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Purpose: Optic nerve head astrocytes (ONAs) normally support and protect the axons of retinal ganglion cells exiting the eye. Along with effects related to elevated intraocular pressure (IOP), proliferation and activation of ONAs, known as 'astrogliosis', is also thought to contribute to the pathophysiology of glaucoma by disrupting axonal transport and preventing axon regeneration. Concentrations of endothelin-1 (ET-1) are elevated in glaucomatous eyes and in animal models of glaucoma. ET-1 injection into the eye causes reduction of ocular blood flow. ET-1 causes a time-dependent proliferation of human ONAs. Tumor necrosis factor-α (TNF-α), a cytokine, which is also elevated in glaucomatous optic nerve head, promotes ET-1 release from ocular cells and could potentially stimulate ET-1 secretion from the ONAs. Hypoxia resulting from ischemia, which is produced by the elevation of IOP or vasospasm in the retinal vasculature, is considered a significant factor contributing to the stress at the glaucomatous optic nerve head. **Methods:** Concentrations of ET-1 secreted by hONAs into cell culture media after hypoxia and TNF-α treatment was measured using an enzyme-linked immunosorbent assay (ELISA). Proliferation of hONAs was measured using a proliferation assay (formazan assay), performed at the end of various time periods of incubation with TNFα and ET-1 under normoxia or hypoxia. The involvement of mitogen activated protein kinase (MAPK) in hONA proliferation was examined using MAPK inhibitors and Western blot analyses. **Results:** Cell culture media collected from hONAs after 24-hour hypoxia with concurrent TNF-α treatment showed a 500% increase in the irET-1. Under

normoxia, both TNF-α and ET-1 caused moderate proliferation of hONAs. Under hypoxia, TNF-α-induced proliferation was greatly increased. **Conclusion:** Hypoxia augments TNF-a and ET-1 induced growth of optic nerve head astrocytes, by way of increasing ET-1 synthesis and release as well as mitogenesis. Therefore reactive ONAs could be the common denominator underlying optic nerve damage in glaucoma since their localization makes them susceptible to mechanistic and ischemic influences in addition to influences of ET-1 and TNF-a.

Keywords: astrocyte; endothelin-1; tumor necrosis factor-α; hypoxia; proliferation; astrogliosis; glaucoma; optic nerve

ENDOTHELIN-1-INDUCED PROLIFERATION OF HUMAN OPTIC NERVE HEAD ASTROCYTES UNDER HYPOXIA

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Endothelin-1-Induced Proliferation of Cultured Human Optic Nerve Head Astrocytes under Hypoxia

Thesis

Presented to the Graduate School of Biomedical Sciences

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By

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Endothelin-1-Induced Proliferation of Cultured Human Optic Nerve Head Astrocytes under Hypoxia

CHAPTER I

Background

Glaucoma is the second leading cause of blindness in the world, affecting over 66 million people worldwide (Quigley et al., 1996). Glaucoma is an optic neuropathy, characterized by cupping of the optic disc, progressive loss of retinal ganglion cells and slow degeneration of optic nerve, resulting in blindness (Quigley et al., 1996). Increase in intra ocular pressure (IOP) is widely used for the clinical diagnosis of glaucoma and drugs that lower elevated IOP are the primary therapy.

A. Optic Nerve Head Astrocytes (ONAs)

Astrocytes are the major glial cells in the optic nerve head, the region where the axons of the retinal ganglion cells (RGCs) converge and exit the eye to form the optic nerve. Astrocytes are quiescent and quasi-differentiated under normal conditions. ONAs envelope the axons of RGCs and maintain the extracellular milieu for the axons by providing neurotrophic support as well as buffering of potassium and glutamate levels (Morgan, 2000; Hernandez, 2000). Astrocytes form glial tubes in the lamina cribrosa providing a supporting meshwork for the RGC axons exiting the eye. The optic nerve contains Type I astrocytes expressing glial fibrillary acidic protein (GFAP), a structural protein Type I are further classified as Type Ia and Ib. Type Ia express only GFAP, while Type Ib express both the astrocytic markers GFAP and neural cell adhesion molecule

(NCAM). Well-characterized Type Ib human optic nerve head astrocytes (hONAs) will be used for this study (Clark et al., 1995; Lambert et al., 2001; Wordinger et al., 2003).

Several observations have led to a wide acceptance of the possibility that elevated IOP leads to reduced blood flow, which could result in hypoxia/ischemia at the ONH (Carter et al., 1990; Flammer, 1994; Hayreh, 1994, Van Buskirk and Cioffi, 1992; Geijer and Bill, 1979). Vasospastic disorders such as migraine headache or cold hands and feet are more commonly seen in patients with both primary open angle as well as normotensive glaucoma (Gasser and Flammer, 1991; Phelps and Corbett, 1985). Also, chronic ischemia of the primate anterior optic nerve induced with endothelin-1 infusion produced a diffuse loss of axons without a change in the intraocular pressure (Cioffi and Sullivan, 1999). Increased immunoreactivity for GFAP and NCAM has been shown in the glaucomatous optic nerve head (Morgan, 2000) and GFAP, S-100 and vimentin in the inner retina (Lam et al., 2003). Ischemia/hypoxia due to elevated IOP and/or vasospasm in the retinal vasculature may reactivate the local astrocytes with subsequent proliferation and hypertrophy, being responsible for disruption of axonal transport, prevention of axon regeneration and degeneration of the optic nerve in glaucoma (Hernandez, 2000; Morgan, 2000).

Changes in the extracellular matrix (ECM) profile have been observed in the glaucomatous optic nerve head. These include increased expression of elastin, TGF-ß and collagens (Pena et al., 2001; Hernandez, 2000; Tanihara et al., 1997). Lam et al have recently studied the response of glial cells to short term elevation in IOP in rats, demonstrating a gradual and sustained increase in GFAP and S-100 immunoreactivity

with a transient increase in vimentin immunoreactivity (Lam et al., 2003). ET-1 causes changes in ECM by increasing collagen and fibronectin in various conditions including intimal hyperplasia following balloon angioplasty (Barolet et al., 2001), systemic sclerosis (Shi-Wen et al., 2001) and renal fibrosis (Evans et al., 2000). ET-1 has also been shown to upregulate the expression of ets-1, a transcription factor that activates expression of matrix-degrading proteinases such as collagenase and stromelysin (Naito et al., 1998). However, the effect of ET-1 on ECM changes in ONAs has not been studied.

B. Endothelin-1

Endothelin-1 (ET-1) is the first of the three known isoforms of a group of vasoactive peptides: ET-1, ET-2 and ET-3. ET-1, a 21-amino acid peptide secreted by the vascular endothelial cells and various ocular cell types, is a potent vasoconstrictor (Brain et al., 1989). ET-1 is synthesized and secreted by retinal pigmented epithelial cells (Narayan et al., 2002) and human non-pigmented ciliary epithelial cells (Prasanna et al., 1998). ET-1 immunoreactivity has been observed in the retina and the glial region of the optic nerve head (Ripodas et al., 2001; Wollensak et al., 1998). Clinically, ET-1 is elevated in the aqueous humor of patients with primary open angle glaucoma (POAG), in which the IOP is elevated and in the plasma of patients with normal tension glaucoma (NTG), a subtype of glaucoma often considered as a vasospastic disorder, in which IOP is apparently normal (Noske et al., 1997, Cellini et al., 1997; Sugiyama et al., 1995). In the Beagle model of glaucoma, ET-1 levels were 4-fold higher than control (Kallberg et al., 2002) and measurements of aqueous humor ET-1 levels in the Morrison model of elevated IOP in Brown Norway rats was elevated 2-3 fold (Prasanna et al., ARVO 2003

abstract). Furthermore, injection or perfusion of ET-1 into the optic nerve or retina causes optic neuropathy similar to that seen in glaucoma (Cioffi et al., 1995; Stokely et al., 2002). ET-1 was elevated in rat cortical astrocytes in association with astrogliosis in conditions such as Alzheimer's disease, neurotrauma and brain ischemia (Cintra et al., 1989; Hama et al., 1992; Zhang et al., 1994; Barone et al., 1994). Besides its association with glaucoma, ET-1 is a potent mitogen for optic nerve head astrocytes (Prasanna et al., 2002) and this action may be further potentiated by hypoxia at the optic nerve head induced by elevated IOP and/or vasospasm in the retinal vasculature (Figure 2).

C. Tumor Necrosis Factor-α (TNF-α)

TNF-α is a cytokine secreted by several different cell types in response to various stimuli including antigens and stress. Previously known as cachectin, TNF-α was first discovered in 1975 in the search for the cancer-cachexia inducing factor (Carswell et al., 1975). TNF-α has been studied widely since and has been found to exert cytolytic or cytostatic activity against tumor cells, stimulate proliferation of normal cells, and has been implicated in numerous processes including inflammation and immunoregulation (Rothe et al., 1992). TNF-α signals through two distinct cell surface receptors, i.e. TNFR-1 (p55/p60) and TNFR-2 (p75/p80) (Tartaglia and Goeddel, 1992 and Vandenabeele et al., 1995). TNF-α is upregulated in a number of neuropathological conditions such as ischemia, neurotrauma, multiple sclerosis, HIV-dementia, and Alzheimer's disease (Venters et al., 2001). TNF-α is among the cytokines believed to be powerful regulators of glial cell activation, which is a consistent feature of various neuropathologies (John et al., 2003).

TNF-α promotes ET-1 synthesis and release in systemic inflammatory responses by several cell types including human umbilical vein endothelial cells (Scalera et al., 2003), monocytes (Ehrenreich et al., 1993), renal epithelium (Ohta et al., 1990). One recent observation is particularly interesting: TNF-α induced the production of ET-1 in an *in-vitro* co-culture model of the human blood brain barrier (BBB). ET-1 thus produced, increased the permeability of the BBB by inducing IL-1ß overexpression in astrocytes, the effect on the BBB being eliminated in the absence of astrocytes (Didier et al., 2003). TNF-α levels are elevated in the glaucomatous eye (Tezel et al., 2001). After the exposure to stress such as ischemia and elevated hydrostatic pressure, glial cells in the optic nerve head were shown to secrete TNF- α as well as other potential damaging agents such as nitric oxide (Tezel and Wax, 2000). In glaucomatous optic nerve heads, the expression of TNF-α and TNFR-1 were upregulated; TNF-α expression was primarily observed in glial fibrillary acidic protein (GFAP)-positive astrocytes, and was proportional to the progression of optic nerve degeneration (Yuan and Neufeld, 2000). All these observations support the hypothesis that TNF-α may cause ET-1 release from ONAs in the glaucomatous optic nerve head, which may further cause proliferation of ONAs in addition to the proliferation caused by TNF-a itself.

D. Hypoxia

Hypoxia or low oxygen supply has long been recognized as an important regulatory factor in various tissues, especially the brain in both health and disease. In development, oxygen tension regulates the maturation of the blood brain barrier by influencing the proliferation of astrocytes (Song et al., 2002). A high prevalence of sleep-

disordered breathing (SDB) has been reported in patients with POAG. Chronic hemodynamic changes and recurrent severe hypoxia resulting from SDB is thought to contribute to anoxic optic nerve damage, implicated in glaucoma (Onen et al., 2000). Also, normal-tension glaucoma has been associated with sleep apnea syndrome, a disease characterized by repetitive upper airway obstructions during sleep, inducing hypoxia and sleep disruption with the risk of cardiovascular and neurological implications (Mojon et al., 2002). Hypoxia upregulates the expression of ET-1 and the ET_B receptor in cultured astrocytes as well as in vascular endothelial cells (Tsang et al., 2001; Shibaguchi et al., 2000). ET-1 itself, which is secreted by various ocular cells, is a potent vasoconstrictor and can cause hypoxia to ocular tissues by a possible vasospastic effect on the retinal vasculature. All these observations justify the need to study the contribution of hypoxia to glaucomatous optic neuropathy.

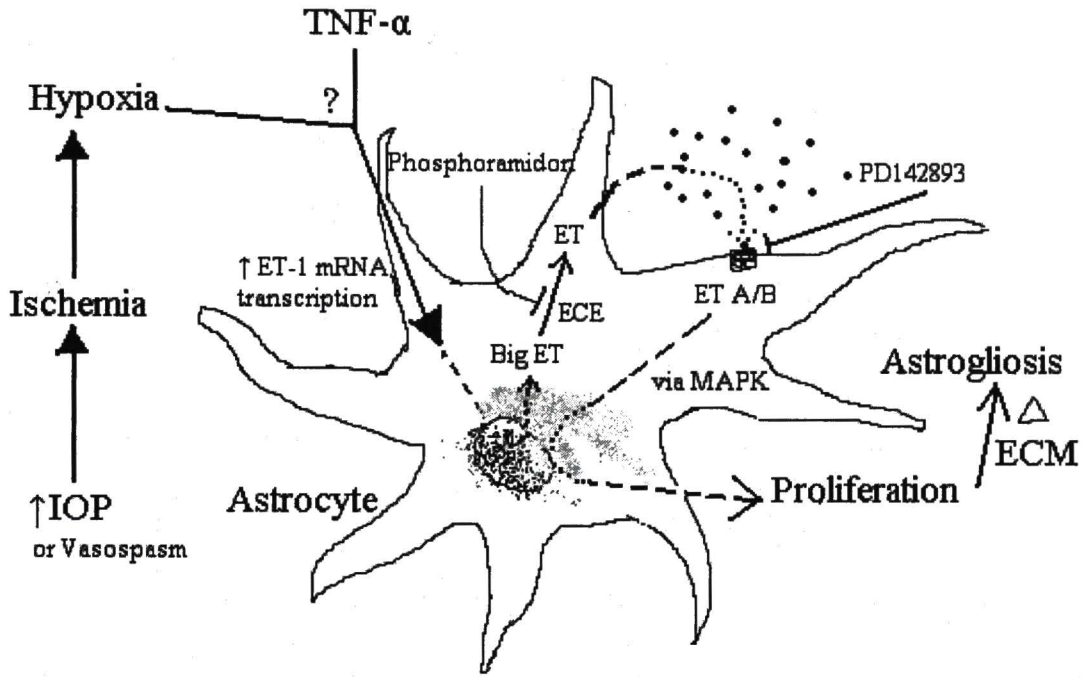

Figure I-1. Hypothetical Representation of Endothelin-1 Synthesis and Astrocyte Proliferation in Response to Hypoxia and TNF-α

(Modified from Yorio et al., 2001)

Hypothesis and Specific Aims

Elevated IOP in glaucoma, may lead to ischemia at the optic nerve head, which would include diminished oxygen supply i.e. hypoxia. Hypoxia/Ischemia is a potent stimulator for ET-1 induced astroglial activation in brain astrocytes, following neurotrauma. ET-1 is a potent mitogen for various cells and its actions are mediated through two independent G-protein coupled receptors, ET_A and ET_B that are expressed in astrocytes (Marsault et al., 1990; Ehrenreich et al., 1991; Hosli and Hosli, 1991; Lazarini et al., 1996). ET-1 and TNF-α levels are elevated in the glaucomatous eye (Noske et al., 1997; Tezel et al. 2001). ***The hypothesis for this study is that hypoxia together with higher levels of TNF-α increases the production of ET-1 by the optic nerve head astrocytes*** **and that** ***ET-1 and TNF-α induces proliferation of astrocytes via the MAPK signaling pathway.*** Proliferation of astrocytes may lead to astrogliosis characterized by hypertrophic "reactive" astrocytes, which ultimately damage the axons (Fig.1).

The following specific aims were pursued to examine this hypothesis.

Specific Aim 1: *To study the effects of Hypoxia and TNF-α on ET-1 secretion from optic nerve head astrocytes (ONAs).*

Specific Aim 2: *To study the effect of ET-1 and TNF-α on proliferation of ONAs under normal and hypoxic conditions.*

Specific Aim 3: *To demonstrate the involvement of MAPK signaling pathway in ET-1 induced proliferation of hONA under normoxia and hypoxia.*

Rationale and Experimental Approach

Specific Aim 1: To study the quantitative effects of Hypoxia and TNF-α on ET-1 secretion from optic nerve head astrocytes (ONAs).

Rationale

Ocular cells such as retinal pigmented epithelial cells synthesize and secrete ET-1 (Narayan et al., 2003). ET-1 immunoreactivity has been observed in the glial region of the optic nerve head (Wollensak et al., 1998). Brain astrocytes release ET-1 under experimental hypoxia (Pluta et al., 1997). Trauma to the brain especially ischemia causes ET-1 release from glial cells (Siren et al., 2000). TNF-α has been shown to induce ET-1 release from various tissues including vascular endothelium, human vascular smooth muscle cells, human non-pigmented ciliary epithelium and brain astrocytes (Patel et al., 2002, Woods et al., 1999, Prasanna et al., 1998). In glaucomatous optic nerve heads, the expression of TNF-α and TNFR-1 were upregulated (Tezel and Wax, 2000; Yuan and Neufeld, 2000). There is evidence for the transcriptional regulation of ET-1 gene by TNF-α (Marsden and Brenner, 1992). Recently, an association of tumor necrosis factor alpha-308 gene polymorphism with primary open-angle glaucoma in Chinese populations has been demonstrated (Lin, 2003). Although functional implications of this polymorphism remain to be examined, TNF-α may be considered a candidate marker for glaucoma. Primary hONAs used in the present study express mRNA for preproET-1 (Prasanna et al., 2002). PreproET-1 is converted to Big ET-1 by diaminopeptidases. Endothelin converting enzyme (ECE) then converts Big ET-1 to ET-1. ECE is involved in ET-1 synthesis in human non-pigmented ciliary epithelium cells (Prasanna et al., 1998;

1999). Hypoxia/ischemia has been shown to upregulate ET-1 mRNA and ET_B receptor in cultured astrocytes and vascular endothelial cells (Tsang et al., 2001; Shibaguchi et al., 2000). Since both hypoxia and TNF-α act as stimuli for ET-1 synthesis, there may be interactions between TNF-α and ET-1 system that occur when tissues are subjected to hypoxic conditions.

Experimental Approach

Primary human optic nerve head astrocytes from 62 and 81 years old donors at early passages (p5-12) and brain astrocytoma cell line U373MG (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium containing 10% FBS. Cells grown to confluence in 24 well- and /or 96 well plates were serum starved for 12 to 24 hrs before commencing the experiment. Experimental hypoxia was generated using an incubator perfused with 95% N_2 and 5% CO_2 and a control set of culture plates was placed in a tissue culture incubator with 95% Air and 5% CO_2. The chamber was perfused with N_2-CO_2 mixture overnight to remove all air from the incubator. Cells were subjected to hypoxia for various time periods (4, 6, 12, 24, 48 or 96h). TNF-α (Peprotech, NJ; 1, 10, or 100nM) was added to one set of wells. ET-1 released into the cell culture media was determined using a commercially available ET-1 ELISA kit (ALPCO, Windham, NH). Involvement of ECE in hypoxia and/or TNF-α induced ET release was determined by pre-treatment of cells with an ECE antagonist phosphoramidon (1μM). A separate set of cells was treated with only phosphoramidon as a control to determine if the compound itself affects the cells or measurement of ET in the media. All raw values were normalized using respective control values at 100%. Statistical analysis was

performed using One Way ANOVA and Student-Newman-Keuls multiple comparison tests to examine statistical significance between various treatment groups; $p<0.05$.

Specific Aim 2: To study the effect of ET-1 and TNF-α on proliferation of ONAs under normal and hypoxic conditions.

Rationale

Astrocyte proliferation is a common phenomenon in conditions such as neurotrauma, nerve crush and toxicity, and is also observed in the glaucomatous optic nerve head. Traumatic injury to the adult CNS results in a rapid response from astrocytes, a process often referred to as *reactive astrogliosis* or *glial scarring*, in which the astrocytes de-differentiate, proliferate and hypertrophy i.e. become *"reactive"*. The functional role of glial scarring is not entirely understood, but it is thought to be an attempt made by the CNS to restore homeostasis by isolation of the damaged region (Fitch and Silver, 1997). Immunostaining for GFAP, an astrocytic marker was shown to increase in response to ET-1 injection into the rat neostriatum (Ishikawa et al., 1997). ET-1 also induced proliferation of rat cortical astrocytes (Hama et al., 1992). TNF-α induces ET-1 release in human non-pigmented ciliary epithelium cells (Prasanna et al., 1998). Since both ET-1 and TNF-α are elevated in the glaucomatous eye in response to ischemic insult, TNF-α together with hypoxia/ischemia may be responsible for astrocyte proliferation in the optic nerve head.

Experimental Approach

Hypoxia is a stimulus for astrocyte proliferation. Both TNF-α and ET-1 can induce astrocyte proliferation (Prasanna et al., 2002; Selmaj et al., 1990; 1991; Barna et al., 1990). TNF-α can increase ET-1 secretion, which in turn will cause further proliferation. Therefore, hypoxia together with ET-1 and TNF-α may be expected to be a very potent stimulus for the proliferation of hONAs. If this is an ET receptor mediated effect, a non-selective ET receptor antagonist may be expected to block the proliferative effect of ET-1, and partially block the effect of TNF-α. Since ET-1 is a potent mitogen, mitogen activated protein kinase may be involved in ET-1 mediated proliferation. An inhibitor of MAPK, PD98059 or MEK1/2 inhibitor U0126 should block the proliferation signal. To test this hypothesis, two different techniques namely ^{3}[H]-Thymidine uptake assay and Formazan assay were utilized. The ^{3}[H]-Thymidine uptake assay measures DNA synthesis within the cells, while the Formazan assay measures the dehydrogenase enzyme activity in metabolically active cells. A predetermined number of cells (between 1000 and 5000) were seeded in 96 well culture plates, allowed to settle overnight, and then serum starved for 12-24 h. For the experiment, fresh serum-free medium containing ET-1 (1, 10 or 100nM), TNF-α (1, 10 or 100nM) or a non-selective ET receptor antagonist PD142893 (1μM) were used. One set of wells was treated with serum containing medium as a positive control for proliferation, as serum is a known stimulus for proliferation. Each experiment was repeated two or three times with 4 to 8 well per treatment. Cells were pre incubated with antagonists/inhibitor for 30 min before the experiment and appropriate controls were used for each antagonist treatment. Cells were

placed in a hypoxia chamber, and a separate plate with the same cell density and treatment groups was placed under normoxia. In case of ^{3}[H]-Thymidine uptake assay described earlier for hONAs (Prasanna et al, 2002), a known amount of ^{3}[H]-labeled Thymidine was added to the serum free medium used for the experiment, and following cytolysis, radioactivity incorporated in the newly synthesized DNA of dividing cells was measured at the end of the incubation period using a scintillation counter. For the Formazan assay, 100µl fresh media was added to the cells along with 20µl of the MTS compound at the end of the incubation period. The cells were incubated at 37°C for 30 minutes and the absorbance was read at 490nm in a kinetic microplate reader (Molecular Devices, Sunnyvale, CA). All raw absorbance values were normalized using respective control values at 100%. Statistical analysis was performed using One Way ANOVA and Student-Newman-Keuls multiple comparison tests to examine statistical significance between various treatment groups at $p<0.05$.

Specific Aim 3: To examine the involvement of MAPK signaling pathway in ET-1 induced proliferation of hONAs.

Rationale

This specific aim focuses on the possible mechanism that may be involved in the actions of ET-1 and TNF-α on hONAs, examined in Specific Aim 1 and 2. Typically, signals for cell survival and death in response to various stimuli are transduced via a series of protein kinases, which phosphorylate subsequent proteins in the cascade. These effector-proteins are then responsible for carrying out the survival promoting or death

inducing function (Liu et al., 1996). Mitogen activated protein kinases (MAP Kinases or MAPKs) are an important family of protein kinases involved in cell proliferation (Robinson and Cobb, 1997; Windmann et al., 1999). Among the members of MAPK family, extracellular signal-regulated kinases (ERK; p44 MAPK/ERK1 and P42 MAPK/ERK2) and the stress-activated protein kinases (SAPK), including the c-Jun amino (N)-terminal kinase (JNK; SAPK1), and the p38 kinase (SAPK2) have been extensively studied. They are activated by phosphorylation of their tyrosine and threonine residues (Davis, 1993; Boulton et al., 1991). The MAPK cascade is activated by mitogens and survival factors such as neurotrophins via surface receptors, leading to activation of the cellular transcriptional machinery, ultimately responsible for growth and/or differentiation (Seger and Krebs, 1995). Mitogens have a weak effect on SAPK activation, but environmental stress such as UV radiation, osmotic shock (Raingeaud et al., 1995) and cytokines, such as TNF-α, cause upregulation and activation of proteins responsible for cell death (Mielke and Herdegen, 2000; Kummer et al., 1997). Thus cell fate is believed to be determined by a delicate balance between the survival-promoting ERK pathway and the death-promoting JNK and p38 pathways (Xia Z 1995). ET-1 causes activation of Erks via the ET_B receptor, leading to DNA synthesis in rat astrocytes (Lazarini et al., 1996). Recently, Tezel et al have demonstrated the prominent and persistent activation of ERK in activated glial cells, which suggests that this signaling pathway is probably associated with the induction and/or maintenance of the activated glial phenotype as seen in glaucoma (Tezel et al., 2003). The following schematic

diagram illustrates the possible role of MAPK signaling involved in ET-1 mediated human optic nerve head astrocyte proliferation.

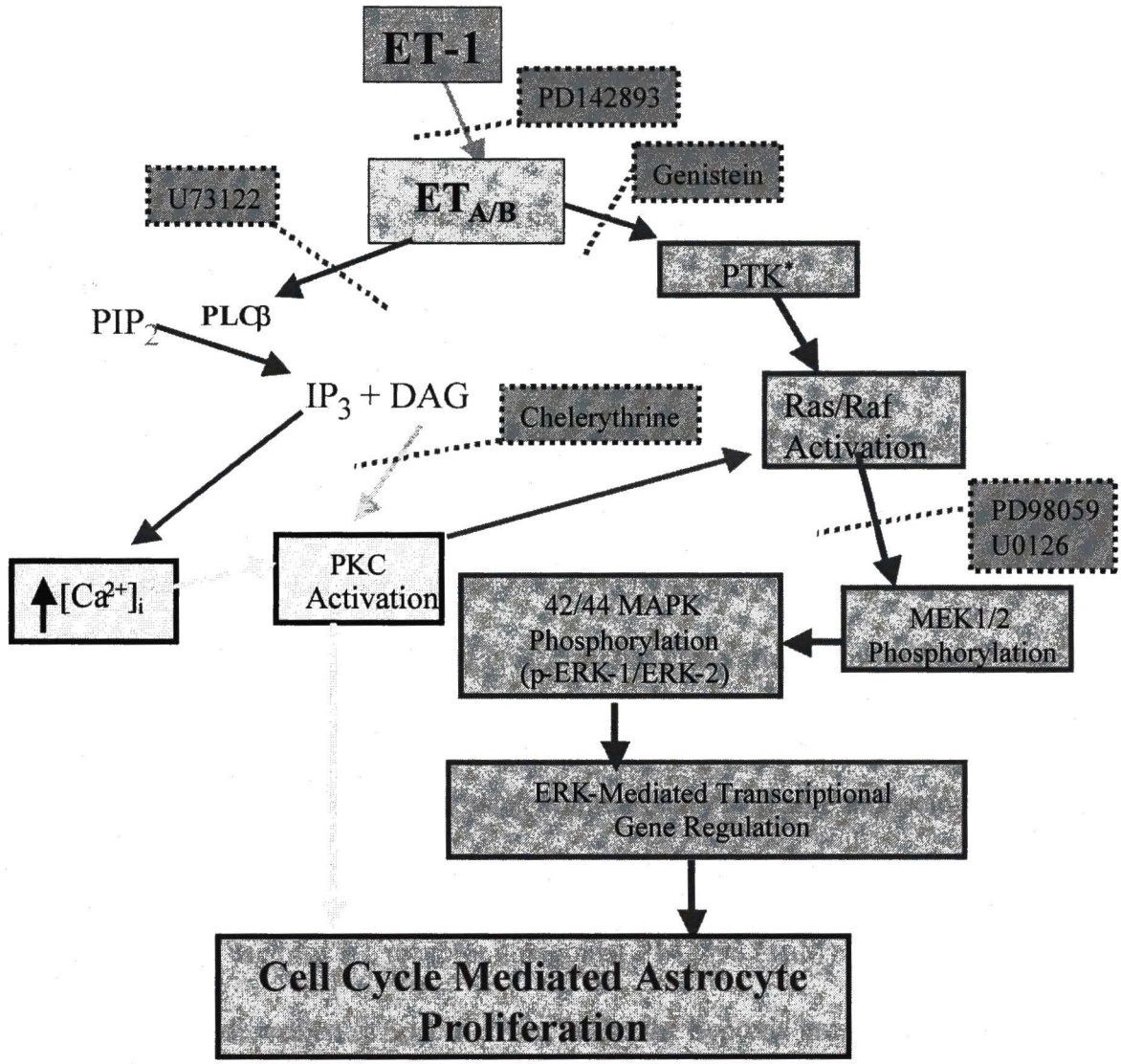

Figure I-2. Hypothetical Representation of ET Receptor Activated Signaling Pathways

Experimental Approach

To test the involvement of the MAPK signaling cascade in ET-1 and TNF-α-induced astrocyte proliferation, two different techniques namely ^{3}[H]-Thymidine uptake assay and Formazan assay were utilized. The ^{3}[H]-Thymidine uptake assay measures DNA synthesis within the cells, while the Formazan assay measures the dehydrogenase enzyme activity in metabolically active cells. MEK1/2 inhibitor U0126 (10μM) was incorporated in proliferation assays, described under Experimental Approach in Specific Aim 2.

To ascertain the phosphorylation of Erk, hONAs grown to confluence on 100mm culture dishes were treated with ET-1 (1, 10, 100 nM) for various time periods (0, 0.5, 1, 5, 10, 15, 30 and 60 min). Protein from the cell lysates was separated using SDS-PAGE. Western blot analysis was performed using commercially available monoclonal antibodies against phosho-Erks ($p42^{MAPK}$ and $p44^{MAPK}$; Cell Signaling Technology, CA). Separate sets were used for ET receptor antagonist P142893 (1μM) and MAPK inhibitors U0126 (10μM) as described in Specific Aim 2. For the hypoxia group, these treatments were performed at the end of 12 or 24 h hypoxia.

CHAPTER II: RESULTS

Endothelin-1-Induced Proliferation of Cultured Human Optic Nerve Head Astrocytes under Hypoxia

Research Article (Submitted to *Glia*, Nov 2003)

Chapter II

Manuscript:

Hypoxia Augments TNF-α-Mediated Endothelin-1 (ET-1) Release and Cell Proliferation in Cultured Human Optic Nerve Head Astrocytes (hONAs)

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Footnotes: * This manuscript comprises of research done by DD in partial fulfillment towards a Masters degree in Biomedical Sciences from UNTHSC.

Abstract:

Hypoxia/ischemia at the optic nerve (ON) could be attributed to insufficient blood supply caused by elevated intraocular pressure (IOP) and/or vasospasms in the microvasculature, contributing to retinal ganglion cell death and optic nerve damage in glaucoma. The mechanisms however remain poorly understood. Optic nerve head astrocytes (ONAs) are important regulators of axon function in the normal ON and in glaucoma, respond to changes in IOP and/or hypoxia by undergoing astrogliosis, which results in ON damage. Endothelin-1 (ET-1), a potent vasoactive peptide and TNF-α, a cytokine, which are both elevated in glaucoma, have been increasingly implicated in glaucomatous neuropathy. ET-1 could participate in optic neuropathy at least in part by exacerbating astrogliosis in the optic nerve head in response to changes in IOP and/or hypoxia, similar to the findings in brain neurotrauma studies. ET-1 was recently shown to promote mitogenesis in cultured human ONAs (hONAs). Furthermore, TNF-α is a known agonist for stimulating ET-1 release from ocular cells. In the present study, the effect of experimental hypoxia on ET-1 synthesis and release as well as on TNF-α and ET-1-mediated hONA proliferation was assessed using ELISA, Q-RT-PCR, and MTT-formazan assays. It was found that hypoxia enhanced TNF-α-mediated ET-1 synthesis and release and also significantly increased TNF-α- and ET-1-mediated hONA proliferation. PD142893, an $ET_{A/B}$ receptor antagonist blocked ET-1-mediated hONA proliferation both under normoxia and hypoxia, while doing so only under normoxia following TNF-α treatment. Moreover, it was determined using U0126, an inhibitor of p42/44 mitogen activated protein kinase (MAPK) pathway that TNF-α-mediated hONA

proliferation was partially blocked under normoxia and hypoxia while completely blocking ET-1's effects only in normoxia. Therefore, it has been shown for the first time that hypoxia augments TNF-α and ET-1 responses in optic nerve head astrocytes, by way of increasing ET-1 synthesis and release as well as mitogenesis. Such an agonist-induced hONA proliferation following hypoxia could result in astrogliosis in glaucoma.

Introduction:

In glaucoma, insufficient blood supply at the optic nerve head (ONH) caused by elevated intraocular pressure and/or dysregulation in the microcirculation (mediated by vasospasms and/or abnormal autoregulation) results in hypoxia which could contribute to optic neuropathy (Flammer et al. 2001; Michelson et al., 1998). Human glaucomatous eyes which had visual field loss also showed microvascular changes including capillary filling defects and avascular areas in the juxtapapillary, choroidal, and retinal vascular beds (Zhao and Cioffi, 2000). These findings suggest that hypoxic/ischemic changes may be partly responsible for retinal ganglion cell (RGC) death, RGC axon loss, and progressive optic nerve damage. The exact mechanisms by which hypoxia-induced optic neuropathy occurs are not clearly delineated. However, optic nerve head astrocytes (ONAs) could be playing an important pathophysiological role (Hernandez, 2000; Morgan, 2000).

Astrocytes are the major glial cell type in the central nervous system and optic nerve head. At the ONH, astrocytes are vital for RGC survival (Morgan, 2000). Astrocytes normally regulate extracellular K^+ and glutamate levels and also provide neurotrophic support for nearby neurons (Waniewski and Martin, 1986; Goss et al., 1998). Astrocytes envelop the axons of RGCs and appear as striations in the retinal nerve fiber layer (Radius and de Bruin, 1981), and produce collagens, elastins, and fibrillins in the lamina cribrosa, providing for additional support to the laminar beams (Hernandez et al., 1991; Hernandez, 2000). The optic nerve contains Type Ia and Type Ib astrocytes, which are characterized based on their histochemical labeling for neural cell adhesion

molecule (NCAM) and glial fibrillary acidic protein (GFAP) (Hernandez et al., 1988; Ye and Hernandez, 1995). All astrocytes express GFAP, however, only Type Ib, which predominates in the optic nerve head also express NCAM (Ye and Hernandez, 1995). Functionally, Type Ia is thought to provide structural support for the axons and Type Ib acts as a physiological interface between vitreous connective and vascular tissues. The localization of ONAs in the lamina cribrosa and at the optic nerve head therefore makes them susceptible both to mechanical effects caused by elevated IOP as well as ischemic insults and hypoxic insults to the optic nerve which may initiate axon damage (Trivinio et al., 1996).

Like brain astrocytes which become reactive when subjected to neurotrauma (Hatten, 1991; Ridet et al., 1997), ONAs also respond to changes in the physiological state of neuronal system especially under conditions of injury (ischemia and/or pressure-related) as seen in glaucoma (Hernandez, 2000). ONAs are usually the first cells to become metabolically active from a quiescent state and begin to rapidly proliferate and migrate to the site of injury and subsequently become hypertrophic (Minckler and Spaeth, 1981; Agapova et al., 2001). There are marked changes in the glaucomatous optic nerve head morphology due to extensive remodeling of the extracellular matrix (ECM) proteins, which result in an environment non-conducive for axon growth or repair (Hernandez, 2000). In glaucoma, hypoxia-mediated ONA activation and optic nerve damage could occur due to abnormal or distinct cellular responses (e.g. proliferation/hypertrophy/changes in ECM) to elevated levels of endogenous substances like tumor necrosis factor-α (TNF-α), a potent cytokine (Yuan and Neufeld, 2000; Tezel

and Wax, 2000; Tezel et al., 2001) and vasomodulators, like nitric oxide (Liu and Neufeld, 2000; 2001), a potent vasodilator, and endothelin-1 (ET-1), a potent vasoconstrictor (Noske et al., 1997; Haefliger et al., 1999; Prasanna et al., 2002; Yorio et al., 2002). Such elevated levels of TNF-α, nitric oxide, and ET-1 have previously been shown in glaucomatous eyes, in the retina, optic nerve head, or aqueous humor (Yuan and Neufeld, 2000; Liu and Neufeld, 2000; 2001; Noske et al., 1997).

Normally, ET-1, a 21 amino acid peptide is present in aqueous humor (AH) at 2-3 times greater levels compared to that in plasma (Lepple-Weinhues et al., 1992) and is also present in various ocular tissues including ciliary epithelium, retina, and glial cells of the optic nerve (Yorio et al., 2002). In fact, ET-1 is found to be significantly elevated in AH of patients with primary open angle glaucoma (POAG) compared to age-matched controls (Noske et al., 1997) and in the circulating plasma of most patients with normal tension glaucoma (NTG), a vasospastic disease (Sugiyama et al., 1995; Cellini et al., 1997). Cultured human ONAs express ET-1 mRNA and also respond to exogenous ET-1 by elevating intracellular calcium and increasing mitogenesis (Prasanna et al., 2002). TNF-α is a potent stimulator of ET-1 synthesis in ocular cells (Prasanna et al., 1998; Narayan et al., 2003). Hypoxia can also stimulate TNF-α secretion in astrocytes (Tezel and Wax, 2000). Presently, the contribution of hypoxia both to TNF-α-induced ET-1 release from cultured human ONAs and agonist-induced ONA cell proliferation was assessed.

Materials and Methods:

Cell Culture and Treatments:

Primary cultures of human optic nerve head astrocytes (hONAs) originally isolated from donor eyes of different ages (62 and 81 year-old donors) were provided to the authors by Dr. Abbot Clark (Alcon Research Ltd, Fort Worth, TX). These cells were isolated as described previously with some modifications (Hernandez et al., 1988; Clark et al., 1995; Lambert et al., 2000). Briefly, human donor eyes from regional eye banks were received within 24 hours of death, and the lamina cribrosa (LC) was dissected from the remaining ocular tissue. LC tissue was cut into three to four explants and placed in culture plates containing DMEM plus 10% fetal bovine serum (FBS). The majority of cells that grew out of the explants were LC cells that were then cultured in Ham's F-10 medium with 10% FBS and passaged using a 0.25% trypsin solution. Mixed cell populations of hONAs and LC cells were trypsinized and plated in serum-free astrocyte growth medium (AGM). After 24 hours in culture, the medium was changed to AGM containing 5% FBS. LC cells failed to attach in serum-free medium and were removed when the medium was removed.

Subsequently, cultured hONAs were maintained in DMEM plus 10% FBS and passaged as described. All cultures were maintained in 5% CO2-95% O2 at 37°C, and medium was changed every 2 to 3 days. 100% confluency was reached after nearly 3 weeks in culture. Confirmation that these hONAs were Type 1b astrocytes was obtained by detecting the presence of GFAP and NCAM. [6,38] The hONAs were grown in collagen

Type I-coated 100-mm culture dishes (Becton Dickinson Labware, Bedford, MA) or T-75 culture flasks and were maintained at 37°C in DMEM media (Gibco, Grand Island, NY) supplemented with 44 mM $NaHCO_3$, 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and antibiotics (Gibco). Cell passages between 6-12 were used in this study. Most experiments were performed using hONAs from at least two different age groups. After confluency was reached, cells were trypsinized and seeded in serum-containing DMEM on a) 1,000 cells/well in a 96-well culture plate for formazan cell proliferation assay, b) 2,000 cells/well in a 96 well plate for ET-1 ELISA, and c) 100-mm dishes for RNA isolation and Q-RT-PCR analysis. Most of the experiments were performed under serum-free culture conditions unless specified. All experiments were replicated two times on collagen coated plates with multiple wells (between 4-8 wells for cell proliferation assays and ET release) for each treatment and three times on regular plastic cell culture plates. Cells were pre-incubated for 30 minutes with phosphoramidon (an inhibitor of endothelin converting enzyme – ECE; 1 μM) to demonstrate ECE involvement in TNF-α-mediated ET-1 secretion. PD142893, an $ET_{A/B}$ mixed antagonist (Sigma-Aldrich, St. Louis, MO) was included to demonstrate ET receptor involvement. The hONAs were pretreated with PD142893 or U0126 (10 μM; a MEK1/2/MAPK inhibitor) (EMD Biosciences, La Jolla, CA), for 30 minutes prior to the addition of ET-1. Statistical analyses were performed to determine significance of treatment over controls using One-way ANOVA with multiple comparison tests. The level of significance was set at $p<0.05$.

In vitro Hypoxia:

Cultured hONAs grown in 96-well plates or 100-mm dishes in complete DMEM were switched to fresh serum-free (SF) DMEM (containing 5 mM glucose) following 2-3 SF-DMEM washes and treated with appropriate agonists in the presence and absence of antagonist/inhibitors. Following the treatments, 96-well plates (without the lids) were placed in a sterile tissue culture incubator, which was perfused with 95%N_2/5% CO_2 for 24 hours at 37°C. The atmospheric air inside the incubator was removed by overnight perfusion with 95% N_2/5% CO_2. Oxygen level was found to be <3% as measured before and after the experiment using FYRITE® Gas analyzer O_2 Indicator (Bacharach Inc, Pittsburgh, PA). Control cells from an identical passage of cells were incubated simultaneously in a regular tissue culture incubator at 95% air/5% CO_2 at 37°C for 24 hours. Following the incubation period, immunoreactive ET-1 released into the culture media following hypoxia (± TNF-α, phosphoramidon, etc.) was measured using an ELISA kit. Independent experiments were performed to determine hONA cell proliferation following hypoxia (± TNF-α, ET-1, etc.) using the MTT-formazan assay.

Endothelin-1 ELISA:

Concentration of immunoreactive ET-1 (ir-ET-1) in cell-culture supernatants from hONAs treated under various conditions was determined using a commercially available ET-1 ELISA kit (Alpco, Windham, NH). The assay involves use of a polyclonal capture antibody specific for ET-1 (1-21) coated on a microtiter plate and a monoclonal detection

antibody added to each well along with the samples. After binding of ET-1 between the two antibodies, a peroxidase- conjugated antibody that detects the bound detection antibody is added, followed by tetramethylbenzidine as the substrate. The enzyme catalyzed color change is detected at 450nm, the absorbance being directly proportional to the concentration of ET-1 in the sample. For this experiment, hONAs were seeded on 96 well tissue culture plates and allowed to grow to a confluence in DMEM with 10% FBS. The medium was changed to serum free (SF-DMEM) overnight before the experiment. At time zero, 200μl of fresh SF-DMEM containing respective agonist/antagonist (e.g. 10nM TNF-α , 1 μM phosphoramidon etc) was added to each well. For inhibitor treatments, the cells were incubated with the respective antagonist 30 minutes (1 hour in case of Actinomycin D and Cycloheximide) before adding TNF-α. The plates were then placed in an incubator perfused with 95% N_2 and 5% CO_2 for hypoxia and normoxia control plate was placed simultaneously in an incubator with 95% Air-5% CO_2.

At the end of the treatment period, media from each well were collected in separate microfuge tubes and centrifuged at 3000 rpm to separate any cells that might be present in the media. ET-1 ELISA was performed using these cell culture media according to the manufacturer's instructions. Absorbance was measured at 450nm using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA). The absorbance corresponded to the concentration on ET-1 in the samples, which was obtained from a standard curve. Concentrations were plotted as percent of control.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (Q-RT-PCR) for Preproendothelin-1 (ppET-1) mRNA:

Total RNA was isolated as previously described (Prasanna et al., 2002). Briefly, total RNA was isolated from hONAs subjected to normoxia or hypoxia and in the absence and presence of TNF-α (10 nM),using Trizol-B reagent (Life Technologies) (phenol/chloroform/ethanol extraction) according to the manufacturer's protocol. All cDNA syntheses were carried out at 42°C for 30 min with 5 μg of total RNA using random primers and AMV reverse transcriptase from Promega (Madison, WI). Real time quantitative PCR to measure the mRNA expression of ppET-1 was performed using SYBR® Green PCR Core Reagents Kit (PE Biosystems, Warrington, UK) with a Cepheid Smart Cycler System (Version 1.2b). ß-Actin expression was used as internal control to normalize ET-1 expression. The reaction is based on the quantification of PCR product by measuring the increase in fluorescence as SYBR Green Dye binds to newly synthesized double stranded DNA. Previously published primer pairs were used for detection of mRNA expression of ppET-1 and ß-Actin (Zhang et al., 2003). The PCR product size for pET-1 was 180 bp and ß-Actin product size was 514 bp. Oligos were purchased from Sigma Genosys, TheWoodlands, TX, USA; the sequences are as follows:

ET-1 Sense Primer: TATCAGCAGTTAGTGAGAGG;

ET-1 Antisense Primer: CGAAGGTCTGTCACCAATGTGC;

ß-Actin Sense Primer: 5'-TGTGATGGTGGGAATGGGTCAG-3'

ß-Actin Antisense Primer: 5'-TTTGATGTCACGCACGATTTCC-3'

The PCR reaction (25µl) contained 2.5µl template and 3mM $MgCl_2$, 5µM of each primer, TaKaRa Ex Taq™ R-PCR DNA Polymerase (5 units/µl), dNTP mix and 10X SYBR® Green I buffer. The amplification was performed with a hot start of 95°C for 2 min followed by 50 cycles at 95°C for 30sec, 56°C for 30sec and 72°C for 30 sec. 'NoTemplate' Blank (Sterile Water) was used as a negative control. Also, 'NoEnzyme' control was used to verify that the dye did not bind to any other component of the reaction mixture except newly formed double stranded DNA.

Immediately after the real-time PCR run, the melting curves were generated to detect the melting temperatures of the specific products (data not shown). The quantitative analysis of PCR products was carried out based on the automatically created baseline of the amplification plots, exponential phase, and plateau in a logarithmic plot of cycle number and ΔRn. The algorithm from the graph calculates the cycle at which each PCR amplification reaches a significant threshold (Ct; i.e., usually ten times the standard deviation of the baseline). The calculated Ct values are a quantitative measurement for the mRNA levels of various genes tested. Quantification of relative ET-1 mRNA expression was achieved by using the comparative Ct method and was normalized to that of β-actin mRNA expression (as described in PE Biosystems User Bulletin#2: http://docs.appliedbiosystems. com/pebiodocs/04303859.pdf). Q-RT-PCR data are presented as the mean percentage to the value of its corresponding untreated control. The relative value of treated eye versus control eye (set at 100%) was calculated. The Q-RT-PCR product purity was also confirmed by analyzing the products by 2% agarose gel electrophoresis (data not shown).

Formazan Cell Proliferation Assay:

The formazan assay was performed as described previously (Prasanna et al., 2002). A commercially available CellTiter 96 Aqueous one-solution cell proliferation assay with the tetrazolium compound MTS (Promega, Madison, WI), was used to evaluate ET-1's mitogenic effects on hONAs. The MTS tetrazolium compound is bioreduced to formazan by NADPH or NADH produced by metabolically active dehydrogenase enzymes of cells and this can be detected at 490 nm. Following cell seeding (2,000 cells/well in quadruplicate wells for each treatment) the cells were maintained in serum containing DMEM overnight. The following day medium was changed to SF-DMEM and allowed to stand overnight. On the day of the experiment, hONA were treated with fresh SF-DMEM containing with ET-1 (100 nM) for a period of 24 hours. In some experiments, hONAs were also pre-treated with 1 μM of PD142893, an $ET_{A/B}$ mixed receptor antagonist prior to the incubation with ET-1.

Following treatments, the culture media was discarded and to each well 100 μl of fresh serum-free DMEM along with 20 μl of CellTiter 96 Aqueous One Solution was added and incubated at 37°C for 30 minutes. The 96-well plate was then placed in a kinetic microplate reader (Molecular Devices, Sunnyvale, CA) and the absorbance was read at 490 nm. In order to determine the actual cell number from the absorbance values of the experimental samples, a standard curve was generated with known numbers of hONAs seeded per well. Cells grown in serum-containing DMEM were used as a positive control while those grown in SF-DMEM were used as a negative control.

Results:

Hypoxia and Cell Morphology in Cultured Human Optic Nerve Head Astrocytes (hONAs)

Cultured hONAs grown on tissue culture plates were exposed separately to experimental hypoxia and normoxia for 24 hours and gross changes in cell morphology was examined qualitatively under both conditions. From Figure 1, it can be seen that no dramatic change in overall morphology could be observed and the cells appeared healthy in both conditions. Normally, hONAs appeared as flat and polygonal process-bearing cells, which under hypoxia conditions appeared to have fewer processes compared to that seen in normoxia.

TNF-a-Mediated ET-1 Release From hONAs is Augmented Following Hypoxia

Brain astrocytes when subjected to experimental hypoxia/ischemia over express either mRNA or protein levels of cytokines like IL-1α and TNF-α (Yu and Lau, 2000) as well as vasoactive peptides, including ET-1 (Ho et al., 2001). Yuan and Neufeld (2000) have shown that the optic nerve heads of patients with primary open angle glaucoma exhibit elevated levels of TNF-α. Therefore, it was determined if a relationship existed between hypoxia and TNF-α on ET-1 release from hONAs following experimental hypoxia.

Previously it was shown that the cytokine, TNF-α was a potent stimulator of ET-1 release from various ocular cells following treatment for 24 hours (Prasanna et al., 1998;

Narayan et al., 2003). Therefore, a similar 24-hour time point was chosen and hONA were subjected to TNF-α treatment either in normoxia or hypoxia conditions and immunoreactive ET-1 (ir-ET-1) released into the culture media was detected by ELISA. Under normoxic conditions, TNF-α caused a 2.6-fold increase in ir-ET-1 levels from hONA cells, which was found to be statistically significant. Interestingly, TNF-α-mediated ir-ET-1 levels were significantly increased by 5-fold following exposure to hypoxic conditions (**Figure 2**). There was no change in basal release of ir-ET-1 in control cells exposed either to normoxia or hypoxia. In order to determine if TNF-α's effects on ET-1 release were occurring due to its actions on endothelin converting enzyme (ECE), hONAs were pre-incubated with phosphoramidon (ECE inhibitor) followed by TNF-α. Phosphoramidon almost completely blocked TNF-α-mediated ET-1 release from hONA cells under normoxia and hypoxia, while having no effects on its own (**Figure 2**,).

In order to ascertain if the augmented rise in ET-1 levels following TNF-α under hypoxia was due to *de novo* transcription and translation of preproET-1 mRNA, hONAs were treated separately with TNF-α in the presence and absence of actinomycin-D (5 μM; Act-D), a transcriptional inhibitor and cycloheximide (10 μM; CHEX), a protein synthesis inhibitor. The cells were pretreated with these inhibitors for 1 hour prior to exposure to hypoxia and normoxia for 24 hours. Based on **Figure 3**, it can be seen that under both normoxia and hypoxia, TNF-α-induced ir-ET-1 release was blocked by Act-D and CHEX pre-treatment. However, basal ir-ET-1 levels did not change in the presence of these inhibitors alone. TNF-α induced cell proliferation may contribute only

minimally to the increased ET-1 secretion from hONAs because ET-1 synthesis from newly formed cells will be at later time points since the S-phase of the cell cycle requires about 16 hours.

Q-RT-PCR Analysis of Preproendothelin-1 (ppET-1) mRNA

Since an increase in amount of ET-1 levels in culture media was observed following 24-hour TNF-α treatment, which was significantly augmented following hypoxia and that this was due to *de novo* transcription and translation, it was determined using Q-RT-PCR if a similar change could be observed in the mRNA levels on hONAs under these conditions. Based on **Figure 4**, it can be observed that in normoxia, TNF-α treatment for 24 hours caused a significant 32% increase in ppET-1 mRNA levels over the control levels (set at 100%). However, in case of hypoxia, TNF-α did not cause any apparent increase in ppET-1 mRNA levels compared to control levels. Hypoxia alone did not increase ppET-1 mRNA and this was similar to that observed for ET-1 protein levels.

Hypoxia Potentiates TNF-α and ET-1-Induced hONA Proliferation

Hypoxia is considered as a strong stimulator of astrocyte proliferation and hypertrophy, resulting in astrogliosis and nerve damage (Brambilla and Abbracchio, 2001). It is also known that TNF-α and ET-1 are potent mitogens for hONAs. Therefore it was determined if hypoxia can further influence hONA cell proliferation in the presence of these agonists. The figures shown are representative data from one experiment run in quadruplicate, in which all treatments were carried out simultaneously on equal number of cells in one 96 well plate for each normoxia and hypoxia. Each

experiment was repeated at least 3 times using cells seeded on regular uncoated plastic cell culture plates and two times on collagen coated plates with cell passages ranging from 6 to 12. As seen from **Figure 5**, in normoxia conditions, both ET-1 and TNF-α treatment for 24 hours caused a significant increase in hONA proliferation by 20% and 40% over control (set at 100%) respectively. In case of hypoxia exposure, both ET-1 and TNF-α caused statistically significant augmentation of cell proliferative response. The augmentation of ET-1 release under hypoxia ranged between 10-20% in different sets of experiments. Albeit small, ET-1-mediated hONA proliferation in hypoxia was 25% greater ($p<0.001$) than control while that for TNF-α was much greater and was 80% over control (**Figure 5**, $p<0.001$). There was a statistically significant effect of hypoxia on the mitogenic actions of ET-1 and TNF-α compared to those observed under normoxia (**Figure 5**, $ symbol, $p = 0.003$ for ET-1, $p<0.001$ for TNF-α). Interestingly, 24-hour hypoxia condition by itself did not stimulate hONA proliferation nor did it cause cell death, as the formazan assay absorbance values for normoxia and hypoxia controls were similar.

Initial experiments were performed using hONAs seeded on regular uncoated plastic cell culture plates, whereby a similar trend of hONA proliferation was observed. ET-1 induced increase in cell numbers ranged between 14-23% over baseline under normoxia and 25-35% under hypoxia. TNF-α induced proliferation ranged between 140-150% over baseline under normoxia and 80-90% under hypoxia (data not shown). The disparity in cell numbers may be attributed to the 20-25% cell death observed after 24 hours of hypoxia when cells were seeded on plastic surface (data not shown). Later

experiments were performed using collagen I coated culture plates as no significant reduction in cell numbers were seen when hONA were subjected to hypoxia for 24 hours when grown on collagen.

In order to demonstrate that the effects of ET-1 were occurring via the activation of ET_A and ET_B receptors, hONA cells were pre-treated with PD142893 (1 μM), a mixed receptor antagonist followed by ET-1 (100 nM). ET-1's effects on hONA cell proliferation were completely blocked both under normoxia and hypoxia (**Figure 5**, ** and $^{\#\#}$ symbols for PD + ET-1). Treatment of hONAs with PD142893 alone did not produce any effects. However since it was shown in Figures 2 and 3 that hONAs could be stimulated by TNF-α to secrete ET-1, it was also likely that this secreted ET-1 could also influence TNF-α's actions on hONA proliferation. Therefore, in order to delineate TNF-α-mediated, ET-1 release effects from TNF-α's ET-independent effects on hONA proliferation, hONA cells were treated with PD142893 followed by TNF-α. It can be seen that under normoxia, PD142893 completely blocked TNF-α-induced hONA proliferation compared to TNF-α alone (**Figure 5**, ** symbol for PD + TNF, $p<0.001$). However, in case of hypoxia, PD142893 did not completely block TNF-α-mediated hONA proliferation compared to control but decreased it significantly compared to TNF-α alone (**Figure 5**, $^{\#}$ and $^{+}$ symbols for PD + TNF for hypoxia, $p<0.001$).

Proliferation of hONAs by ET-1 and TNF-α Treatment is Dependent on Mitogen Activated Protein Kinases (MAPKs)

MAPK immunostaining has been recently shown to be increased in glaucomatous human retinas as compared to non-glaucomatous retinas and this increased immunostaining is associated mainly with hypertrophic astrocytes and Muller cells (Tezel et al., 2003). Similarly, ET-1-mediated brain astrocyte proliferation involves the activation of p42/44 MAPKs (Lazarini et al., 1996; Stanimirovic et al., 1995). Therefore it was presently determined if a similar MAPK pathway is activated following ET-1 and TNF-α-induced hONA proliferation. Since an augmented proliferation response was observed in hypoxia condition following treatment with ET-1 and TNF-α, the role of hypoxia and MAPK activation was further explored using an upstream MAPK inhibitor, U0126 (10 μM). Under normoxia, U0126 completely blocked ET-1-induced hONA proliferation (**Figure 6**, ** symbol for U0126 + ET-1 in normoxia; $p = 0.004$ compared to ET-1), however in hypoxia, U0126 was unable to block ET-1-induced hONA proliferation (**Figure 6**, $p = 0.634$ versus ET-1). In case of TNF-α treatment, unlike that for ET-1, U0126 partially blocked TNF-α-induced hONA proliferation both in normoxia and hypoxia (**Figure 6**, $p<0.001$ versus TNF-α).

Discussion:

In the present study it has been demonstrated for the first time that cultured human optic nerve head astrocytes (hONAs) synthesize and release endothelin-1 (ET-1) and that ET-1 levels (both mRNA and protein) can be elevated significantly by TNF-α. Also being reported for the first time is the finding that TNF-α-induced ET-1 release from hONAs is augmented by hypoxia. As seen with reactive brain astrocytes, the mitogenic role of ET-1 and TNF-α acting on hONAs either as autocrine or paracrine agonists is also augmented following exposure to hypoxia. The involvement of mitogen activated protein kinase (MAPK) signaling pathway with ET-1 and TNF-α, particularly, p42/44 MAPK has also been shown for the first time in hONAs.

ET-1 mRNA expression has been previously shown in astrocytes of the retina as well as optic nerve both in tissues and cultures, suggestive of astrocytes being "ET-source" cells (Ripodas et al., 2001; Prasanna et al., 2002). Presently, an increase in ET-1 protein levels in hONA culture media was observed only in the presence of TNF-α, which was significantly enhanced in case of hypoxia. In hONAs, hypoxia for 24 hours by itself did not affect basal ET-1 levels, similar to the findings of Hasselblatt et al. (2001) in rat brain astrocytes who also found no change in ir-ET-1 levels even up to 15 hours of hypoxia. Hasselblatt et al. (2001) attributed this lack of ir-ET-1 increase in hypoxia to the role of ET_B receptors as clearance receptors for ET-1, which get upregulated in hypoxia (Shibaguchi et al., 2000; Hasselblatt et al., 2001). In the present study, the level of ET_B expression (mRNA and protein) in hONAs subjected to normoxia was not evaluated. However in contrast to our findings, in neonatal rat brain astrocytes a 1.5 fold increase

and in murine embryonic cortical astrocytes a 2-fold increase in ir-ET-1 levels were observed following 24 hour hypoxia (Schmidt-Ott et al., 2001; Ho et al., 2001). Also in hONA exposed to normoxia and hypoxia, there was no change in preproendothelin-1 (ppET-1) mRNA levels as assessed by Q-RT-PCR which was also in contrast to the findings of Ho et al. (2001) and Schmidt-Ott et al. (2001). A possible reason as to why there was no apparent change ppET-1 mRNA level in hONA could be attributed partly due to the instability of ppET-1 mRNA itself (Inoue et al., 1989). Moreover the lack of inhibition of Act-D and CHEX on basal ppET-1 mRNA and on basal ET-1 release from hONA suggests that tonic production of ET-1 may occur due to the enzymatic conversion of abundant levels of pre-made ppET-1 into ET-1 perhaps involving an ECE-dependent mechanism.

With regards to TNF-α-induced ET-1 synthesis and release in hONAs, it is known from previous reports that TNF-α is a potent stimulator of ET-1 synthesis and secretion in several cell types including those of the eye (Prasanna et al., 1998; Narayan et al., 2003). In ciliary non-pigmented epithelial cells TNF-α stimulation induced ET-1 secretion via *de novo* ppET-1 synthesis, and the process involved the endothelin converting enzyme (ECE) system (Prasanna et al., 1998). In the present study, it appears that *de novo* ppET-1 transcription and translation processes are activated both in normoxia and hypoxia and that TNF-α acts in an ECE-dependent manner. This is supported by the finding that ppET-1 message does not change while ET-1 levels increase several fold. Rat cortical astrocytes exposed to acute hypoxia resulted in an increase in ECE-1 immunostaining (Ehrenreich et al., 1999). Surprisingly, an increase in

ppET-1 mRNA levels was observed only for TNF-α treatment in normoxia condition while no such increase was seen in case of hypoxia even though a 500% increase in ET-1 protein levels were detected. This could be due to an increased rate of translation of ppET-1 mRNA into protein, which coupled with the instability factor, is reflected as if there was no increase in ppET-1 mRNA as seen in Q-RT-PCR assay.

In some early reports, Minckler and Spaeth (1981) observed astrocytic hyperplasia (proliferation) in human optic nerve heads associated with early to moderate stages of glaucoma, specifically at the prelaminar and pars scleralis regions. It has been shown in the Morrison rat model of glaucoma that ONAs proliferate, migrate, and exhibit extensive astrogliosis in the optic nerve (Johnson et al., 2000). In glaucomatous human optic nerve heads, hypertrophic astrocytes have been detected (Hernandez, 2000) and exposure of hONAs to elevated hydrostatic pressure, results in migration of ONAs (Tezel et al., 2001), suggesting that a change in morphology appears to be necessary for astrocyte activation. However in cultured brain astrocytes, there is no significant change in astrocyte morphology when subjected to experimental hypoxia (Schmidt-Ott et al., 2001), although in a rat model of hypoxia, astrocytic processes are withdrawn from associated dendrites, resulting in neuronal deterioration (Goshgarian and Yu, 1990). In the present study, only subtle changes in morphology were observed in hONAs subjected to 24-hour hypoxia. Fewer astrocytic processes and a more spindle shaped phenotype were observed as compared to that seen in normoxia cultures. Under 24-hour hypoxia condition, there was also no cell death observed for hONAs seeded on collagen (data not shown). Perhaps a combination of hypoxia/hypoglycemia could have caused cell death in

these cells. ET-1 causes a significant alteration of cytoskeletal organization in brain astrocytes (Koyama and Baba, 1996), promotes tyrosine kinase phosphorylation of focal adhesion kinase and paxillin (Koyama et al., 2000) and reverses dibutryl cAMP-induced astrocyte stellation (Schmidt-Ott et al., 2001). These morphological changes are probably responsible for astrocyte proliferation, migration, and hypertrophy in the glaucomatous optic nerve head. It is of interest to note that neurons also influence astroglial proliferation and if the neuron:astroglia ratio is four to one then glial proliferation is inhibited but in conditions where the ratio is lower, then glia are free to proliferate (Hatten et al., 1991). Therefore in case of glaucoma wherein retinal ganglion cell loss is accompanied by axon loss, the likelihood of astroglia to proliferate is quite high which may initiate astrogliosis early on in glaucoma.

ET-1 (100 nM) causes hONA proliferation, suggesting that exogenous ET-1 (probably from retinal pigmented epithelium and other retinal sources) could act as a mitogen for hONAs (Prasanna et al., 2002). While ET-1's contribution to hONA proliferation under hypoxia was a modest 25% over baseline, TNF-α's ability to augment this proliferative response up to 80% over baseline in hypoxia is interesting. TNF-α is also known to cause astrocyte proliferation in bovine brain astrocytes (Selmaj et al., 1990; 1991) and human astrocytic cell lines (Barna et al., 1990) but not in human fetal brain astrocytes (Moretto et al., 1993). In glaucomatous human optic nerve heads an increase in TNF-α, its receptor TNFR1, and an associated induction of nitric oxide synthase-2 (known to promote neurodegeneration) are all observed in ONAs (Yuan and Neufeld, 2000). Recently, a significant association of the TNF-α allele-308

polymorphism with POAG patients in Chinese populations has been reported, making it a potentially important candidate marker for glaucoma (Lin et al., 2003). Considering that glaucoma is a progressive optic neuropathy, chronic glial activation (which presumably commences with proliferation, migration, and ends with hypertrophy) could occur in the presence of constantly elevated levels of ET-1 and TNF-α. These actions could be manifested via combinatorial signaling cascades via direct effects on astroglial activation and/or on optic nerve circulation. The possibility of an ET-1-induced feedback mechanism involving changes in pO_2 levels in the circulation is therefore likely to further induce astroglial activation.

As reported previously in normoxia cultures of hONAs, both ET_A and ET_B receptors contributed to ET-1-induced cell proliferation and a mixed ET receptor antagonist, PD142893 was used to block both receptors (Prasanna et al., 2002). ET-1's mitogenic actions under hypoxia were blocked by PD142893, suggesting that both ET_A and ET_B receptors may participate (Prasanna et al., 2002). PD142893 also completely blocked TNF-α's mitogenic effects in normoxia, suggesting that TNF-α's actions were likely mediated via ET-1 release and the resultant ET-1-mediated hONA proliferation. However in case of hypoxia, TNF-α was found to initiate discrete/direct mitogenic signaling effects since only a partial blockade was offered by PD142893 on TNF-α-induced hONA proliferation.

Both ET-1 and TNF-α-associated signaling in astrocytes have been shown to involve the mitogen activated protein kinase (MAPK) pathway, specifically involving p42/44 MAPK activation (Marcus et al., 2003; Lazarini et al., 1996). In astrocytes,

activation of p42/44 MAPK pathway in most cases results in cell proliferation and are also associated with neuronal injury and repair following hypoxia/ischemia (Wang et al., 2003; Carbonell and Mandell, 2003; Teixeiria et al., 2001). Presently, the involvement of p42/44 MAPK in hONA proliferation under normoxia and hypoxia following ET-1 and TNF-α treatment was implicated using a MEK1/2 inhibitor, U0126. ET-1's effects on cell proliferation were blocked by U0126 only under normoxia but not under hypoxia. But U0126 treatment partially blocked TNF-α-induced hONA proliferation both in normoxia and hypoxia. Collectively, these data suggest that p42/44 MAPK signaling is activated in both conditions by TNF-α but only under normoxia by ET-1. In hypoxia, ET-1's mitogenic effects on hONAs while being ET receptor mediated appear to involve hitherto unidentified signaling cascades and may include intracellular calcium (MacCumber et al., 1990; Supattapone et al., 1989) and p38 MAPK (Schinelli et al., 2001).

The role of ET-1 in astroglial proliferation and astrogliosis is receiving increasing attention since this peptide is elevated in reactive astrocytes in many neurological disorders including Alzheimer's disease, brain infarcts, and neurotrauma (ischemia, stroke, hemorrhage etc.) (Nie and Olsson, 1996). Normally astrocytes are quiescent and do not express elevated levels of ET and its related proteins including $ET_{A/B}$ receptors and ECE), however in response to pathophysiological stimuli, there is a dramatic increase in the levels of ET components which results in reactive astrogliosis (Jiang et al., 1993; Nie and Olsson, 1996). Specifically in clinical situations and in ischemia models, decreased cerebral blood flow leads to the elevation of ET-1 levels, mainly in astrocytes

and is thought to contribute to neuropathy (Jiang et al., 1993; Lampl et al., 1997; Macrae et al., 1993; Viossat et al., 1993). In glaucoma, while the exact amount of ET-1 in the optic nerve has never been measured thus far in humans, the immunolabeling for ET-1 is significantly greater in optic nerve head of an experimental rat model of glaucoma exposed to elevated pressure compared to that seen in controls (Prasanna et al., 2003, submitted). Also the increase in ET-1 immunolabeling is mainly associated with GFAP-positive ONAs (Prasanna et al., 2003, submitted). Furthermore, several studies have shown that constant perfusion or intravitreal injection of low doses of ET-1 to the optic nerve resulted in axon loss, gliosis, and reduction of blood flow to the optic nerve (Cioffi et al., 1995; Orgul et al., 1996; Oku et al., 1999). Circulating ET-1 levels are elevated in normal tension glaucoma patients which could be associated with vasospasms in optic nerve head microvasculature and is further suggestive of a role for ET-1 and hypoxia/ischemia in glaucoma (Flammer et al., 2001; Cellini et al., 1997; Sugiyama et al., 1995). In the present study, since TNF-α in hypoxia conditions is able to significantly increase ET-1 release from hONAs, it is also likely that this ET-1 released can further promote vasospasms and ischemia/reperfusion in the capillaries of the posterior ciliary artery and central retinal artery located at the lamina cribrosa and optic nerve head.

In conclusion, it has been shown for the first time that hypoxia augments TNF-α and ET-1 responses in optic nerve head astrocytes, by way of increasing ET-1 synthesis and release with a resultant mitogenic effect. A common denominator underlying optic nerve damage in glaucoma therefore could be reactive ONAs since their localization

makes them susceptible to mechanical and ischemic influences in addition to their responsiveness to ET-1 and TNF-α.

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Figure Legends:

Figure 1. Cultured human optic nerve head astrocytes (hONAs) exposed to normoxia or hypoxia/ischemia for 24 hours. The overall morphology of hONAs for up to 24 hours of hypoxic conditions appears to be similar to that seen in normoxia however the cells exposed to hypoxia appear to have fewer astrocytic processes (Arrowheads). Primary hONAs were seeded at similar densities (2,000 – 3,000 cells/well) in serum containing DMEM and later switched to serum-free DMEM and cultured either in an incubator at 37°C with 5%CO_2 – 95% air for normoxia conditions or 5%CO_2 – 95% N_2 for hypoxia conditions. A 20X objective lens was used to capture the DIC images.

Figure 2. Release of endothelin-1 (ET-1) from primary human optic nerve head astrocytes (hONAs) into the culture media as determined by ELISA. Hypoxia (24 hours) causes a significant increase in TNF-α-mediated immunoreactive ET-1 (ir-ET-1) levels in the culture media compared to that seen under normoxia. Cultured hONAs (5000 cells/well) were initially seeded in a collagen-coated 96-well plate and subjected to hypoxia (95% N_2/ 5% CO_2 at 37°C) in serum-free conditions for 24 hours in the presence or absence of TNF-α (10 nM) and phosphoramidon (1 μM; endothelin converting enzyme inhibitor). Another set of cells was exposed to normoxia for 24 hours. Cells were pre-incubated with phosphoramidon for 30 minutes prior to the addition of TNF-α. * and $^{+}$ Denote statistical significance of TNF-α-mediated ir-ET-1 levels under hypoxia and normoxia versus their respective controls. ** and $^{++}$ Denote statistical significance of ir-ET-1 levels following TNF-α treatment compared to those following phosphoramidon

alone or phosphoramidon ± TNF-α under normoxia and hypoxia respectively. *** Denotes statistical significance of TNF-α-mediated ET-1 release between normoxia and hypoxia. Statistical significance for all conditions were determined by One-way ANOVA and Student Newman Keuls multiple comparison test ($p<0.001$) (n = 4 wells/treatment).

Figure 3. TNF-α-mediated endothelin-1 release from cultured primary human optic nerve head astrocytes (hONAs) is dependent on *de novo* transcription and translation. Cultured hONAs seeded on a collagen-coated 96-well plate were pre-treated separately with protein synthesis inhibitors, actinomycin-D (Act-D; 5 μM) and cycloheximide (CHEX; 10 μM) for 1 hour followed by TNF-α (10 nM) and exposed to normoxia and hypoxia for 24 hours. Culture media was collected and ir-ET-1 levels were quantitated by ELISA. * and $^{+}$ denote statistical significance of ir-ET-1 levels following TNF-α treatment under normoxia and hypoxia compared to respective controls. ** and $^{++}$ denote statistical significance of ir-ET-1 in the presence of TNF-α compared to other treatments (Act-D, CHEX, Act-D + TNF-α, and CHEX + TNF-α) under normoxia and hypoxia. Statistical significance for all conditions were determined by One-way ANOVA and Student Newman Keuls multiple comparison test ($p<0.001$) (n = 4 wells/treatment).

Figure 4. Quantitative RT-PCR analysis of preproendothelin-1 (ppET-1) mRNA levels in hONAs treated with TNF-α (10 nM) in normoxia and hypoxia for 24 hours. Cultured hONAs were seeded in 100-mm dishes (n = 3 per condition) and subjected to hypoxia as described in the Materials and Methods section. Following treatments, cDNA

was synthesized from total RNA extracted from hONAs and Q-RT-PCR was performed using primers against human ppET-1 and β-actin (used as a control gene).

Figure 5. Effect of hypoxia on endothelin-1 (ET-1) and TNF-α-mediated cell proliferation of cultured human optic nerve head astrocytes (hONAs). Cultured hONAs seeded on collagen-coated 96 well plates (1,000 cells/well) were treated serum-free DMEM containing ET-1 (100 nM) or TNF-α (10 nM) in the presence and absence of PD142893 (an ET_A/ET_B receptor antagonist) in normoxia or hypoxia for 24 hours. Cell proliferation was assessed using the MTT-formazan assay as described in the Materials and Methods section. Columns representing normoxia and hypoxia controls and ET-1 and TNF-α treatments in this figure represent the same data set as shown in figure 6. * Denotes statistical significance of % hONA cell proliferation of ET-1 and TNF-α versus respective controls *under normoxia* while $^{\#}$ denotes the same for ET-1 and TNF-α treatments versus their respective controls *under hypoxia*. ** Denotes statistical significance of hONA cell proliferation of agonists (ET-1 and TNF-α) *under normoxia* versus other treatments (PD142893, PD + ET-1, and PD + TNF-α) while $^{\#\#}$ denotes the same *under hypoxia*. $^{\$}$ Denotes statistical significance of agonist-induced hONA proliferation in hypoxia versus normoxia and $^{+}$ denotes statistical significance of TNF-α versus PD + TNF-α *under hypoxia*. Statistical significance for all conditions were determined by One-way ANOVA and Student Newman Keuls multiple comparison test ($p<0.001$) (n = 4-8 wells/treatment).

Figure 6. Effect of mitogen activated protein kinase (MAPK) inhibitor, U-0126 (10 μM) on ET-1 and TNF-α-induced hONA cell proliferation under normoxia and hypoxia as determined by MTT-Formazan assay. Cultured hONAs seeded on collagen-coated 96-well plates (1,000 cells/well) were serum starved and treated with agonists, ET-1 and TNF-α in the presence and absence of U-0126 for 24 hours in normoxia and hypoxia. Columns representing normoxia and hypoxia controls and ET-1 and TNF-α treatments in this figure represent the same data set as shown in figure 5. * Denotes statistical significance of % hONA cell proliferation *under normoxia* following agonist treatment while # denotes the same except *under hypoxia* treatment. ** Denotes statistical significance versus agonist treatments *under normoxia* whereas ## denotes the same under *hypoxia condition.* *** Denotes statistical significance of U0126 versus U0126 + TNF *under normoxia* while ### denotes the same *under hypoxia.* Statistical significance for all conditions were determined by One-way ANOVA and Student Newman Keuls multiple comparison test ($p<0.001$) ($n = 4$-8 wells/treatment).

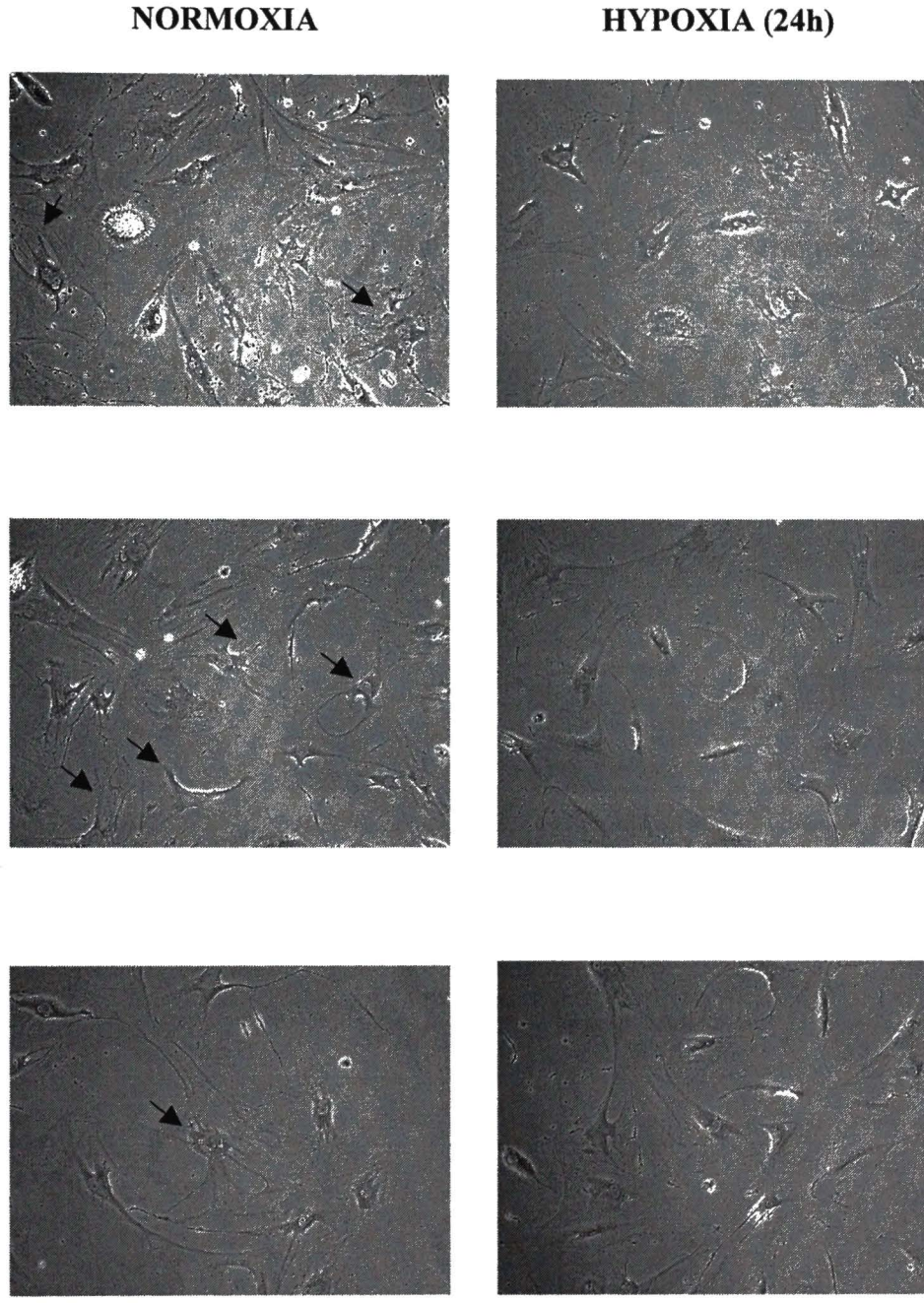
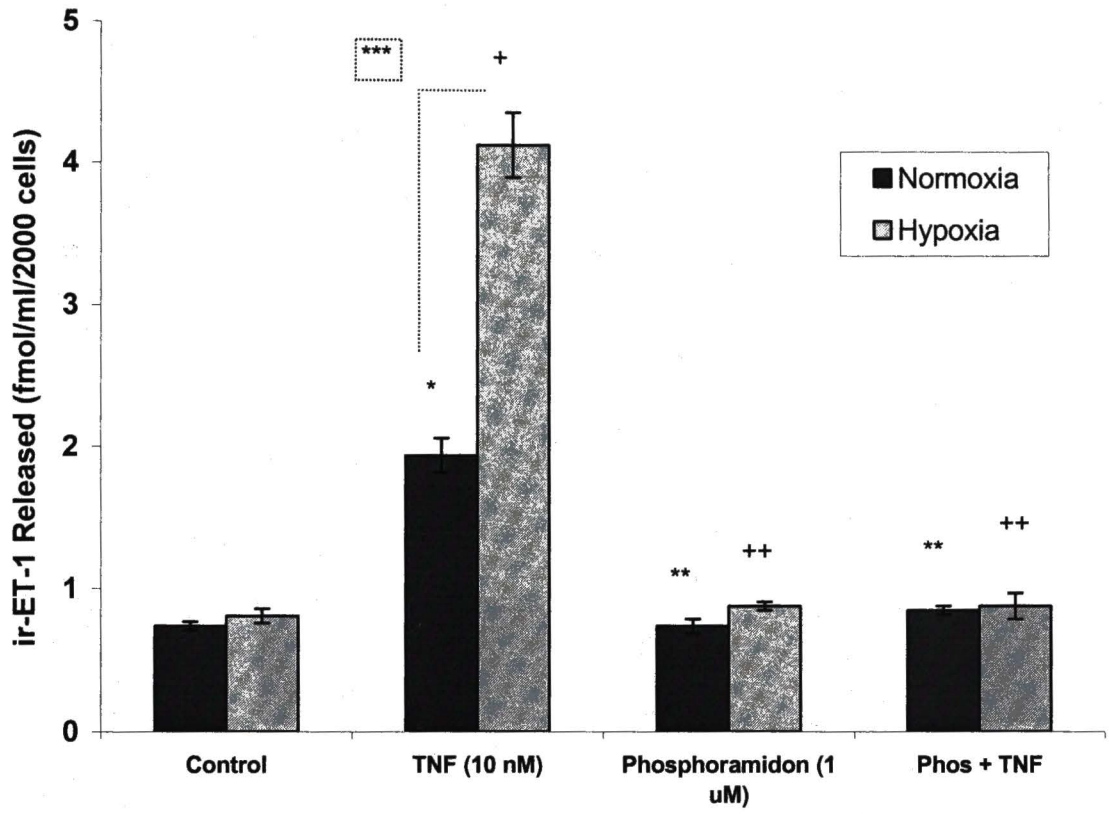


Figure 1.


ir-ET-1 Released (fmol/ml/2000 cells)
5
4
3
2
1
0
***
+
*
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++
**
++
Normoxia
Hypoxia
Control
TNF (10 nM)
Phosphoramidon (1 uM)
Phos + TNF

Figure 2.

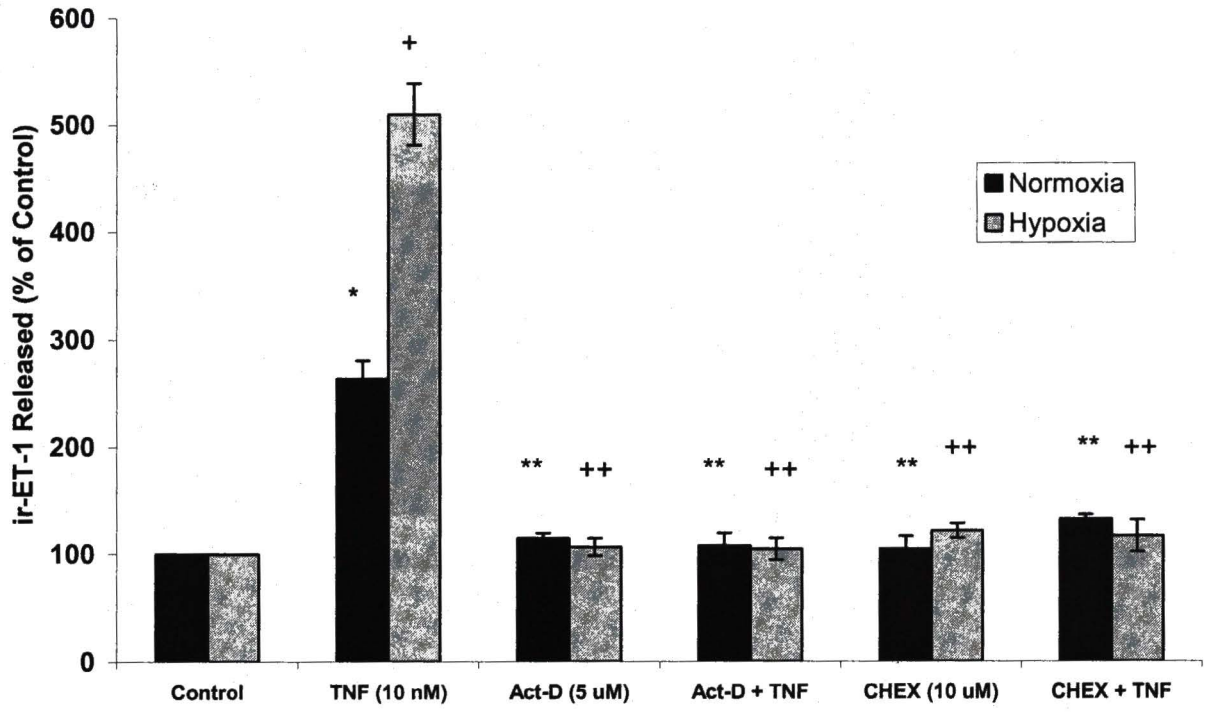

600
500
400
300
200
100
0
ir-ET-1 Released (% of Control)
Normoxia
Hypoxia
*
+
**
++
**
++
**
++
**
++
Control
TNF (10 nM)
Act-D (5 uM)
Act-D + TNF
CHEX (10 uM)
CHEX + TNF

Figure 3.

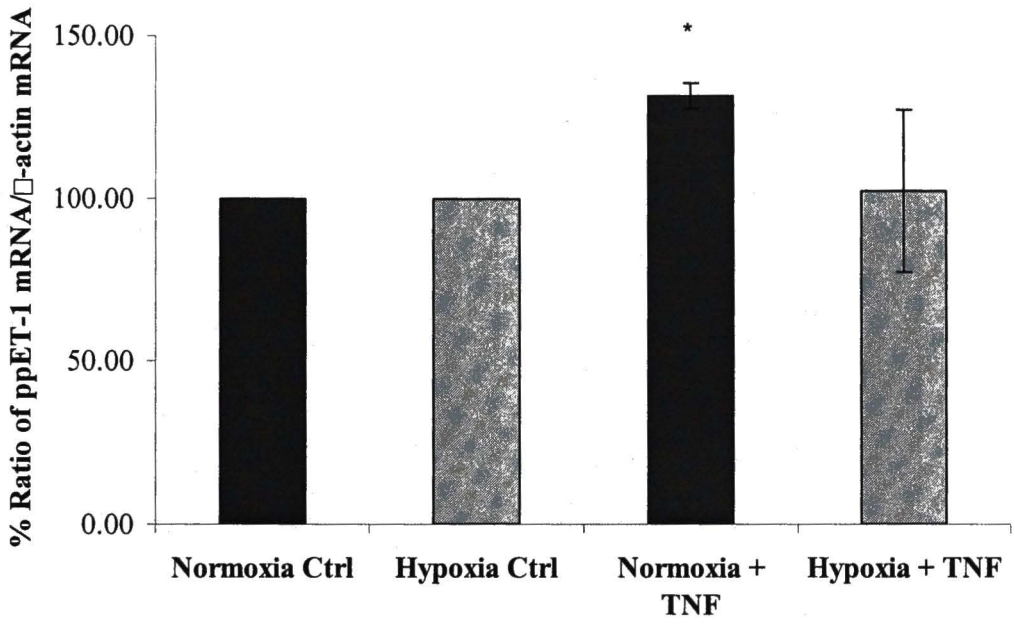
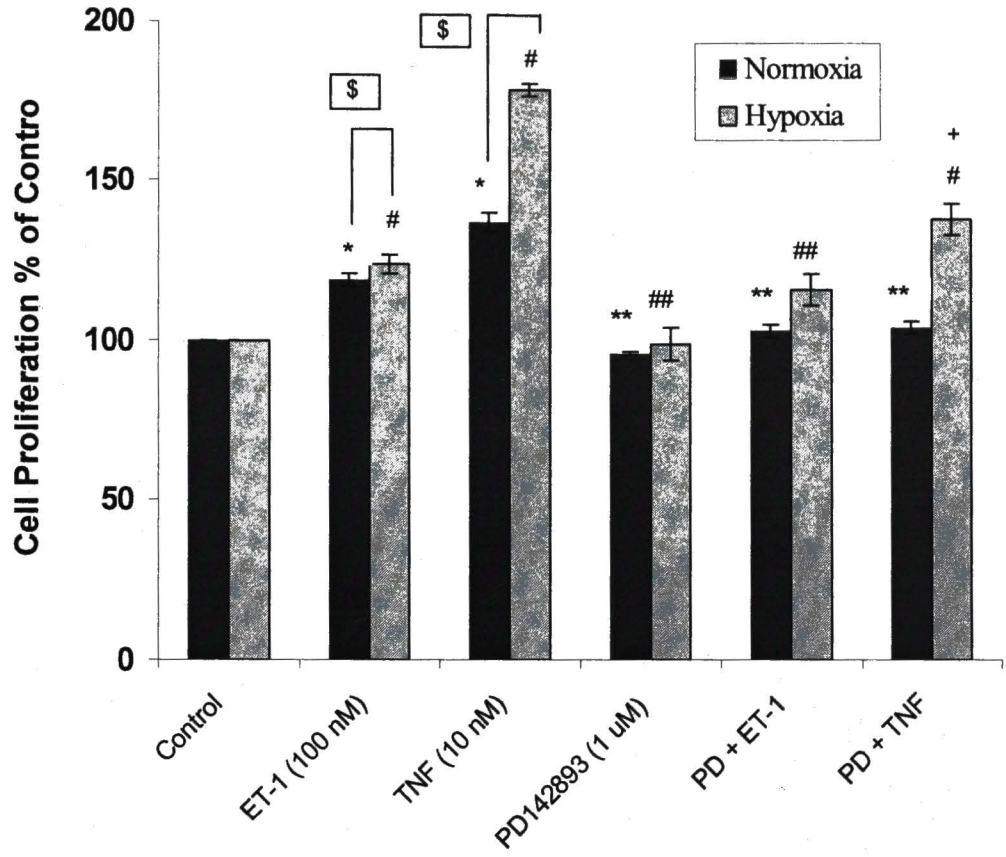

% Ratio of ppET-1 mRNA/□-actin mRNA
150.00
100.00
50.00
0.00
*
Normoxia Ctrl
Hypoxia Ctrl
Normoxia + TNF
Hypoxia + TNF

Figure 4.


Cell Proliferation % of Contro
200
150
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Normoxia
Hypoxia
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+
Control
ET-1 (100 nM)
TNF (10 nM)
PD142893 (1 uM)
PD + ET-1
PD + TNF

Figure 5.

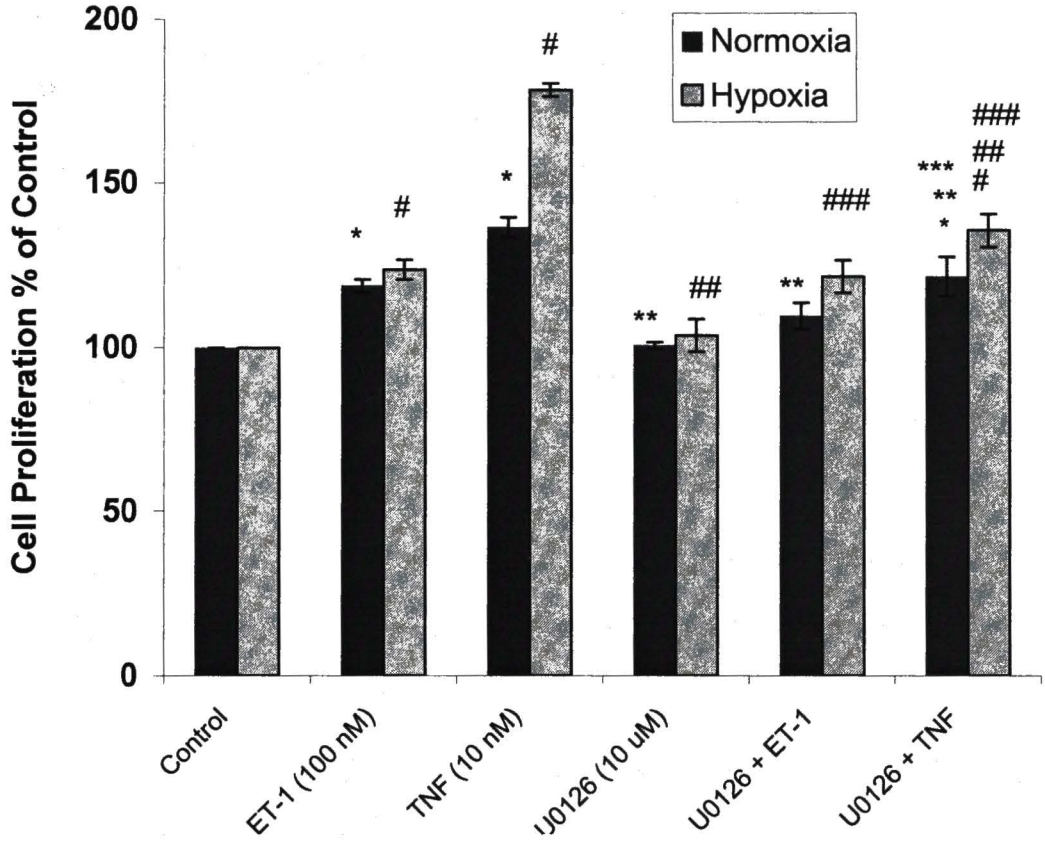
Cell Proliferation % of Control
200
150
100
50
0
Normoxia
Hypoxia
Control
ET-1 (100 nM)
TNF (10 nM)
U0126 (10 uM)
U0126 + ET-1
U0126 + TNF

Figure 6.

CHAPTER III : DISCUSSION

Endothelin-1-Induced Proliferation of Cultured Human Optic Nerve Head Astrocytes under Hypoxia

CHAPTER III

Discussion

In the present study it has been shown that primary cultures of human optic nerve head astrocytes (hONAs) synthesize and secrete endothelin-1 (ET-1) into the cell culture media constitutively. The synthesis of ET-1 in terms of both protein and mRNA is increased significantly upon treatment of hONAs with tumor necrosis factor-α (TNF-α). Hypoxia further augments the increase in ET-1 secretion when applied together with TNF-α treatment, but does not increase the ET-1 mRNA levels. This study also confirms the earlier observation that ET-1 is mitogenic for hONAs (Prasanna et al., 2002). Furthermore, hypoxia significantly increases the proliferation of hONAs in response to ET-1 and TNF-α treatment.

Alteration in morphology is an important step prior to the migration of reactive astrocytes as seen in glaucoma. Hypoxia treatment for 24 hours caused subtle morphological alterations in hONAs in the present study. The cells appear to have withdrawn some astrocytic processes, and show a spindle shaped phenotype as compared to untreated cells. However, these observations may be considered subjective in case of cultured hONAs and more morphometric studies are needed to confirm the changes in the cells.

At normal oxygen tension, the levels of ET-1 secreted in the culture media by hONAs were significantly higher than control when the cells were treated with TNF-α for

24 hours. This finding is in keeping with previous observations that TNF-α promotes ET-1 synthesis and release in systemic inflammatory responses by several cell types including human umbilical vein endothelial cells (Scalera et al., 2003), monocytes (Ehrenreich et al., 1993), renal epithelium (Ohta et al., 1990). In ciliary non-pigmented epithelial cells TNF-α stimulation induced ET-1 secretion via *de novo* ppET-1 synthesis and the secretion process involved the endothelin converting enzyme (ECE) system (Prasanna et al., 1998). The involvement of ECE in TNF-α mediated ET-1 secretion under both normoxia and hypoxia and also active transcription and translation has been demonstrated in this study.

Hypoxia treatment for 24 hours alone did not elevate irET-1 levels in hONA culture media significantly. This is in contrast with earlier findings where hypoxia alone increased ET-1 secretion from neonatal rat brain astrocytes (Schmidt-Ott et al., 2001). The lack of increase in irET-1 levels in this study may be explained by previous reports that hypoxia/ischemia upregulates not only ET-1 but also the clearance receptor for ET-1, the ET_B receptor, in cultured astrocytes and vascular endothelial cells (Tsang et al., 2001; Shibaguchi et al., 2000). hONAs express the ET_B receptors (Prasanna et al., 2002); however, it remains to be seen if hypoxia and/or TNF-α affect the expression of ET_B receptors in these cells, as has been shown in other cell types. Cell culture media from hONAs subjected to 24-hour hypoxia with concurrent TNF-α treatment showed a 500% increase in the irET-1 levels over baseline. TNF-α stimulates ET-1 synthesis in brain astrocytes. However, this is the first report demonstrating that hypoxia induced further increase in ET-1 secretion. Hypoxia inducible factor-1α (HIF-1α), a transcription factor

upregulated under hypoxia, binds to the promoter region of ET-1 gene, inducing transcription. HIF-1α alone is not sufficient for transcription of the ET-1 gene, but requires the activation of at least one more transcription factor out of activator protein-1, GATA-2 and p300/CBP (Yamashita et al., 2001). TNF-α activates the transcription factor AP-1 in brain astrocytes (Kordula et al., 2000). Thus, in the presence of both TNF-α and hypoxia, both HIF-1α and AP-1 may be activated, leading to a marked increase in ET-1 synthesis and secretion.

An increase in pre-pro-ET-1 (ppET-1) mRNA expression was also seen upon TNF-α treatment under normoxia, but there was no further increase in ppET-1 mRNA under hypoxia. The reason for this may lie in the short intracellular half-life of ppET-1 mRNA (only 15-20 minutes) due to the presence of three AUUUA motifs in its 3'-nontranslated region, which may mediate selective translation-dependent destabilization of the mRNA (Inoue et al., 1989). The increased rate of translation of ppET-1 mRNA into protein may also help explain the surprisingly large (500%) increase in irET-1 levels in culture media upon TNF-α treatment under hypoxia causing no apparent change in ppET-1 mRNA or even a possible decrease.

Astrocytes can act as a source of ET-1 in the optic nerve head in response to insult such as hypoxia together with TNF-α. Hypoxia itself did not cause significant increase in ET-1 synthesis, but required the presence of TNF-α. An increase in both of these factors at the optic nerve head is a reasonable possibility since astrocytes have also been shown to produce cytokines in response to similar stresses. The expression of TNF-α and TNFR-1 is upregulated in the glaucomatous optic nerve head with TNF-α expression being

primarily observed in glial fibrillary acidic protein (GFAP)-positive astrocytes (Yuan and Neufeld, 2000;Yan et al, 2000). The presence of ET-1 at the optic nerve head may lead to a vicious circle in which ET-1 secreted in response to hypoxia may cause local vasoconstriction and further hypoxia/ischemia at the ONH and retina. Several earlier reports have shown increased ET-1 levels in glaucoma patients and animal models of glaucoma (Noske et al., 1997, Kaiser et al., 1995; Kallberg et al., 2002). Also, ET-1 immunoreactivity has been observed in the retina and the glial region of the optic nerve head (Ripodas et al., 2001; Wollensak et al., 1998). Taken together, these observations suggest that astrocytes act as a source of ET-1 in glaucoma. The effects of ET-1 in tissues such as the retina and the optic nerve are thought to be mostly detrimental as injection or perfusion of ET-1 into the optic nerve or retina causes optic neuropathy similar to that seen in glaucoma (Cioffi et al., 1995) as well as disruption of axonal transport (Stokely et al., 2002). Moreover, the involvement of ECE in secretion of ET-1 from astrocytes suggests that ECE may be a possible target for future drug development.

The possible role of astrocytes in the progression of glaucomatous optic nerve damage was recognized in earlier reports where proliferation of astrocytes was observed at the optic nerve head and the extent of proliferation was linked to the progression of the disease (Minkler and Spaeth, 1981). Increased immunoreactivity for astrocytic markers GFAP and NCAM was shown in the glaucomatous optic nerve head (Morgan, 2000), suggesting that the cells may have become hypertrophic and/or have proliferated and migrated into areas previously occupied by the nerve fiber bundles. What causes astrocyte proliferation is not known. However, ischemia/hypoxia due to elevated IOP

and/or local vasospasm is thought to affect the astrocytes in this region by causing "reactivation", i.e. proliferation and hypertrophy. Reactivation of astrocytes causes loss of neurotrphic support to the axons, which can cause axon degeneration. Thus, reactive astrocytes at the ONH may be responsible for disruption of axonal transport, prevention of axon regeneration and degeneration of the optic nerve in glaucoma (Hernandez, 2000; Morgan, 2000). In the present study, cultured astrocytes did not exhibit major alteration in phenotype upon exposure to hypoxia for 24 hours, which is similar to the findings of Schmidt-Ott et al. (2001). Though subtle changes in the lengths and numbers of astrocytic processes were observed, it is hard to equate these changes with those that might be occurring *in-vivo*. These may be due to the effect of hypoxia on the ATP levels in astrocytes. Although brain astrocytes have been shown to upregulate their glycolytic capacity in response to hypoxic stress, thus providing sufficient ATP for cell survival and critical functions (Marrif and Juurlink, 1999), they may withdraw astrocytic processes under such stress. Also, TNF-α induced release of ET-1 by hONAs into the cell culture media may affect the shape since ET-1 is known to cause significant alteration in cytoskeletal organization in brain astrocytes (Koyama and Baba, 1996) and retinal pigmented epithelium cells (Narayan et al., 2003). Hypoxia has also been shown to reverse dibutryl cAMP-induced astrocyte stellation via activation of ET-1 system (Schmidt-Ott et al., 2001). Thus, hypertrophy and proliferation of astrocytes at the optic nerve head due to various factors may be responsible for axonal damage as seen in glaucoma.

The effect of two agents that are elevated in the glaucomatous eye, namely ET-1 and TNF-α on proliferation of cultured human optic nerve head astrocytes under hypoxia is being reported for the first time the present study. A high concentration of ET-1 at the optic nerve head results as the astrocytes themselves release ET-1 in response to insults such as hypoxia and cytokines such as TNF-α. Interestingly, TNF-α is also known to cause astrocyte proliferation in bovine brain astrocytes (Selmaj et al., 1990; 1991) and human astrocytic cell lines (Barna et al., 1990) but not in human fetal brain astrocytes (Moretto et al., 1993). A significant association of the TNF-α -308 gene polymorphism with POAG patients in Chinese populations has been reported recently, and TNF-α may be considered a candidate marker for glaucoma (Lin et al., 2003). Although functional implications of this polymorphism remain to be examined, TNF-α may be considered a candidate marker for glaucoma. In the present study, ET-1 (100nM) caused a moderate proliferation, whereas proliferation in response to TNF-α was more pronounced. However, the degree of proliferation induced by ET-1 treatment in this study is comparable with that reported earlier (Prasanna et al., 2002). Under hypoxia, ET-1 induced proliferation was not dramatically increased (25% over baseline), but was significantly higher than that seen under normoxia, whereas, TNF-α-induced proliferation under hypoxia was greatly increased (80% over baseline). Initial experiments were performed using hONAs seeded on regular uncoated plastic cell culture plates, whereby a similar trend of hONA proliferation was observed. ET-1 induced increase in cell numbers ranged between 14-23% over baseline under normoxia and 25-35% under hypoxia. TNF-α induced proliferation ranged between 140-150% over baseline under normoxia and 80-

90% under hypoxia (data not shown). The disparity in cell numbers may be attributed to the 20-25% cell death observed after 24 hours of hypoxia when cells were seeded on plastic surface (data not shown). Later experiments were performed using collagen-I coated culture plates as no significant reduction in cell numbers were seen when hONA plates on collagen were subjected to hypoxia for 24 hours. The significance of small increase in ET-1 induced proliferation under hypoxia can be appreciated in the context of glaucoma, taking into consideration the chronic nature of the disease. TNF-α and hypoxia applied together provide an immensely effective stimulus for proliferation of hONA. Therefore chronic glial activation (proliferation, migration and hypertrophy) in glaucoma could be a combined effect of constantly elevated levels of ET-1 and TNF-α along with hypoxia/ischemia that may occur at the optic nerve head due to elevated IOP or vasospasms in the retinal vasculature. In normal tension glaucoma (NTG), where IOP is not elevated, the high levels of ET-1 in the plasma may cause an ischemic insult at the ONH, further leading to glial activation.

The expression of ET-1 receptors ET_A and ET_B in hONAs and their involvement in ET-1-induced proliferation of these cells under normoxia has already been demonstrated (Prasanna et al., 2002). Under hypoxia, a non-selective $ET_{A/B}$ receptor antagonist PD142893 significantly blocked hONA proliferation, indicating the involvement of ET-receptors. Interestingly, PD142893 also significantly blocked the effect of TNF-α, almost entirely under normoxia but to a lesser extent under hypoxia. This suggests that TNF-α induces ET-1 release, which in turn may be responsible for the proliferation under normoxia, whereas under hypoxia TNF-α may activate other

mitogenic signaling pathways in addition to the endothelin system. Activation of the ET_B receptor by endothelin has been linked to glial activation in the brain. Further studies using selective ET_A and ET_B receptor antagonists such as BQ610 and BQ 788, respectively, are needed to evaluate the involvement and specific roles these receptors in ONA proliferation. An endothelin receptor antagonist may provide at least partial protection against glial activation and is a possible target for drug treatment of glaucoma.

ET-1 has been shown to cause activation of extracellular signal regulated kinases (Erks p42/44) via the ET_B receptor, leading to DNA synthesis in rat astrocytes (Lazarini et al, 1996). TNF-α-associated signaling also involves these mitogen activated protein kinases (erks p42/44) (Marcus et al., 2003). In astrocytes, activation of p42/44 MAPK pathway in most cases results in cell proliferation and is also associated with neuronal injury and repair following hypoxia/ischemia (Wang et al., 2003; Carbonell and Mandell, 2003). Recently, prominent and persistent activation of Erk in activated glial cells has been demonstrated, which suggests that this signaling pathway is probably associated with the induction and/or maintenance of the activated glial phenotype as seen in glaucoma (Tezel et al, 2003). In this study, the involvement of p42/44 MAPK in hONA proliferation under normoxia and hypoxia following ET-1 and TNF-α treatment was observed using a MEK1/2 inhibitor, U0126. The proliferative effect of ET-1 was blocked by U0126 pre-treatment under normoxia, but not under hypoxia. In addition, U0126 also partially blocked TNF-α-induced hONA proliferation both in normoxia and hypoxia. Collectively, these data suggest that p42/44 MAPK signaling is activated under both normal and hypoxic conditions by TNF-α but only under normoxia by ET-1. In hypoxia,

mitogenic effects of ET-1 on hONAs while being receptor mediated also appear to involve other signaling cascades and may include intracellular calcium (MacCumber et al., 1990; Supattapone et al., 1989) and p38 MAPK (Schinelli et al., 2001).

ET-1 causes activation of Erks via the ET_B receptor, leading to DNA synthesis in rat astrocytes (Lazarini et al., 1996). Recently, Tezel et al. have demonstrated the prominent and persistent activation of ERK in activated glial cells, which suggests that this signaling pathway is probably associated with the induction and/or maintenance of the activated glial phenotype as seen in glaucoma (Tezel et al., 2003). A time course western blot analysis of erk1/2 activation following 100nM ET-1 treatment showed that the highest activation was achieved at 5 minutes. These results are preliminary and more experimentation is required to validate these data. The phosphorylated forms of erk-1/2 were found to decrease at15 minutes and returned to baseline at 60 minutes. Early activation followed by decrease in phosphorylation of ERK-1/2 is characteristic of a proliferative response, whereas delayed onset of ERK phosphorylation, followed by persistent activation for several hours is indicative of apoptotic cell death signaling. MEK1 inhibitor PD98059 blocked the activation of ERK-1/2, confirming the results of the proliferation assays. It remains to be examined how TNF-α and hypoxia influence MAPK activation in hONAs. As seen in the proliferation studies, U0126 only partially blocked proliferation in response to TNF-α treatment under hypoxia, and further studies are required to elucidate this phenomenon.

Several observations have led to wide acceptance of the possibility that elevated IOP leads to reduced blood flow, which could result in hypoxia/ischemia at the ONH

(Carter et al., 1990; Flammer, 1994; Hayreh, 1994, Van Buskirk and Cioffi, 1992; Geijer and Bill, 1979). Vasospastic disorders such as migraine headache or cold hands and feet are more commonly seen in patients with both primary open angle as well as normotensive glaucoma (Gasser and Flammer, 1991; Phelps and Corbett, 1985). Moreover, chronic ischemia of the primate anterior optic nerve induced with endothelin-1 infusion was shown to result in diffuse loss of axons without a change in the intraocular pressure (Cioffi and Sullivan, 1999). Increased immunoreactivity for GFAP and NCAM has been shown in the glaucomatous optic nerve head (Morgan, 2000) and GFAP and S-100 in the inner retina (Lam et al., 2003), suggesting proliferation and/or hypertrophy of astrocytes. ET-1 being a potent vasoconstrictor is also capable of causing hypoxia at the optic nerve head by inducing local spasms. Hypoxia can in turn induce ET-1 secretion by astrocytes via hypoxia inducible factor-1 (HIF-1) in association with one or more other transcription factors such as AP-1, GATA-2 and CBP (Yamashita et al., 2001). The findings of the present study coupled to these above observations support the possible role of hypoxia in astrocyte proliferation at the optic nerve head in glaucoma. Very little is known about the blood oxygenation levels in the retinal vasculature. Real time measurement of blood oxygenation at the ONH and retina with the help of novel optical imaging systems may prove to a useful tool in both animal models of glaucoma as well as in the clinical setting. Nevertheless, current knowledge suggests the involvement of hypoxia in glaucomatous optic nerve damage.

ET-1 immunoreactivity has been observed in the retina and the glial region of the optic nerve head (Ripodas et al., 2001; Wollensak et al., 1998). Clinically, ET-1 levels

are elevated in the aqueous humor of patients with primary open angle glaucoma (POAG), in which the IOP is elevated and in the plasma of patients with normal tension glaucoma (NTG), a subtype of glaucoma often considered as a vasospastic disorder, in which IOP is apparently normal (Noske et al., 1997, Kaiser et al., 1995). In the Beagle model of glaucoma, aqueous ET-1 levels were 4-fold higher than control (Kallberg et al., 2002). Furthermore, injection or perfusion of ET-1 into the optic nerve or retina causes optic neuropathy similar to that seen in glaucoma (Cioffi et al., 1995; Stokely et al., 2002). High ET-1 levels have been reported in rat cortical astrocytes and astrogliosis in other conditions such as Alzheimer's disease, neurotrauma and brain ischemia (Cintra et al., 1989; Hama et al., 1992; Zhang et al., 1994; Barone et al., 1994). All these observations justify the increasing acceptance of the contribution of ET-1 in the development of glaucomatous optic neuropathy via astrocyte activation.

TNF-α levels are also elevated in the glaucomatous eye (Tezel et al., 2001). After the exposure to stress such as ischemia and elevated hydrostatic pressure, glial cells in the optic nerve head were shown to secrete TNF-α as well as other potential damaging agents such as nitric oxide (Tezel and Wax, 2000). In glaucomatous optic nerve heads, the expression of TNF-α and TNFR-1 were upregulated; TNF-α expression was primarily observed in GFAP-positive astrocytes, and was proportional to the progression of optic nerve degeneration (Yuan and Neufeld, 2000). Also, TNF-α significantly increases ET-1 release from hONAs in hypoxic conditions as shown in this study and may exacerbate the effects of ET-1.

In conclusion, it has been shown for the first time that hypoxia augments TNF-α and ET-1 mediated proliferation of optic nerve head astrocytes, via increased ET-1 secretion and mitogenesis. Therefore reactive ONAs could be partly responsible for optic nerve damage in glaucoma since their localization makes them susceptible to mechanical and hypoxic/ischemic stress compounded with influences of ET-1 and TNF-α.

Future Directions:

ET-1 release from hONAs in response to TNF-α is greatly augmented by hypoxia as seen in this study. Elevated hydrostatic pressure and ischemia have been shown to induce TNF-α synthesis and release from glial cell in the optic nerve head (Tezel and Wax, 2000). It remains to be examined if under hypoxia hONAs act as a source of TNF-α, which then stimulates ET-1 secretion and proliferation in an autocrine fashion. TNF-R1 is also upregulated in the glaucomatous ONH (Yuan and Neufeld, 2000) and its involvement in the synthesis and secretion of ET-1 from hONAs as well as in TNF-α induced proliferation of ONAs needs to be examined. Hypoxia may potentially upregulate TNF-R1 in ONAs and may increase the sensitivity of these cells to TNF-α treatment. This might be a factor contributing to the dramatic increase in ET-1 secretion (~500%) upon exposure to hypoxia as seen in this study. Hypoxia-inducible factor-1a (HIF-1a) is a transcription factor implicated in the alteration of ET-1 expression in the rat brain during chronic hypoxia (Hu et al., 1998; Chavez et al., 2000). The human ET-1 promoter contains a hypoxia-inducible factor-1 (HIF-1) binding site which contributes to the activation of ET-1 expression in endothelial cells and the molecular regulation of ET-1 gene by hypoxia involves HIF-1a in association with AP-1 and GATA-2 transcription factors (Yamashita et al., 2001). In a recent proteomic analysis, TNF-α has been shown to induce AP-1 transcription factor in an epithelial cell line HeLa (Jiang et al., 2003). It may be hypothesized that hypoxia provides the stimulus for upregulation of HIF-1a while TNF-α stimulates AP-1 and these factors together greatly increase the transcription of ET-1 gene in ONAs.

In an interesting observation regarding the interplay between TNF-α and the endothelin system, TNF-α was shown to induce the production of ET-1 in an *in-vitro* co-culture model of the human blood brain barrier (BBB), which increased the permeability of the BBB. This increased permeability was due to ET-1 mediated IL-1ß overexpression in astrocytes and the effect on the BBB was eliminated in the absence of astrocytes (Didier et al., 2003). Also, TNF-α was found stimulate ET-1 secretion from retinal pigmented epithelium cells (ARPE-19) and also increased the permeability of the blood-retinal barrier by disruption of tight junctions between the RPE (Narayan et al., 2003). Optic nerve head astrocytes are partially responsible for the integrity of the blood brain barrier at the optic nerve. On the basis of these findings and the results of the present study it may be hypothesized that TNF-α induced ET-1 secretion may lead to optic nerve damage by increasing the permeability of the blood-brain barrier and that hypoxia contribute to this damage since it greatly increases the synthesis of ET-1 from astrocytes. Studies using *in-vitro* co-culture models of the blood brain barrier and the blood retinal barrier may help understand how hypoxia influences this interplay between TNF-α and the endothelin system.

In all these studies, *in-vitro* testing is necessary because of limited resources for measurement of tissue oxygenation at the retina and the optic nerve. Color Doppler imaging remains the only option, and can provide information mainly about blood flow parameters, not the oxygenation levels. Recent advances in medical imaging systems utilizing near infrared or visible spectra for measurement of oxygenation levels of blood may be put to use here. This would provide a very powerful, yet relatively inexpensive

tool for the scientist as well as the clinician. For example, ET-1 is a potent and vasoconstrictor and is capable of producing vasospasms in the retinal vasculature. The direct effect of ET-1 perfusion or injection can be measured using an imaging system capable of detecting oxygenation of blood at the ONH or retina.

Proliferation of astrocytes in response to ET-1 and TNF-a treatment was significantly increased upon hypoxia treatment in this study. Astrocyte activation in response to stress factors such as hypoxia/ischemia and cytokines also causes remodeling of the ECM at the ONH. ET-1 has been shown to cause changes in ECM by causing increased collagen and also fibronectin in various conditions including intimal hyperplasia following balloon angioplasty (Barolet et al., 2001), fibroblasts in systemic sclerosis (Shi-Wen et al., 2001) and renal fibrosis (Evans et al., 2000). ET-1 has also been shown to upregulate the expression of ets-1, a transcription factor that activates expression of matrix-degrading proteinases such as collagenase and stromelysin (Naito et al., 1998). A proteomic analysis of glial protein expression following CNS injury showed alteration in proteins involved in cytoskeletal reorganization (caldesmon, calponin, alpha B-crystallin, stathmin, collapsing response mediator protein-2), cell adhesion (vinculin, galectin-1), signal transduction (RACK-1) and astrocyte differentiation (glutamine synthetase). However, the effect of ET-1 on ECM changes in ONAs has not been studied.

The proliferation induced by ET-1 in hONAs in this study appeared to be receptor mediated. The role of individual ET receptors remains to be elucidated. It has been hypothesized that the ET_B receptor in responsible for transducing the mitogenic signal in

hONAs (Yorio et al., 2003). Further studies using selective ET_A and ET_B receptor antagonists such as BQ610 and BQ 788 are needed to evaluate the involvement and specific roles of these receptors in ONA proliferation. An endothelin receptor antagonist may provide at least partial protection against glial activation and is a possible target for drug treatment of glaucoma.

Increasing evidence points to the role of ET-1 in the development of glaucomatous optic neuropathy, with most of it's effects in the posterior segment of the eye being detrimental. The endothelin system offers various potential drug targets, which may be targeted for developing new drugs for glaucoma. More specifically, ECE inhibitors and ET receptor antagonists may prove useful in blocking the detrimental effects of ET-1 in the posterior segment of the eye. Prevention of astrogliosis may retard the progression of optic nerve degeneration in glaucoma.

CHAPTER IV: APPENDIX

Endothelin-1-Induced Proliferation of Cultured Human Optic Nerve Head Astrocytes under Hypoxia

APPENDIX

^{3}[H]-Thymidine Uptake Assay to Determine the Effect of ET-1 and TNF-α on Proliferation of U-373MG Astrocytoma Cell Line Under Hypoxia

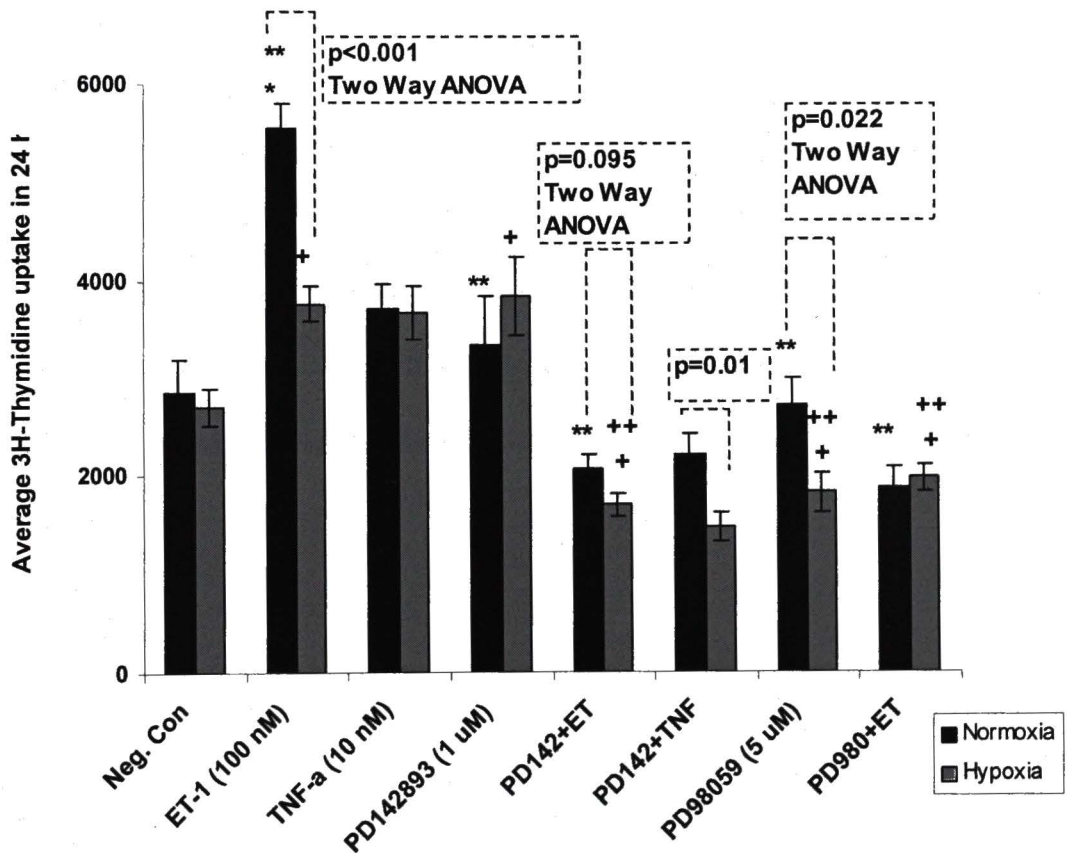

Figure A-1.

Results of ^{3}[H]-Thymidine uptake assay (Figure A-1) show the effect of 24 hours hypoxia treatment on ET-1 and TNF-a induced proliferation of U373MG astrocytoma cell line. ET-1 (100nM) alone increased cell numbers twofold within 24h under normoxia, while under concurrent hypoxia there was only a moderate increase in cell numbers. TNF-a induced proliferation was also moderate as compared to control and there was no significant difference between hypoxia and normoxia treated groups. Pre-treatment with a non-selective ET-A/B receptor antagonist PD142893 significantly blocked the proliferation in response to ET-1 and TNF-a, suggesting that proliferation of U373MG in response to both ET-1 and TNF-a involved ET receptors. Also, pre-treatment with a MAPK inhibitor PD98059 significantly blocked the proliferation in response to both ET-1 and TNF-a under normoxia as well as hypoxia. There was a significant reduction in cell numbers in cells treated with PD98059 alone under hypoxia but not under normoxia, suggesting that basal proliferation was blocked by PD98059 under hypoxia.

Western Blot Analysis of Erk-1/2 Activation in hONAs Treated with ET-1

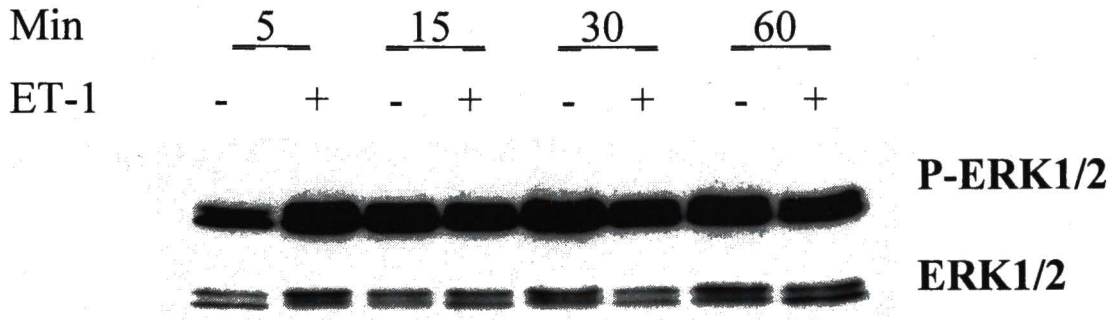

Figure A-2.

Effect of MEK1/2 inhibitor PD98059 (5 mM) on phosphorylated ERK1/2 in hONA after 5 min application of ET-1 (100 nM).

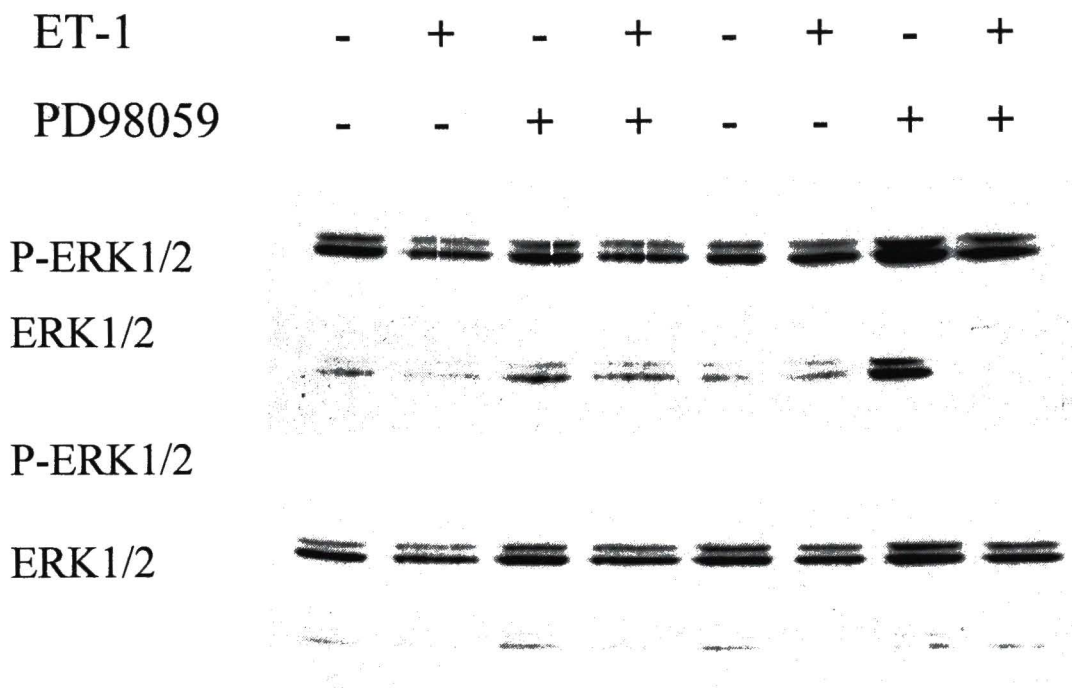

Figure A-3.

Results (Figure A-2 and A-3):

To ascertain the phosphorylation of Erk, hONAs grown to confluence on 100mm culture dishes were treated with ET-1 (1, 10, 100 nM) for various time periods (0, 0.5, 1, 5, 10, 15, 30 and 60 min). Protein from the cell lysates was separated using SDS-PAGE. Western blot analysis was performed using commercially available monoclonal antibodies against phosho-Erks ($p42^{MAPK}$ and $p44^{MAPK}$; Calbiochem, CA). A time course western blot analysis of erk1/2 activation following 100nM ET-1 treatment showed that the highest activation was achieved at 5 minutes (Figure A-2). The phosphorylated forms of erk-1/2 were found to decrease at at 15 minutes and returned to baseline at 60 minutes. Early activation followed by decrease in phosphorylation of erks1/2 is characteristic of a proliferative response, whereas delayed onset of erk phosphorylation, followed by persistent activation for several hours is indicative of apoptotic cell death signaling. MEK inhibitor PD98059 blocked the activation of erk-1/2 (Figure A-3), confirming the results of the proliferation assays.

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Endothelin-1-Induced Proliferation of Cultured Human Optic Nerve Head Astrocytes under Hypoxia

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