Braun, Brittany M., Identification and Characterization of Natriuretic Peptides and Natriuretic Peptide Receptors in Human Lens Epithelial Cells, Master of Science (Biomedical Sciences), December, 2009, 49 pp, 2 tables, 7 illustrations, bibliography, 38 titles.

Purpose: The natriuretic peptide (NP) family consists of three peptides; atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) as well as three receptors; natriuretic peptide receptor A (NPR-A), natriuretic peptide receptor B (NPR-B) and natriuretic peptide receptor C (NPR-C). The natriuretic peptide family, although well examined in some systems, has not been extensively studied in ocular systems. The purpose of this study was to demonstrate both existence and functionality of components of the natriuretic peptides and natriuretic peptide receptors in a human lens epithelial cell line, HLE-B3.

Methods: HLE-B3, a virally transformed human lens epithelial cell line, was used for all experiments. The effects of stress on the expression of natriuretic peptide and receptor mRNA and protein was determined with the use of a step down procedure. The cells were grown to confluence in Eagle's minimal essential medium (MEM) containing 20% fetal bovine serum (FBS) and subsequently, stepped down into 2% FBS MEM, followed by serum free MEM. After 24hr of serum free MEM the cells were placed into hypoxic conditions for 24 hours and then subjected to re-oxygenation. In order to determine the presence and expression profile of messenger RNA (mRNA) for the natriuretic peptides and their associated receptors reverse transcriptase PCR (RT-PCR) and quantitative real time PCR (QRT-PCR) were used. Protein expression for the natriuretic peptide receptors was observed using Western Blotting. Immunohistochemistry along with confocal imaging was employed in order to illustrate subcellular localization of the natriuretic peptide receptors. Enzyme-linked immunosorbent assay was used to determine cyclic GMP (cGMP) activity in HLE-B3 cells.

Results: Messenger RNA expression was detected for all three natriuretic peptides and their associated receptors. Protein expression was observed for all three natriuretic peptide receptors. Protein expression remained at a relatively constant level, regardless of the cells being subjected to a variety of stressors, the exception being NPR-B. Cellular localization was observed for the three natriuretic peptide receptors with the image density being higher for NPR-A and NPR-B when compared to NPR-C. Localization appeared to be diffuse throughout the cytosol with no indication of nuclear staining. Functionality of all three natriuretic peptides was demonstrated by a cGMP activity assay with the rank order of potency of activation being CNP»ANP≥BNP.

Conclusions: This article is the first demonstration that all members of the natriuretic peptide family, including both peptides and receptors, are expressed and are functional in the human lens epithelial cell line, HLE-B3.

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IDENTIFICATION AND CHARACTERIZATION OF NATRIURETIC PEPTIDES AND NATRIURETIC PEPTIDE RECEPTORS IN HUMAN LENS EPITHELIAL CELLS

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CHAPTER I

INTRODUCTION

The Human Lens

The crystallin lens of the human eye is a transparent, highly organized complex structure that transmits light and aides in its focusing on the retina. Lens cells are organized into two contiguous but morphologically very distinct cell sub-populations. The lens cells facing the anterior chamber of the eye, bathed in aqueous humour, are a monolayer of epithelial cells. The lens cells facing the posterior chamber of the eye, bathed in vitreous humour, are dramatically elongated into fibers. It is at the boundary between aqueous and vitreous humours, around the equatorial region of the lens, that the changes from one morphological type into the other occur [1]. Lens transparency results from appropriate architecture of lens cells and tight packing of their proteins, resulting in a constant refractive index over distances approximating the wavelength of light [2].

Introduction to the Natriuretic Peptide Family

The natriuretic peptide (NP) family is believed to have evolved for the homeostatic regulation of volume, osmosis and pressure regulation of the circulatory system. Natriuretic peptides are best known as a group of hormones that are primarily released from the heart and play an important role in cardiovascular homeostasis specifically, control of fluid volume and blood pressure [3]. Recently, evidence has demonstrated that this family of cardiovascular

natriuretic peptides plays an autocrine and paracrine role in the control of myocardial structure and function [4]. These peptides play an important role in the regulation of electrolytes and water balance through their diuretic and natriuretic effects. They also inhibit the renin-angiotensin-aldosterone system and regulate the permeability of systemic vasculature as well as inhibit the activity of the sympathetic nervous system [3]. The natriuretic peptide system is comprised of three structurally related peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Corresponding with the peptides are three distinct subtype natriuretic peptide receptors: natriuretic peptide receptor A (NPR-A), natriuretic peptide receptor B (NPR-B) and natriuretic peptide receptor C (NPR-C).

The Natriuretic Peptides

The natriuretic peptide family was first described with the demonstration of atrial natriuretic peptide, in the 1980's by de Bold, et al who demonstrated natriuretic responses in rats when injected with atrial myocardial extract, later referred to as ANP [5]. The discovery of ANP was soon followed by the finding of an almost homologous although distinct peptide in the porcine brain, brain natriuretic peptide or BNP by Sudon, et al [6]. Two years later Sudon reported the discovery of the third natriuretic peptide, CNP [7].

The natriuretic peptides and their receptors are found across species. Of the peptides, ANP and CNP have highly conserved sequences across the species whereas the structure of BNP varies greatly [8]. The natriuretic peptides are all comprised of a shared 17-member disulfide ring structure [9]. The precursor prohormone for each of the peptides is encoded for by a separate gene which, for ANP and BNP, is located on human chromosome 1 [10,11]. All three of the peptides are synthesized as prepropeptides. A signal peptide from the prepropeptides is

removed converting them into propeptides. The propeptides then undergo proteolytic cleavage which converts them to their active peptide form [12]. In its active form, ANP is a 28 amino acid peptide with a hairpin structure caused by a cysteine bridge between residues 7 and 23. BNP exists as a 32 amino acid structure, with a hairpin similar to that of ANP. CNP, although containing the hairpin loop structure, is the smallest of the three natriuretic peptides consisting of 22 amino acids and a truncated carboxy terminus [11].

ANP and BNP are circulating peptides mainly produced in the heart and that are involved in the regulation of natriuresis, diuresis and blood flow. These two peptides have preferentially bind the NPR-A receptor isoform. Unlike ANP and BNP, CNP predominantly acts as a vasodilator and is found principally in the central nervous system and endothelial cells and displays an affinity for the NPR-B receptor isoform [10,13]. Gene expression of the peptides is controlled by numerous factors including: α adrenergic agonists, endothelian, growth factors, Vitamin D, retinoids, mechanical strain and hypoxia [14].

The Natriuretic Peptide Receptors

The natriuretic peptides exert their effects through interaction with the natriuretic peptide receptors (NPRs). NPR-A is the target receptor for both ANP and BNP. Structurally, NPR-A consists of a glycosylated, extracellular ligand-binding domain linked to a hydrophobic transmembrane spanning segment. In the intracellular region, a non-catalytic ATP-binding kinase-like domain is thought to regulate the receptor activity. NPR-B is structurally similar to NPR-A but preferentially binds to CNP [14]. The actions of the natriuretic peptides are mediated by their selective interaction with their cognate receptors, leading to the generation of second messengers; cyclic AMP (cAMP), cyclic GMP (cGMP), or the modulation of ion

channels [8]. The NPR-A and NPR-B natriuretic peptide receptors are both single transmembrane spanning proteins containing particulate guanylyl cyclase (pGC) activity in their intracellular domain [13]. Regulation of NPR-A can be accomplished by ANP and, presumably, BNP. NPR-A contains a cGMP response element situated between base pairs 1396 to 1307 on the promoter region of the receptor gene. ANP and BNP can down regulate and inhibit the activity of NPR-A through the action of their second messenger, cGMP [11].

Unlike NPR-A and NPR-B, NPR-C has no GC activity. Instead, it is known as a "clearance receptor." NPR-C is thought to signal through the inhibition of adenylate cyclase in some cells [15]. Structurally, NPR-C has a similar extracellular domain to NPR-A and NPR-B [14]. In many tissues NPR-C is the most abundant of the natriuretic peptide receptors [16]. NPR-C binds all three natriuretic peptides with a high and comparable affinity. The receptor may participate in mediating some of the cellular actions of the natriuretic peptides via coupling to Gi proteins and negative modulation of adenylyl cyclase activity [17]. However, its main action in the cardiovascular system seems to be the modulation of circulating and local natriuretic peptide concentrations that are available to bind NPR-A and NPR-B [18]. The clearance of the natriuretic peptides via NPR-C is accomplished by receptor-mediated endocytosis and subsequent lysosomal hydrolysis which occurs in the extracellular region of the receptor [14,19].

Functions of the Natriuretic Peptides and Natriuretic Peptide Receptors in Ocular Systems

Recently, molecular evidence has been presented for presence and functionality of natriuretic peptide system in the ciliary epithelium, the trebecular meshwork and the human retina [8,10,20]. In the eye, natriuretic peptides are reported to regulate intraocular pressure and stimulate guanylate cyclase. Also, all three receptors, particularly the NPR-C receptor, have

been shown to adjust the NP concentration of the aqueous humor [21]. Recently, the NP family has been shown to modulate neuronal activities by changing levels of cGMP. Their involvement in both cGMP production and neuronal activities allows for speculation that they may be involved in image processing in the retina [13]. It has also been suggested that the NP system might be involved in the development of glaucoma and diabetic retinopathy [10]. To date, there have been no reports of the presence of the natriuretic peptide system in human lens epithelial cells.

Studies of the natriuretic peptides in human ocular models is limited, however, there are a considerable number of reports using various animal models. Rabbits and rats are common models used to study the peptides and their function within various regions of the eye. In rabbits many of these studies have focused on the effects of the natriuretic peptides on intraocular pressure (IOP). Research has demonstrated that basal levels of all three natriuretic peptides can be detected in the aqueous humor of rabbits with normal IOP, with BNP having the highest concentration [22]. More recent studies have shown that all three NPs are effective, to different degrees, in lowering IOP in rabbit eyes. It has also been elucidated that guanylyl cyclase activity in the rabbit ciliary process can be stimulated by all three natriuretic peptides thus confirming the presence and functionality of NPR-A and NPR-B. In rat models binding sites for all three peptides have been demonstrated in the ciliary process [21]. Also, it has been shown that ANP, BNP and CNP are distributed within the rat Müller cells. Distinct expression profiles are seen and it has been suggested that they may play neuromodulatory functions at different retinal layers [13]. Studies in the turtle retina have also revealed the presence of all three natriuretic peptides and have demonstrated their ability to increase cGMP levels within the eye [23].

Studies of NPR-A in bullfrog Müller cells have shown the receptor to be both present and functional by demonstration of an induced inward current with the addition of BNP [24].

Purpose of the Project

This project was designed to demonstrate the presence and functionality of the natriuretic peptides and their associated natriuretic peptide receptors in a human lens epithelial cell line, HLE-B3. Data obtained by reverse transcriptase PCR, quantitative real-time PCR, Western blotting, immunohistochemistry and cGMP activity assay demonstrates the presence of the natriuretic peptide system in the lens as well as how the peptides and receptors respond to different stressors. The results show that the natriuretic peptide receptors are functional by their ability to elicit a cGMP response with the addition of the natriuretic peptides. We suggest that lenticular natriuretic peptides and associated receptors function as novel autocrine/paracrine mediators, which, under appropriate conditions play a crucial role in lens mitochondrial protection by activating guanylyl cyclase and resulting in a cGMP response, deterring from downstream mitochondrial permeability transition and cell death.

CHAPTER 2

RESULTS

Methods

Cell Culture

An SV-40 virus immortalized human lens epithelial cell line, HLE-B3, was used for all experiments [25]. The cells were maintained in Eagle's minimal essential medium (MEM) containing 20% fetal bovine serum (FBS) and 2 mM L-glutamine, nonessential amino acids, and 0.02g/l gentamycin solution and maintained at 37°C and 5% CO_2 as previously described in Flynn, et al [26].

In order to demonstrate how the cells respond to stressful conditions a step down method was utilized and will be referenced throughout the article. In brief, HLE-B3 cells were grown to confluence in 20% FBS MEM and stepped down into 2% FBS MEM for a period of 24 hours. This was followed by a step down into serum free MEM for an additional 24 hours. The cells were then placed in a hypoxia chamber (5.0% O_2) in serum free MEM for up to 24 hours. Following the hypoxic incubation the medium was replaced with fresh oxygenated serum free MEM and the cells placed back into normoxia (5.0% CO_2) for re-oxygenation collections.

PCR

Reverse transcriptase-PCR (RT-PCR) was performed on populations of HLE-B3 cells. The cells were lysed, collected and purified according to instructions supplied with the Illustra RNAspin Mini kit (GE Healthcare, Piscataway, NJ). RNA concentration was determined spectophometrically and RT-PCR was performed to obtain cDNA. RT-PCR was carried out with cDNA and synthesized primers (Sigma-Aldrich Inc., St.Louis, MO) using previously published primers for the natriuretic peptides and their receptors as reported by Rollín, et al [10]. The PCR product was electrophoresed on 2% agarose gels, the bands excised, and sent off for third-party sequencing (Northwoods DNA, Inc., Solway, MN).

Quantitative RT-PCR was performed on synthesized cDNA in a Smart Cycler thermocycler using a Brilliant Sybr Green core reagent kit (Stratagene, La Jolla, CA) and the natriuretic peptide and natriuretic peptide receptor primer pairs were used in the RT-PCR experiments at a final concentration of 200µM. All fold increases in mRNA are based upon average delta CT values calculated from comparisons to an actin standard and to baseline CT values determined from cells maintained under normal (20% FBS MEM, 5.0% CO_2) conditions.

Western Blot Analysis

Western blotting was carried out as described by Flynn, et al. [26]. Briefly, total cell lysates for HLE-B3 cells were collected following subjection to different conditions by rinsing with PBS, pH 7.0, and subsequent cell lysis with a hot extraction lysis buffer, pH 6.8, consisting of 0.12M Tris-HCl, 4% SDS, 20% glycerol. This was conducted by direct addition of 250µl of lysis buffer heated to 100°C to cell monolayers. Cell lysates were immediately scraped and collected in 1.5mL eppendorf tubes. This was followed by sonication and snap freezing in liquid

nitrogen. Samples were stored at -80°C until use. Protein concentration was determined spectrophotometircally with a DC protein assay (Bio-Rad, Hercules, CA). 3x SDS (Laemmli) buffer was added to the lysates, which were subsequently boiled for 10 min. Proteins were resolved by electrophoresis on 12% SDS-polyacrylamide gels (10µ g protein/lane). Proteins were then transferred onto nitrocellulose using a TE 22 Mini Transfer Tank (Hoefer Inc. Holliston, MA).

For Western blot analysis, nitrocellulose membranes were blocked with 0.1% BSA in Tris-buffered saline (TTBS) for 30 min. The membranes were then washed four times with 0.1% BSA and 0.02% Tween-20 in TTBS at 15min per wash. The membranes were probed overnight at 4°C with primary antibodies for either NPR-A, NPR-B, NPR-C or actin (Santa Cruz Biotechnology, Santa Cruz, CA). The following day, blots were then washed 4times for 15min each in TTBS and then incubated with either goat anti-rabbit or goat anti-mouse secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Blots were again rinsed 4times for 15min in TTBS and proteins were detected using a SuperSignal west pico chemiluminescent kit from Pierce (Rockford,IL). Probed membranes were imaged using an alphafluor imager (Alpha Innotech Corporation, San Leandro, CA).

Immunohistochemistry

Immunohistochemistry was conducted as previously described by Flynn, et al [27]. In brief, cells were seeded onto coverslips in 35 mm dishes and maintained in MEM with 20% FBS for 24 h at 37°C, 5% CO_2. Cells were subsequently stepped down to MEM with 2% FBS for 24h at the same atmospheric conditions followed by 24h in serum free MEM. Prior to the step down into serum free MEM cells were rinsed with sterile 0.05M PBS, pH 7.0.

To prepare for imaging the serum free MEM was removed, the cells were rinsed with sterile PBS and fixed in 1% paraformaldehyde in PBS at 4°C for 30min. The fixative was removed and the cells rinsed in 0.05 M PBS, pH 7.0, containing 50 mM NH_4Cl and 0.05 M PBS and (washing buffer) two times at 10 min for each rinse. Washing was followed by incubation with blocking buffer, 2% BSA PBS, for 20min at room temperature. Cells were incubated with primary antibody for NPR-A, NPR-B and NPR-C at a 1:1000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The coverslips were then washed with washing buffer four times at 5min per wash and incubated with a fluorescent secondary antibody, Alexa Fluor 488 Goat-anti-rabbit (Invitrogen Corporation, Carlsbad, CA), at a 1:500 dilution. Controls for immunohistochemical staining included a pure rabbit IgG control and control containing no primary antibody. Imaging was conducted on a Zeiss LSM 410" at 40x magnification.

cGMP Assay

For the cGMP assay cells were plated into 35mm dishes and grown overnight to confluence in 20% FBS MEM. On day two, the cells were rinsed twice with serum free media and then incubated in serum free MEM containing 25µM of IBMX for 15minutes at 37°C. Following the incubation period ANP, BNP or CNP (Sigma-Aldrich, Saint Louis, MO) in concentrations of 0.1µM, 1.0µM, or 5.0µM were added to the individual plates and cells were further incubated for 10minutes. Cells were lysed and the activity assay was carried out according to manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI). Spectrophometric readings were completed using a Spectra Max Pro with Molecular Probes

software. Statistical analysis was completed with the use of the cGMP assay's manufacturer supplied excel spreadsheet.

Results

Expression of messenger RNA for the natriuretic peptides and their receptors in HLE-B3 cells

Reverse transcriptase polymerase chain reaction (RT-PCR) followed by polymerase chain reaction (PCR) was completed using previously published primer pairs [7] to determine ANP, BNP and CNP as well was NPR-A, NPR-B and NPR-C mRNA expression in HLE-B3 cells. (Table 1). All PCR products resolved at the expected fragment size. (Figure 1). The bands were excised from the agarose gels and submitted for sequencing to further verify the accuracy of the experiment and confirm positive identification by sequence of the PCR products. The resulting sequences were entered into NCBI's Basic Local Alignment Search Tool (BLAST) and came back with 100% homology for all three natriuretic peptides and the three natriuretic peptide receptors, thus confirming the authenticity of mRNA for all natriuretic peptides and receptors in the HLE-B3 cell line.

Quantitative Expression of messenger RNA for natriuretic peptides and their receptors in HLE-B3 cells under varying conditions.

Following the determination and authenticity of the that mRNA products for the natriuretic peptides and receptors in HLE-B3 cells, quantitative reverse transcriptase PCR (QRT-PCR) was used to determine if variations in mRNA synthesis occurred due to subjecting the cells to a series of stressful conditions in a step down procedure. The cells were stepped down from 20% FBS MEM to 2% FBS MEM and finally to serum free MEM at 24hr intervals. They were

then placed into hypoxia for 24 hours before being introduced back into normal oxygen conditions.

Messenger RNA levels were measured using cycle threshold (CT) values. Change in CT (delta CT) values was calculated using 20% FBS MEM readings as a baseline and β-actin as a control. Although the majority of the natriuretic peptides and receptors displayed little to no variation in mRNA levels, increases in mRNA under stress were seen for ANP and NPR-B. (Figure 2, Table 2). The increases in message production appeared to begin when the cells were stepped down from 20% FBS MEM into 2% FBS MEM. For example, NPR-B mRNA expression went up 227% compared to baseline control expression when the cells were stepped down into 2% FBS MEM and increased further, to 415%, when stepped down into serum free media.

Protein expression profiles for Natriuretic Peptide Receptors

Protein expression for the natriuretic peptides and their receptors was conducted using Western blotting. Expression profiles were observed for the same conditions as that of the messenger RNA expression experiments: serum step down, hypoxia (4hr and 24hr) and re-oxygenation (4hr). Again, β-actin was used as a control. Natriuretic peptide protein expression was undetectable for ANP, BNP and CNP using Western blotting techniques. However, natriuretic peptide receptor protein expression patterns were able to be detected. (Figure 3). All natriuretic peptide receptors resolved at expected locations: NPR-A and NPR-B at 120, and NPR-C at 64 kDa. Relatively consistent expression levels were seen for NPR-A and NPR-C. The protein expression for NPR-B appeared to increase following the step down into 2% FBS MEM and remains elevated during all subsequent stressors.

Immunohistochemical imaging for localization of natriuretic peptide receptors in HLE-B3 cells

Immunohistochemistry and confocal microscopy were utilized to determine localization of the natriuretic peptide receptors in HLE-B3 cells. HLE-B3 cells stepped down into serum free MEM displayed positive staining for each of the three receptors: NPR-A, NPR-B, and NPR-C. (Figure 4). All three receptors were localized throughout the cytosol of the HLE-B3 cells with an even distribution. There was no nuclear staining observed for any of the three receptors. Of the three receptors both NPR-A and NPR-B had a similar amount of staining intensity whereas NPR-C displayed a considerably lower intensity.

cGMP Activity Assay

The NPR-A and NPR-B receptor isoforms are particulate guanylyl cyclases which mediate the effects of ANP, BNP and CNP [3]. In order to determine functionality of the natriuretic peptide receptors we tested the reaction to addition of ANP, BNP and CNP to the cells and the consequent stimulation of guanylate cyclase activity. HLE-B3 cells were subjected to bolus addition of natriuretic peptides in varying concentrations: 0.1µM, 1.0µM and 5.0 µM and incubated for 10 minutes. Addition of bolus natriuretic peptides in varying concentrations elicited a cGMP response in HLE-B3 cells for all three peptides at all concentrations. (Figure 5).

Although all three peptides elicited a cGMP response, the response provoked by addition of CNP was much higher than that of ANP and BNP. Under minimum concentration of peptide (0.1 µM) CNP displayed a 43- fold and 32-fold higher cGMP production when compared to ANP and BNP, respectively. Whereas ANP and BNP appeared to have a dose dependent effect on cGMP production, CNP addition seemed to elicit a maximal cGMP response at all three concentrations of peptide tested. The order of potency of the peptides in production of a cyclic

GMP response is CNP»BNP≈ANP. Remarkably, this finding mirrors that of Coca-Prados' published results for cGMP response in the ciliary process and those of Pang in the ciliary muscle and trebecular meshwork. In both of their studies, CNP elicited a strikingly higher cGMP response, than either ANP or BNP, in the respective cell types [8,20].

CHAPTER II FIGURE LEGENDS AND FIGURES

Figure 1. Reverse transcriptase PCR analysis of the natriuretic peptides (ANP, BNP and CNP) and receptors (NPR-A, NPR-B and NPR-C) in HLE-B3 cells.

Total RNA was isolated from HLE-B3 cells and subjected to RT-PCR for cDNA synthesis. Polymerase chain reactions were run with the synthesized cDNA with the primer pairs for each of the three natriuretic peptides and natriuretic peptide receptors. PCR products were run on 2.5% agarose gel alongside a 100 bp step ladder (Promega Corporation, Madison, WI). All products resolved in the expected location. Bands were excised after imaging and sent off for sequencing (Northwoods DNA, Inc., Solway, MN) to further verify results. Sequences were entered into NCBI's BLAST and came back with 100% homology for respective natriuretic peptide and receptor genes.

Table 1 and Figure 2. Reverse Transcriptase PCR analysis of the Natriuretic Peptides (ANP, BNP and CNP) and Receptors (NPR-A, NPR-B and NPR-C) in HLE-B3 Cells.

mRNA expression levels were measured by quantitative RT-PCR in an experiment in which HLE-B3 cells were subjected to different stressors. The cells were grown in 20% FBS MEM and stepped down into media containing decreasing amounts of serum after 24hour periods and three different biological populations were collected at each time point. Collection points were 20% FBS MEM, 2% FBS MEM, serum free MEM, 4hr hypoxia, 24hr hypoxia, 4hours re-

oxygenation and 24hours of re-oxygenation. ***Analysis:*** Analysis of quantitative RT-PCR data was completed by calculating delta CT values using a β-actin control and a baseline standard of mRNA expression at 20% FBS MEM in normal oxygen. Values are representative of an n=6 and expressed relative to the respective baselines (20% FBS MEM) which are assigned a value of 1.0.

Figure 3. Western blot analysis of natriuretic peptide receptor protein expression in HLE-B3 cells

Protein expression for the natriuretic peptide receptors was measured by Western blot analysis as HLE-B3 cells were subjected to stress. In the experiment the cells were grown in 20% FBS MEM and stepped down into media containing decreasing amounts of serum after 24hr periods. The cells were then exposed to hypoxia (5.0% O_2) for 24hrs and then reperfused in normal oxygen for 4hrs. Collections were made after 24hrs in 20% FBS MEM, 2% FBS MEM, and serum free MEM as well as at 4 hours and 24hours of hypoxia and 4 hours of re-oxygenation. Western blots were run with an actin control and at a concentration of 10µg protein per lane.

Figure 4. Immunohistochemical analysis of natriuretic peptide receptor expression in HLE-B3 cells

Immunohistochemistry and confocal microscopy was used to determine expression of natriuretic peptide receptors on HLE-B3 cells. Control images shown include HLE-B3 cells incubated with no primary antibody but with a goat-anti-rabbit secondary and cells incubated with a pure rabbit IgG as a primary antibody. For each natriuretic peptide receptor shown, images are representative of four fields of view per receptor per population with two separate populations of cells each.

Figure 5. Analysis of natriuretic peptide induced cyclic GMP production in HLE-B3 cells

In order to determine functionality of the natriuretic peptide system in HLE-B3 cells cyclic GMP activity was measured. Individual cell populations were pre-incubated for 15 min with 25µM IBMX, a phosphodiesterase inhibitor, followed by addition of ANP, BNP and CNP in concentrations of 0.1µM, 1.0µM and 5.0µM and further incubation for 10 min. Following incubation cells were lysed and cyclic GMP activity measured using an enzyme linked immunoassay kit from Cayman Chemical (Ann Arbor, Michigan), as described under Materials and Methods. Values shown are representative of means of two separate experiments with an n=6.

Table 1. Natriuretic peptide and receptor oligonucleotide primer pairs

Gene (gene bank accession #)	Left/Right	Location	PCR Product (bp)
Actin, beta (ACTB) (NM_001101)	5'>CATCCTCACCCTGAAGTACC<3' 5'>GTACAGGGATAGCACAGCCT<3'	273-513	241
Natriuretic peptide precursor A (NPPA) (NM_006172)	5'>GATTTCAAGAATTTGCTGGACCAT<3' 5'>TTGCTTTTTAGGAGGGCAGATC<3'	209-435	227
Natriuretic peptide receptor A (NM_000906.3)	5'>GCAAAGGCCGAGTTATCTACATC<3' 5'>AACGTAGTCCTCCCCACACAA<3'	1131-1228	98
Natriuretic peptide precursor B (NPPB) (NM_002521)	5'>CGGGTTACAGGAGCAGCG<3' 5'>CTCCAGGGATGTCTGCTCCA<3'	225-297	73
Natriuretic peptide receptor B (NM_003995)	5'>CGGGAGGATGGACTTCGA<3' 5'>CATGACAACCAGCCCAGTTACA<3'	1072-1146	75
Natriuretic peptide precursor C (NPPC) (NM_024409)	5'>AGCGTGGGCTCGCCTT<3' 5'>CTTGTTGGCTCCTTTGTATTTGC<3'	249-309	61
Natriuretic peptide receptor C (NM_000908)	5'>GGAAGACATCGTGCGCAATA<3' 5'>GATGCTCCGGATGGTGTCA<3'	950-1028	79

The primers used for all reverse transcriptase and quantitative real time PCR experiments were previously published primers by Rollín, et al. [10].

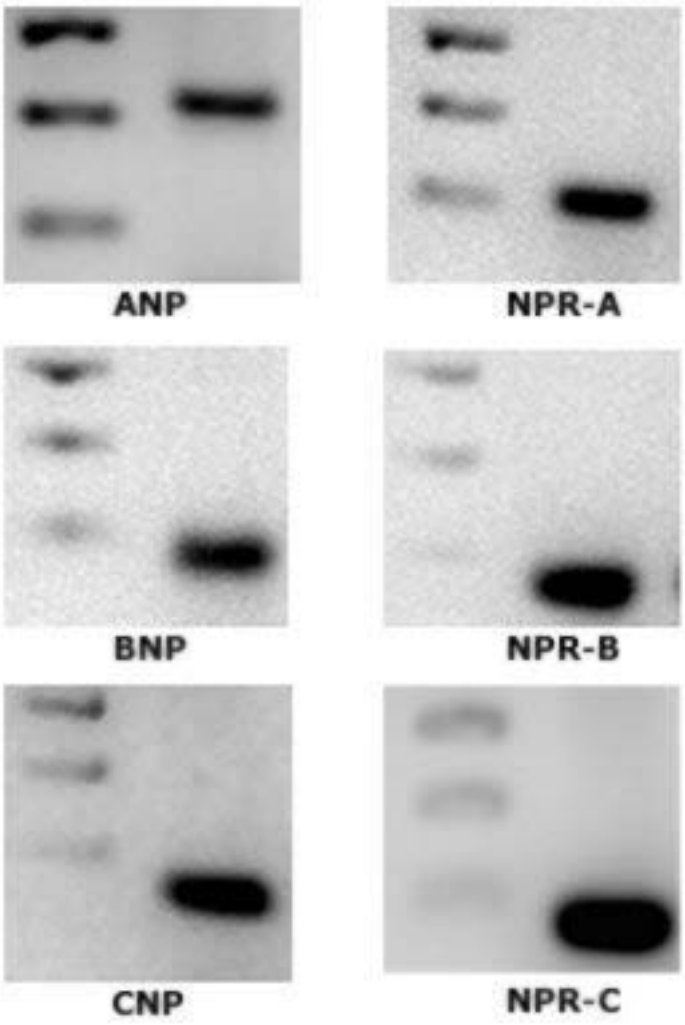

Figure 1. Reverse Transcriptase PCR analysis of the Natriuretic Peptides (ANP, BNP and CNP) and Receptors (NPR-A, NPR-B and NPR-C) in HLE-B3 Cells.

Table 2. Quantitative RT-PCR analysis of mRNA expression for the natriuretic peptides and receptors in HLE-B3 cells

	ANP	BNP	CNP	NPR-A	NPR-B	NPR-C
2% Serum	1.50±0.26	1.09±0.22	1.80±0.09	1.12±0.06	2.27±0.14	1.05±0.26
Serum Free	3.30±0.13	0.42±0.16	2.65±0.17	2.18±0.34	4.15±0.08	1.30±0.22
4hr hypoxia	2.96±0.38	0.44±0.18	2.78±0.31	1.25±0.56	3.51±0.12	1.13±0.29
24hr hypoxia	1.87±0.48	0.51±0.16	2.13±0.14	1.19±0.06	2.38±0.19	0.57±0.09
4hr re-oxygenation	0.90±0.25	0.42±0.43	1.04±0.24	0.50±0.16	0.44±0.16	1.50±0.26
24hr re-oxygenation	1.44±0.51	0.37±0.63	0.99±0.28	0.49±0.15	0.64±0.09	1.08±0.39

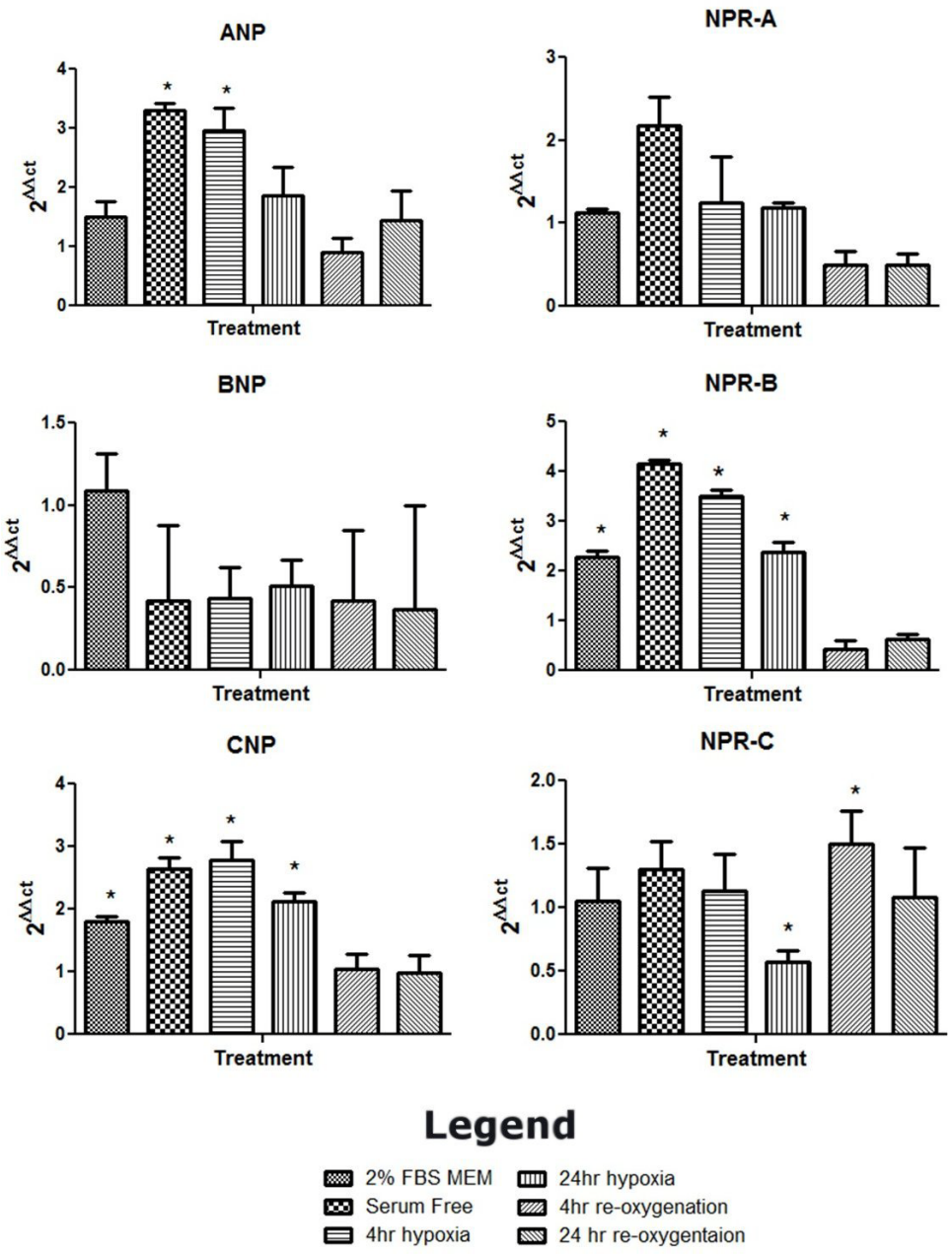

Figure 2. Reverse Transcriptase PCR analysis of the Natriuretic Peptides (ANP, BNP and CNP) and Receptors (NPR-A, NPR-B and NPR-C) in HLE-B3 Cells.

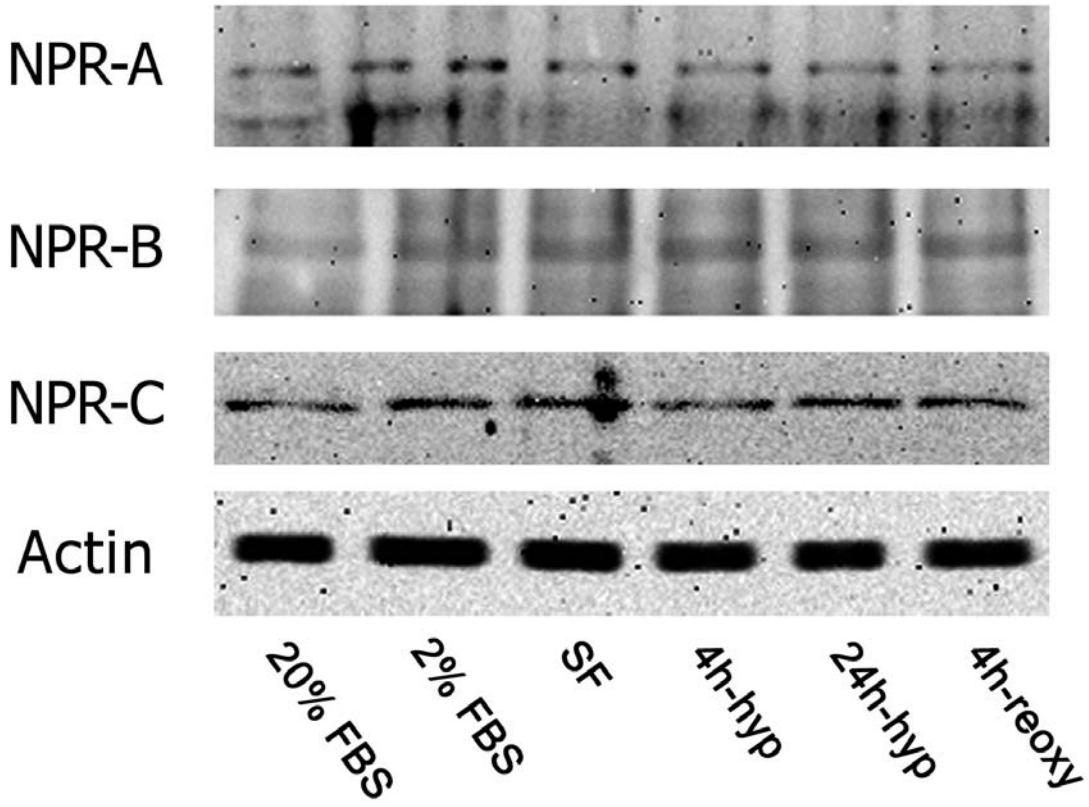

Figure 3. Western blot analysis of natriuretic peptide receptor protein expression in HLE-B3 cells

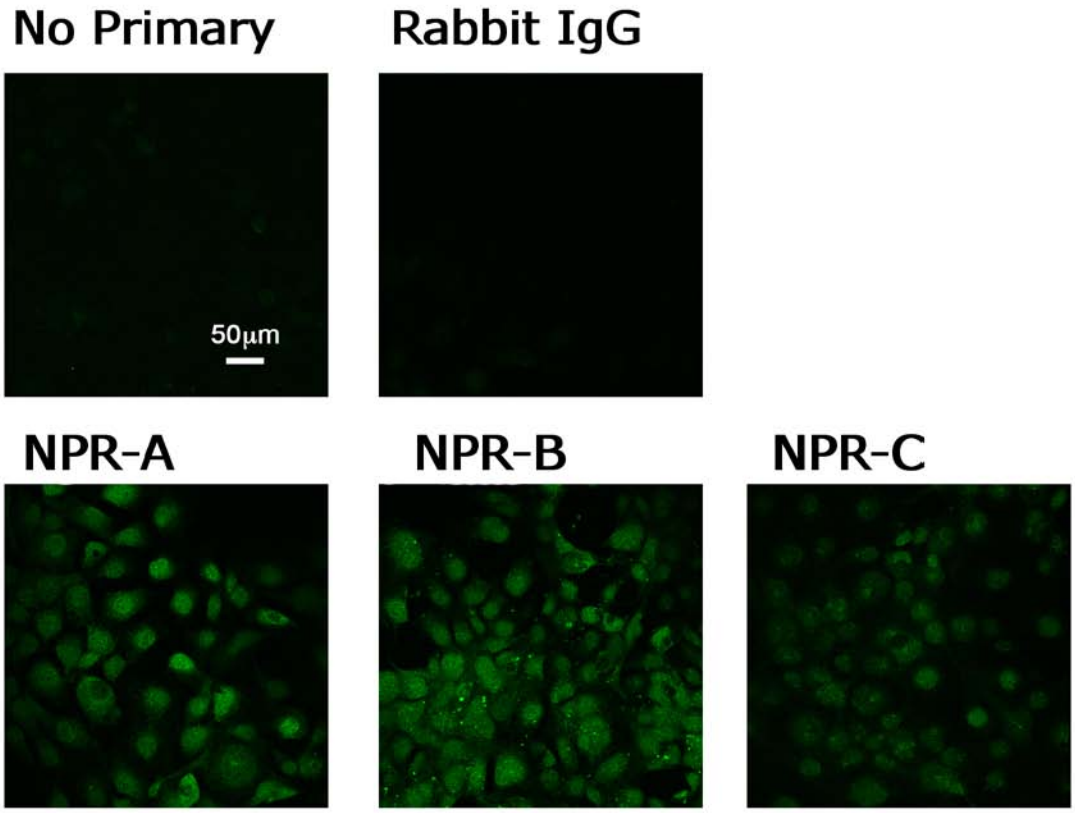

Figure 4. Immunohistochemical analysis of natriuretic peptide receptor expression in HLE-B3 cells

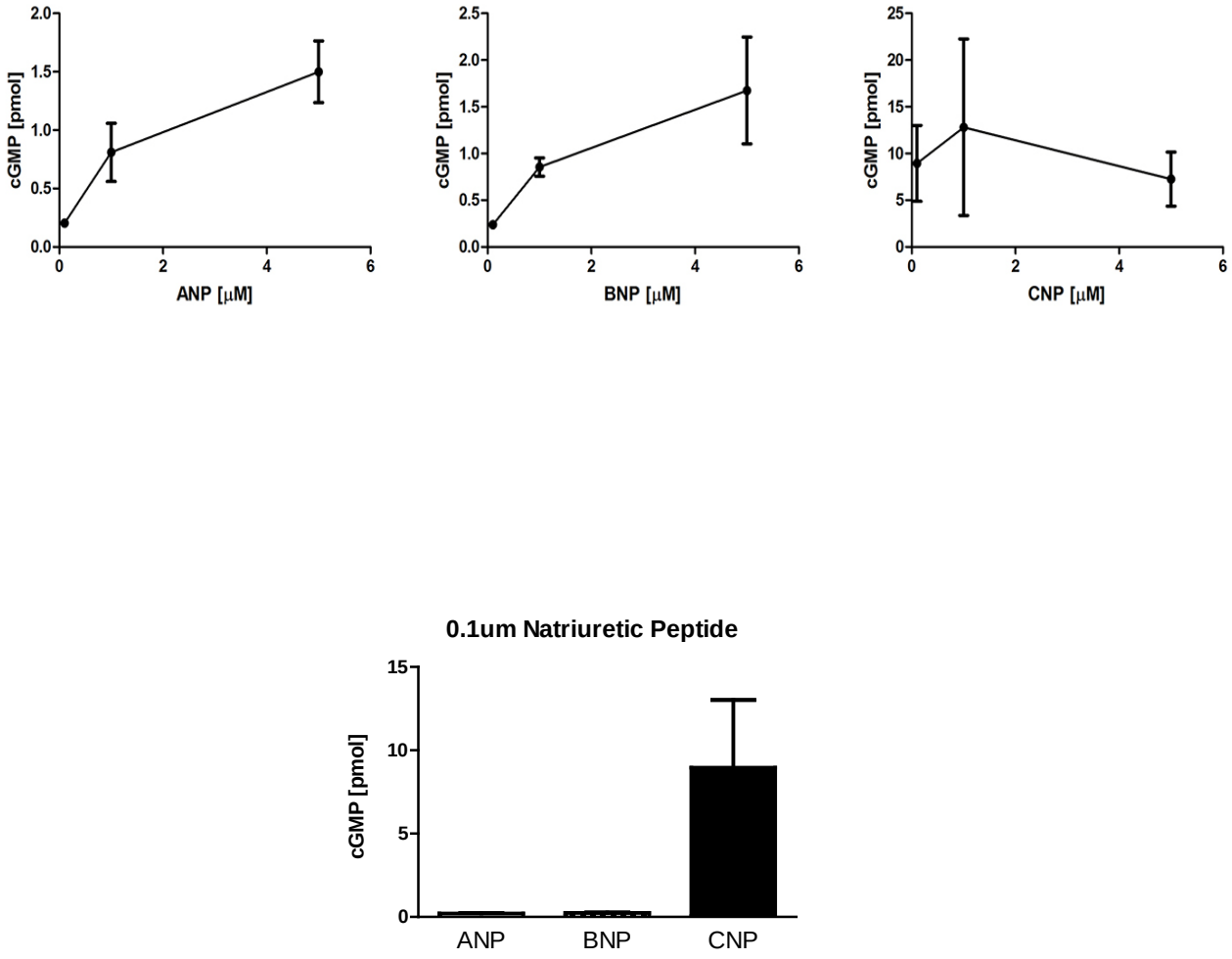

Fig.5. Analysis of natriuretic peptide induced cyclic GMP production in HLE-B3 cells

CHAPTER III

DISCUSSION AND FUTURE DIRECTIONS

Until now, the presence of the natriuretic peptide family was unknown in the human lens. There have been studies of their presence and function in other body systems, but relatively few studies demonstrating their existence in regions in the eye. Reported here is the first demonstration of the incidence and functionality of the natriuretic peptide system in human lens epithelial cells.

The natriuretic peptide family plays an important role in normal human physiology. These polypeptide mediators have a range of roles throughout the body, including their well known role in extracellular volume and blood pressure homeostasis. However, natriuretic peptides effects are not just limited to homeostasis and also involve autocrine and paracrine regulatory actions which affect processes including growth and function of cells [28].

In this study mRNA expression was demonstrated and quantified for all three natriuretic peptides and receptors in cultured human lens epithelial cells. Although most had stable expression a few displayed increases in expression when subjected to stressors. Studies are still needed to identify all of the factors that can affect mRNA expression of the natriuretic peptides and receptors. However, the aforementioned findings are supported by other studies, primarily

in the cardiovascular system, which have demonstrated that transcription of the natriuretic peptides can be affected by, α-adrenergic agonists, endothelin, prostaglandins, growth factors, vitamin D, retinoids, glucocorticoids and other factors. Hypoxia, stretching of cardiac myocytes, and cardiac volume overload also cause changes in natriuretic peptide expression [14].

Western blotting analysis to detect and demonstrate expression profiles for the natriuretic peptide receptors and particularly the natriuretic peptides themselves proved to be difficult. We suspect that the antibody used for the natriuretic peptide receptors did not have strong specificity and that the receptors were found in low levels thus resulting in inconclusive blots. It appears that other researchers have had similar problems in detection as this is the first known demonstration of the receptors by Western blotting in the ocular system. Though attempts were made, the peptides were undetectable by Western blotting, immunohistochemical imaging or enzyme-linked immunoassays. There are several reasons for the inability to find the peptides by these conventional means. One of the possibilities is the potential role of the peptides as neurotransmitters, if this indeed their function it could mean that they are likely to be rapidly synthesized and degraded, resulting in low steady-state concentrations [13]. Also, rates of peptide synthesis is the lens are likely extremely low in general. Other researchers have resorted to the use of RIA to detect the natriuretic peptides in their systems with success, and this method may be implemented in future studies. Finally, although mRNA for the peptides is clearly being transcribed and the lens epithelial cells respond to them, we do not yet know the fate of these transcripts after their production. It is possible that the proteins might act in an either autocrine or paracrine role. It is also reasonable to think that perhaps, although the mRNA is being formed, synthesis of the encoded proteins doesn't occur and instead protein is produced by other ocular cells and then act to exert effects on surrounding cells.

Of the natriuretic peptides, CNP evoked the largest increase in cGMP production. As mentioned previously, this finding mirrors that of Coca-Prados's work in ciliary epithelial cells and Pang's studies in ciliary muscle cells and trebecular meshwork cells [8,20]. A possible reason for CNP producing such a large induction of cGMP production may very well be that the NPR-B receptor subtype is the primary receptor in these ocular systems, as well as in the lens [21]. CNP is of endothelial and renal cell origin and has a wide distribution in the vasculature, brain, bone, epithelium and other tissues. It is thought to act via a paracrine mechanism and lacks the natriuretic and diuretic effects of both ANP and BNP [29]. CNP instead plays a vasoactive and antiproliferative role [30]. CNP has been implicated in regulating transmitter release, synaptic transmission and neuronal survival in the nervous system as well as being potentially involved in neural development. CNPs exact physiological functions in the nervous system and its impact on neural development are not known [31]. However, in unpublished studies we have demonstrated a lack of eNOS in the lens, therefore, cGMP formation by CNP may compensate for the lack of NO production in the lens.

Most of what is known about the structures and functions of the natriuretic peptides is from cardiovascular research. The role of the natriuretic peptides in the ocular system is unknown although possible roles have been suggested including anti-angiogenic factors and regulators of intraocular pressure [32, 33]. In the cardiovascular system the primary role of the natriuretic peptides is in volume and pressure regulation. In the heart ANP is primarily expressed in the atrium whereas BNP is found mostly in the ventricle. CNP is usually described as being present primarily in the CNS but it also exists in the endothelium, macrophages and cardiac fibroblasts. ANP and BNP are released in the heart in response to muscle stretch and are important in regulation of systemic vasculature permeability, cardiac growth, cellular

proliferation, cardiac hypertrophy and renal water and sodium excretion. Aside from acting as circulating hormones they also act as local autocrine and paracrine factors. In heart failure the three NPs have actions of diuresis, natriuresis, vasodilatation, inhibition of aldosterone synthesis and renin secretion as well as playing a role in regulation of blood pressure and volume. The natriuretic peptides increase cGMP in myocytes, myocardial cells, endothelial cells during ischemia and reperfusion exerting cardioprotective effects against myocardial reperfusion injury [34].

As the natriuretic peptides and receptors are not well studied in the ocular system, far less is known about their role in the eye. This study is the first known to demonstrate the presence and functionality of the natriuretic peptide system in the lens. It has been suggested that the natriuretic peptides and receptors may be involved in intraocular pressure regulation, ocular disease pathology and/or imaging processing in the retina [8, 10, 13]. However, of particular interest to this research group though is their possible role as mitochondrial protective agents. This is an especially interesting possibility as the lens, being avascular, thrives in hypoxic conditions.

Stressful conditions, which can include serum starvation and hypoxic conditions, increase formation of reactive oxygen species (ROS). Intracellular accumulation of ROS and calcium can provoke a change in mitochondrial permeability transition (MPT), through a high-conductance pore in the inner part of the mitochondrial membrane. MPT initiates intracellular cascades culminating in cell death [35]. Although the exact mechanism through which necrotic cell death occurs is not known it is thought that the change in MPT leads to a de-energization due to mitochondrial depolarization along with loss of ATP synthesis [36]. The mitochondria, however, are supplied with protective mechanisms to prevent such cell death from occurring.

Protective signals are transmitted to the mitochondria via signaling pathways including those using cGMP, which may interfere with mechanisms of cell death. Specific details of this chemistry have been modeled in an excellent review by Garcia-Dorado et al. [37]. In brief, cGMP can be synthesized by two different types of guanylyl cyclases (GCs): the first is a nitric oxide (NO) sensitive GC which can be cytosolic or soluble. The second type of GC are particulate GCs which are integral membrane proteins of the plasma membrane. The natriuretic peptide receptors NPR-A and NPR-B are particulate GC's and are stimulated by the natriuretic peptides [4]. The cGMP signaling caused by the peptides and receptor combination can be modulated in part by the availability of the natriuretic peptides (as well as by nitrous oxide availability). The third natriuretic peptide receptor, NPR-C, has no GC activity but instead acts as a clearance receptor for the natriuretic peptides, binding to each with varying affinities [23]. Increased production of cGMP can have several effects including: natriuresis, inhibition of renin and aldosterone, vasorelaxation, antihypertensive effect, lusitropism, antihypertrophy, inhibition of fibrosis, cytoprotection, inhibition of platelet aggregation, antiapoptosis and anti-inflammatory effects [28].

This project demonstrated the functionality of the natriuretic peptides and natriuretic peptide receptors by utilization of a cGMP assay. All three peptides elicited an increase in cGMP production with CNP showing the highest potency. Although the role of the natriuretic peptides family in the lens is unknown it is possible that their presence and subsequent cGMP activities are acting to compensate for the lack of eNOS production in the lens. In the current working model of the lab, hypoxic conditions lead to stimulation of erythropoietin and finally to the production of nitric oxide (NO). NO production leads to nitric oxide-sensitive guanylyl cyclase activation producing cGMP and ultimately leading to MPT pore stabilization and thus

mitochondrial protection. (Figure 6). Unpublished studies conducted within the lab it has been demonstrated via Western blotting that eNOS is not present in the human lens epithelial cell lines HLE-B3 or SRA 01/04. (Figure 7). We therefore hypothesize that a plausible role of the natriuretic peptides within the lens is to compensate for the lack of eNOS (thus protection via nitric oxide). That is, natriuretic peptides replace the requirement for eNOS and act to protect the mitochondria against depolarization.

Another theory is that the natriuretic peptides are involved in the regulation of lens volume. As the natriuretic peptides are involved in fluid volume, osmosis and pressure regulation throughout the body it is not unlikely that they are involved in the same processes within the lens [4]. Lens transparency is dependent on an ordered tissue architecture which must be well maintained. Any disruption within the architecture of the lens can cause light to scatter resulting in difficulty in imaging and eventually lens cataract. Hence the volume of the fiber cells that make up the bulk of the lens needs to be tightly regulated if lens transparency is to be preserved. Lens volume is regulated via regulation of ion concentrations. Decreases in volume can be controlled by the loss of K^+ and Cl^- ions and obligatory water loss. Channels and transporters involved in expulsion of these ions include of K^+ and Cl^- channels as well potassium– chloride cotransporters (KCCs). In contrast, increases in lens volume can be driven by the intracellular accumulation of K^+, Na^+, and Cl^- ions. The uptake of these ions is mediated by transporters such as the sodium–potassium–chloride cotransporter (NKCC) [38]. The natriuretic peptides and receptors may be involved in this regulation as this is a primary function of the family.

This study has provided a first look at the identification and characterization of the natriuretic peptide (NP) family; atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP),

and C-type natriuretic peptide (CNP), natriuretic peptide receptor A (NPR-A), natriuretic peptide receptor B (NPR-B) and natriuretic peptide receptor C (NPR-C) in the human lens epithelial cell line, HLE-B3. There is still much work to be done in elucidating the role of the natriuretic peptide system in the lens as well as in the rest of the eye.

In order to further research the natriuretic peptide family in human lens epithelial cells it is important that various methods and techniques are employed. There were difficulties conducting this research that have provided incentive to try other approaches. For instance, although there were problems in determining the presence of both natriuretic peptides and natriuretic peptide receptors by Western blotting techniques we have other methods that can be utilized in future work. In the case of the receptors, the first inclination is to try a different company for antibodies as we had a number of problems with specificity. Using different antibodies would hopefully result in a better blot with cleaner and clearer bands. The larger problem deals with providing evidence of the existence of the peptides themselves. For this situation it would most likely be best to take a cue from other researchers who have shown presence using radioimmunoassay (RIA) techniques as these provide a much higher sensitivity than Western blotting or EIA.

In order to demonstrate functionality of the natriuretic peptide system (and thereby again confirm its presence, albeit indirectly) there are several options. As our primary focus is the possible role as mitochondrial protective role of the natriuretic peptides, JC-1 analysis (which provides information about the mitochondrial membrane potential) would provide a way to determine their effect on the mitochondria. JC-1 is a cationic dye which accumulated in the mitochondria depending on mitochondrial membrane potential. When the mitochondria is polarized the JC-1 dye aggregates in the mitochondria and can be detected by a red fluorescence.

As the mitochondria depolarizes the aggregates change to JC-1 monomers thus shifting the fluorescence from red to green. Therefore, mitochondrial depolarization can be indicated by an increase in the green/red fluorescence intensity ratio [27]. We have the ability to do siRNA knockdowns of the peptides as well as the receptors. By selectively knocking down the peptides and receptors and utilizing JC-1 analysis we can determine if the natriuretic peptides or the natriuretic peptide receptors have any effect on the stability of the lens mitochondria.

In order to show conclusively that there is no eNOS production occurring in lens epithelial cells we could use a selective inhibitor of nitric oxide-sensitive guanylyl cyclases such as ODQ (1H [1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) from Sigma-Aldrich. If inhibition of these GCs has an affect on the cells it would question the validity of the data from the Western blotting for eNOS. We could also use a guanylyl cyclase activator to determine the effects upon the lens cells. All of these methods should be considered for use in future studies. For the present time though, we have provided solid evidence for the presence of the natriuretic peptide and natriuretic peptide receptors and have also demonstrated for further research on the subject.

CHAPTER III FIGURE LEGENDS AND FIGURES

Figure 6. eNOS production in human lens epithelial cell lines HLE-B3 and SRA 01/04

Western blotting analysis demonstrates the lack of eNOS production in two human lens epithelial cell lines: HLE-B3 and SRA 01/04 under multiple conditions including normal oxygen, hypoxia and re-oxygenation. Image printed with permission of P.Cammarata and J. Pack.

Figure 7. Flow chart of the natriuretic peptides and receptors involvement in mitochondrial protection.

This modified flow chart demonstrates the hypothetical process by which the natriuretic peptides and receptors exert their cGMP activities in order to provide mitochondrial protection from mitochondrial permeability transition, resulting in stabilization of the mitochondria.

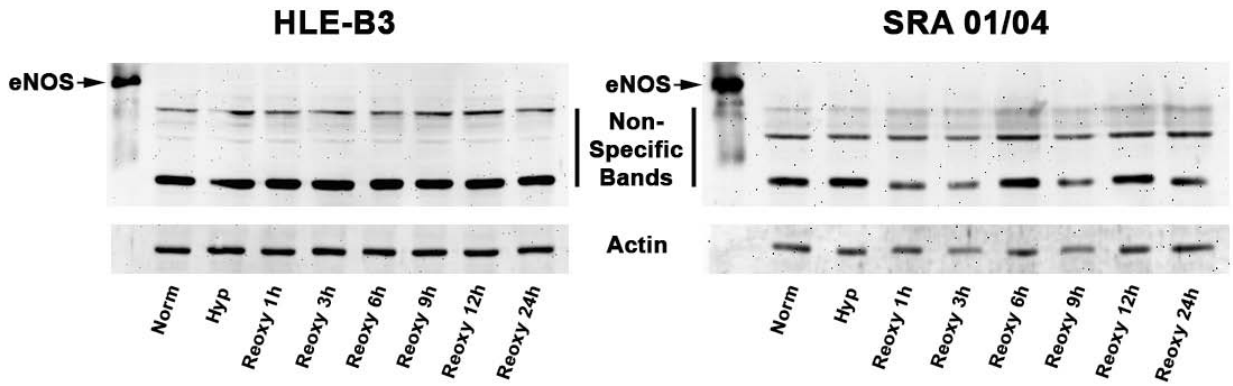

Figure 6. eNOS production in human lens epithelial cell lines HLE-B3 and SRA 01/04

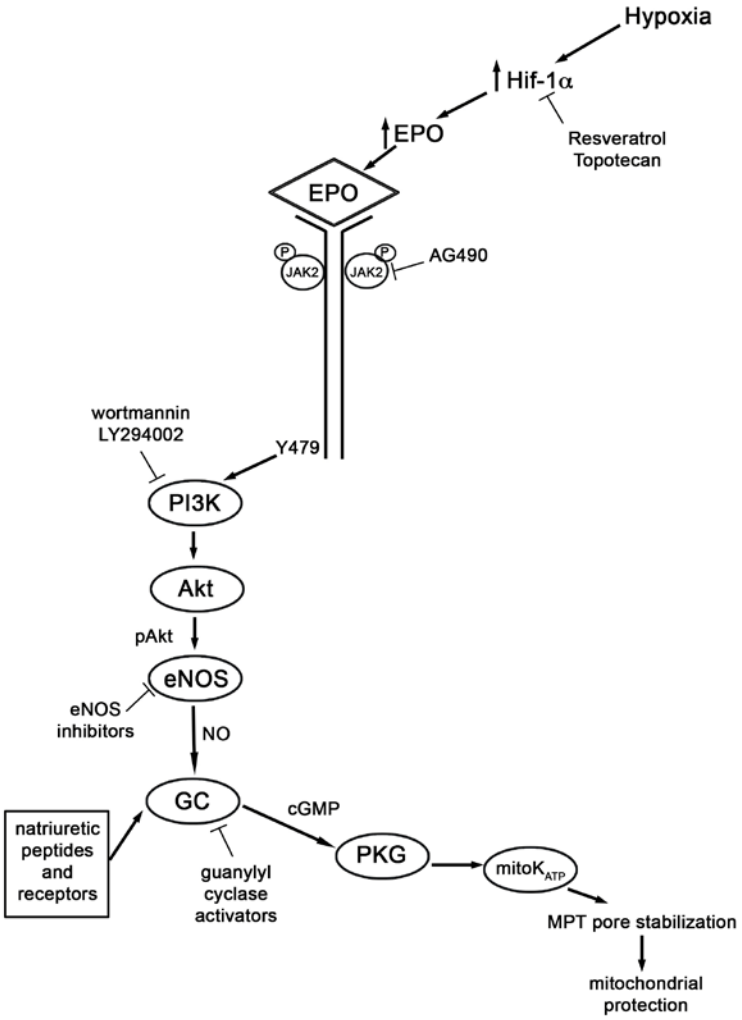

Fig.7. Flow chart of the natriuretic peptides and receptors involvement in mitochondrial protection

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