucoma. In future studies the matrix deposition mediated by ET-1 would be correlated to the loss of retinal ganglion cells by performing a time course experiment and subsequent analysis of collagen deposition and retinal ganglion cells loss (Fig.2.). Alternatively the contribution of ET-1 mediated collagen deposition in retinal ganglion cells loss will be determined by inhibiting collagen synthesis utilizing collagen type I and type VI siRNA or specific collagen synthesis inhibitors such as mithramycin. Similar experiments intended to inhibit collagen synthesis with either endothelin receptor antagonist or collagen synthesis inhibitors in elevated IOP models of glaucoma would further determine the role of collagen deposition in the progression of POAG.

Primary open angle glaucoma continues to be the leading cause of irreversible blindness worldwide. Elevated IOP is a key risk factor and lowering IOP is the only therapeutic intervention available to delay the progression of the disease. Extensive research in past years have demonstrated that the loss of retinal ganglion cells by apoptosis underlies the

loss of vision in glaucoma and has opened up neuroprotection as a novel modality for treating glaucoma. The studies addressed in this dissertation and as well as studies proposed for future direction would further our understanding of the pathology of POAG and broaden the range of anti-glaucoma drugs to include antifibrotic drugs such as endothelin receptor antagonists.

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