INFLUENCE OF ENVIRONMENTAL AND CHEMICAL FACTORS ON CELLULAR SIGNALING IN LENS EPITHELIAL CELLS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

The Degree Doctorate In Philosophy in the

Graduate School of The Ohio State University

By

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The Ohio State University
2007

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ABSTRACT

Cataract is the leading cause of vision loss worldwide and is currently treated only by surgical intervention. Cataract risk is influenced by many variables, including heredity, disease and oxidative stress. Oxidative stress-induced damage to lens biomolecules can accumulate with aging or exposure to environmental and/or chemical stressors. Lens epithelial cells (LEC) comprise the first cell layer exposed to these stressors, and disruption of this monolayer is considered an initiating event in cataract development. The activation of stress-induced cell signaling pathways facilitates the coordination and modulation of the LEC response to injury. Specifically, the mitogen-activated protein kinase (MAPK) family is an important group of cell signaling proteins which, when activated, initiate a cascade of cellular changes leading to differences in cell proliferation, differentiation and death. The MAPK subfamilies, extracellular signal regulated kinase (ERK), c-Jun N terminal kinase (JNK) and p38, are all found within the lens. The first objective of this research was to determine the mechanism(s) of cell death in human lens epithelial cells (HLE) exposed to environmental (ultraviolet B (UVB) radiation) and chemical stressors (hydrogen peroxide, (H_2O_2) and tertiary butyl hydroperoxide, (TBHP)). While all three stressors decreased HLE cell density, the mechanism of cell death was different. Exposure of HLE to environmental (UVB) stress resulted in changes in cell morphology, DNA fragmentation and annexin/propidium

iodide staining that were consistent with apoptosis, whereas H_2O_2 and TBHP increased lactate dehydrogenase and produced changes characteristic of necrosis. In addition, activation of stress signaling proteins such as JNK, c-Jun and DNA fragmentation factor 45 (DFF45) were observed only in UVB-treated cells. Inhibition of JNK activity increased UVB-induced cell death, suggesting that this pathway may serve a prosurvival role in HLE cells.

In addition to oxidative stress, age-related cataract is associated with changes in protein and membrane composition of the lens, including a decrease in transport properties. Gap junction intercellular communication (GJIC) is critical to both nutrient and waste transport in the lens, and can be regulated by cell stress, cell signaling pathways and dietary compounds. The mechanisms of this regulation are not clearly established, although changes in the levels of connexins, specific proteins which comprise gap junction channels, is likely involved. LEC contain connexin 43 (Cx43), a common epithelial connexin, as the major component of gap junctions in this cell layer. Both retionoids and carotenoids have previously been shown to upregulate gap junction intercellular communication (GJIC) and increase Cx43 levels in *in vitro* cell culture models, but have not been investigated in primary LEC. Age-related cataract prevalence in canine species closely parallels that in humans, making the canine lens a suitable model for study. The second objective of this research was to examine the role of cell signaling pathways and the dietary compounds, retinoids and carotenoids, in modulating

GJIC in canine LEC. Treatment with the protein kinase C (PKC) activator, TPA, significantly decreased GJIC in canine LEC by 80% as compared to controls and increased Cx43 phosphorylation as assessed by immunoblotting. Pretreatment of cells with two PKC inhibitors, GF109203X and Gö6976, partially restored TPA-inhibited GJIC by 40% and 60%, respectively, and reduced Cx43 phosphorylation. Expression of calcium dependent PKC isoforms was detected in canine whole lens and LEC. Retinoic acid increased expression of Cx43 and enhanced GJIC in canine LEC.

In summary, LEC utilize stress-induced signaling pathways to respond to environmental and chemical stressors. The activation of cell signaling pathways dictates the response of LEC to these stressors. Gap junctions provide a potential avenue by which regulatory molecules pass between cells of the lens. Cells of the lens epithelium are well coupled by gap junctions and intercellar communication is regulated by cell signaling pathways. In addition, GJIC is regulated by dietary compounds which direct the proliferation and differentiation of these cells. The long term application of this project includes a better understanding of the molecular mechanisms and cell signaling pathways by which various stressors and dietary compounds modulate lens physiology and cataractogeneis in humans and animals.

Dedicated to my parents for their unconditional
love, support and devotion to my well being and for teaching
me to accept and expect only the best

To Chris and Natalie: my role models who stimulate
my thinking and give me hope of reaching all my dreams.

To Will, Nick and Joe for allowing me to never forget
the beauty of innocence and the joy
of finding something "new" in every day.

ACKNOWLEDGMENTS

I wish to thank my adviser, Joshua A. Bomser, for all of his support, guidance and encouragement in the completion of this degree.

I thank the members of my committee, Mark Failla, Carmen M.H. Colitz and Tatiana Oberyszyn for their thoughtful discussions and devotion to increase the quality of my work.

I thank my labmate, Elizabeth Clubbs, and technician, Amanda Agler for their thoughtful contributions to this research.

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1. Long AC, Colitz C.M.H, Bomser JA. (2007). Regulation of gap junction intercellular communication in primary canine lens epithelial cells: role of protein kinase C. Curr Eye Res 32: 223-231.

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3. Long, A.C, Ching S., Quan, N. , Boileau, T. and Bray, T.M. (2001) Prevention of type 1 diabetes with dietary antioxidant cocktails in non-obese diabetic mice. Free Radical Biology and Medicine. 31(S1): S34 (abstract).

FIELD OF STUDY

Major Field: The Ohio State University Graduate Program in Nutrition

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CHAPTER 1

INTRODUCTION

1.1 Background

Cataract development is a complex, multifactorial process, and represents the leading cause of blindness in the world. In addition to age, oxidative stress and nutrition play key roles in cataract development. Various dietary antioxidants may help to combat oxidative-stress induced cataract development, although epidemiological and experimental studies on the identity, quantity and length of supplementation with these compounds have been inconsistent. While conflicting reports make it difficult to establish a clear protective role for any one nutrient or combination of nutrients, dietary intervention appears to be most effective when sustained for several years or decades prior to cataract development. Independent of their antioxidant abilities, several classes of dietary compounds such as carotenoids, retinoids, green tea catechins and soy isoflavones, have been shown to increase gap junction intercellular communication (GJIC). Disruption of GJIC occurs as an age-related consequence in the lens, and can lead to alteration in lens physiology which leads to cataract.

The lens epithelium is the first cell layer exposed to cell stress and plays a key role in maintaining lens homeostasis. Lens epithelial cells (LEC) utilize a variety of cell

signaling pathways including the mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways to respond to stressors and regulate basic cellular functions such as proliferation, differentiation and death. Cultured LEC provide important models by which to study cataractogenesis, and may provide more consistent results than *in vivo* models. Primary canine LEC can be easily maintained in culture with the optimization of several basic conditions and provide a valuable model for the study of GJIC and cataractogenesis.

The overall goal of this work is to understand the cell signaling pathways utilized by LEC to respond to a variety of stressors and the role that nutrition plays in modulating these responses. There are two specific objectives associated with this goal. First, the role of environmental and chemical stress and gap junction communication as mediators of cell death in both immortalized human and primary canine LEC will be examined. A second objective is to determine if retinoids and carotenoids, as well as other ocular antioxidants, can modulate stress-induced cell signaling or gap junction communication in LEC. Our long term objectives are to understand the contribution of environmental and chemical stress to the progression of cataract in humans and animals and to elucidate the potential mechanisms by which retinoids, carotenoids and other dietary antioxidants might exert their protective effects in cataract development.

1.2 Hypotheses

Our central hypotheses include: **a)** Environmental and chemical stress induce damage to the lens epithelium and lead to distinct forms of cell death in human LEC; **b)** Gap junction intercellular communication (GJIC) is present in primary canine LEC and can be modulated by cell signaling pathways; and **c)** retinoids and carotenoids modulate GJIC

via an antioxidant-independent mechanism involving the regulation of connexin 43 protein in primary canine LEC.

1.3 Specific Aims

There are three specific aims of this research.

Specific Aim 1: *Identify and characterize the mechanism of cell death in human lens epithelial cells (HLE) exposed to environmental (UVB) and chemical (H_2O_2 and TBHP) stress.*

Specific Aim 2: *Examine the modulation of gap junction communication by cell signaling pathways in primary canine lens epithelial cells exposed to chemical stress.*

Specific Aim 3: *Determine the role of retinoids and carotenoids in modulating cell proliferation, cellular signaling and GJIC in canine lens epithelial cells.*

CHAPTER 2

LITERATURE REVIEW

2.1 ANATOMY AND PHYSIOLOGY OF THE MAMMALIAN LENS

2.1.1 Biology and Metabolism

The anatomy of the ocular lens is appropriately and exquisitely designed to perform its major biological function of transmitting and focusing light onto the retina for subsequent perception of visual stimuli and regulation of circadian rhythms [1]. In order to perform this function, the lens must remain transparent, a characteristic achieved by a precise arrangement and regulation of volume within lens cells. These cells lack organelles and contain a high concentration of stable proteins, resulting in a higher refractive index than the fluids surrounding the lens. This difference in refractive index enables light to be focused to specific photoreceptors in the retina, where light is converted into an electrical stimulus perceived by the brain.

The lens, along with the cornea, anterior chamber, iris, and posterior chamber, comprises the anterior segment of the eye. The lens is bordered anteriorly by the aqueous humor, posteriorly by the vitreous humor, and is laterally suspended within the eye by zonular fibers, thin extensions of the nonpigmented epithelium of the ciliary body (**Fig 2.1**). The aqueous humor is a complex solution of nutrients, growth factors and mineral

ions, and provides nutrition to the lens. Aqueous fluid is not a simple dialysate of the plasma, but is produced by the ciliary body, which utilizes active transport mechanisms to concentrate specific ions, vitamins, and other substances from the plasma, including ascorbic acid, lactate and amino acids. In contrast to the aqueous humor, the vitreous humor is a viscous, gel-like fluid composed largely of collagen, hyaluronic acid and other matrix proteins. The vitreous humor functions in the maintenance of volume and shape of the globe and may influence differentiation of epithelial cells [1].

The lens is composed of both epithelial cells and fiber cells. A single layer of epithelial cells, collectively referred to as the lens epithelium, is located solely at the anterior pole of the lens. Lens epithelial cells (LEC) are divided into three zones, central, germinative and equatorial (**Fig 2.2**). At the midpoint of the lens, the lens equator, LEC differentiate, lose all intracellular organelles, and elongate to form the second major cell type of the lens, fiber cells. Fiber cells are continually laid down over time, similar to rings on a tree. Newly differentiated, superficial fiber cells are located in the lens cortex, whereas the oldest fiber cells are compacted in the center of the lens. While epithelial and fiber cells are distinct cell types, they are connected both physically and functionally in the lens by a vast network of small pores called gap junctions. These junctions facilitate the transport of water, nutrients and wastes throughout the lens, regulating lens transparency and homeostasis [2, 3]. The lens is an avascular tissue, lacking both blood vessels and innervation by sensory nerves. It is composed of approximately 1/3 protein and 2/3 water, as well as a small amount of lipid, mostly confined to cellular membranes. The protein content of the lens is one of the highest reported in tissues [3]. Ninety percent of lens proteins are water soluble and ten percent are water insoluble, the

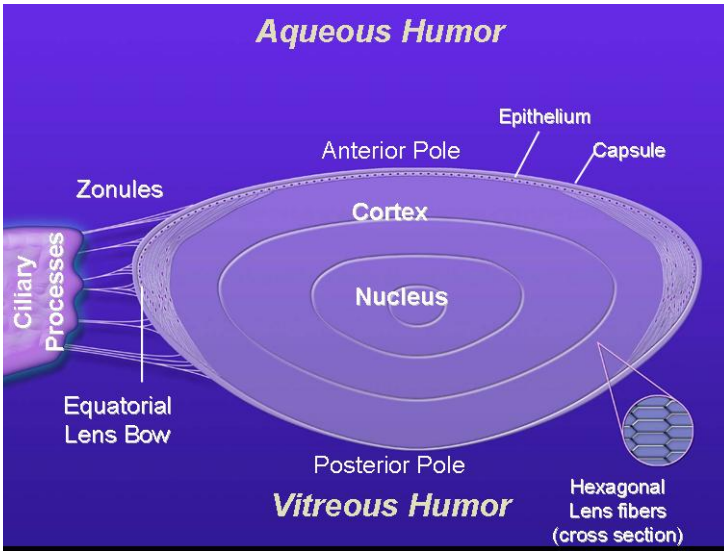

Figure 2.1: Structure of the Ocular Lens
The lens is bordered anteriorly by the aqueous humor, posteriorly by the vitreous humor and is laterally suspended by zonular fibers. The lens is completely encircled by a proteinaceous membrane, the lens capsule. A single monolayer of cuboidal epithelial cells is found at the anterior pole. Epithelial cells migrate along the circumference of the lens and divide and differentiate into lens fiber cells at the equatorial region of the lens, the lens bow. Superficial fiber cells are found within the cortex of the lens, whereas older fiber cells are compacted in the center, the lens nucleus.

latter including membrane proteins, cytoskeletal proteins, and aggregates of crystallin proteins. The crystallins are a unique set of stable proteins divided into alpha, beta and gamma families. In addition to its structural role, alpha crystallin has been identified as a molecular chaperone and may reduce oxidative-induced stress to lens proteins with age or environmental stress. Alpha crystallin is not lens specific, but is located in a variety of other tissues, including skin, lung and brain [5].

The oxygen tension in and around the lens is low, with reported values close to 15mmHg or 2% O_2 [6]. Consequently, most of lens metabolism is achieved via anaerobic respiration. Aerobic respiration, however, is utilized by those cells with mitochondria, including lens epithelial cells and superficial fiber cells [1]. The derivation and characteristics of cell types in the lens are described further.

2.1.2 Embryology and cell types

The lens is embryologically derived from surface ectoderm. Lens morphogenesis is initiated with a thickening in the surface ectoderm, the lens placode, which invaginates and pinches off to form the lens vesicle. Cells lining the anterior surface of the lens vesicle remain as LEC, and will divide and differentiate throughout the life of the organism. Cells at the posterior surface of the lens vesicle elongate and gradually obliterate the hollow of the vesicle. These elongated cells, called primary lens fiber cells, comprise the oldest region of the lens, the embryonic nucleus [2].

Lens Capsule. The lens capsule encircles the entire lens, and its thickness is greater anteriorly than posteriorly. In the adult dog, the thickness of the anterior lens capsule is reported as 50-70μm, while the posterior lens capsule is 2-4μm (Colitz, CM

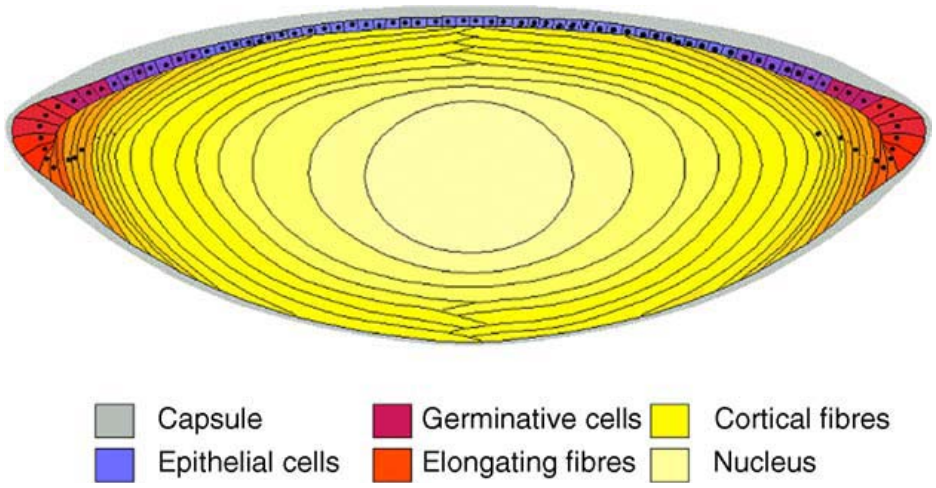

Figure 2.2: Regions of the Lens
The lens is surrounded by a protein capsule (gray) and the lens epithelium is divided into central epithelial cells (blue), germinative epithelial cells (fuscia) and elongating fiber cells (orange). Cortical fiber cells (dark yellow) are superficial to older, fiber cells (nucleus). [4]

personal communication). The lens capsule is composed of structural proteins such as type IV collagen, laminin, entactin and heparin sulfate proteoglycans. It serves as the basement membrane and is synthesized by LEC at the anterior surface. The lens capsule is freely permeable to most compounds and thickens with age [1].

Lens epithelial cells. LEC are polarized epithelial cells oriented such that their apical side faces the cortical fiber cells below, whereas their basal side faces the lens capsule. LEC have typical epithelial characteristics including a cuboidal appearance and tight packing. The diameter of human LEC is reported as 9-17μm [2]. Three major regions of the lens epithelium are defined as central, germinative, and equatorial. Central LECs do not undergo mitosis, however, they are essential for transport of solutes between the lens and aqueous humor and for the secretion of capsular material. Cells in the germinative region of the lens epithelium surround cells of the central region, and divide by mitosis. Daughter cells from this region migrate along the circumference of the lens, until they reach the lens equator, at a region called the lens bow. At the lens bow, equatorial LEC begin to terminally differentiate into lens fiber cells. These cells elongate, lose all intracellular organelles, including nuclei and mitochondria, and become interconnected via unique "ball and socket" type connections [2]. Degradation of organelles occurs in a process similar to cellular apoptosis, involving enzymes such as caspases [7].

All regions of the lens epithelium have similar activity of telomerase, a ribonucleoprotein enzyme complex that adds TTAGGG repeated sequences onto the shortening ends of DNA, maintaining telomere length and preventing cell senescence. Interestingly, increased telomere length has been reported in cataractous lenses as compared to normal epithelial cells [8].

The lens epithelium is the major site of transport, metabolism, detoxification, and defense in the lens. Active transport of ions across the lens epithelium from the aqueous humor creates a gradient of sodium and potassium ions so that potassium is pumped in and sodium is extruded out of the cell. Three isoforms of Na+/K+ ATPase are found in the epithelium, as well as three carrier systems for the transport of basic, neutral, and acidic amino acids [1, 2]. Taurine is a unique amino acid accumulated by LEC may have antioxidant properties in the lens [9]. LEC contain an active ubiquitin-dependent proteolysis pathway and a high concentration of glutathione and NADPH [10].

Lens fiber cells. Mature lens fiber cells, lacking organelles, are metabolically inactive cells **(Fig 2.1)**. Lens fiber cells are continually formed throughout life, and, in contrast to organs such as the skin, older cells are not sloughed but compacted in the lens center. The oldest fiber cells were laid down early in embryological development, in the lens nucleus [3]. Lens fiber cells accumulate a group of long-lived proteins called crystallins, which have half-lives in the decades. Lens fiber cells rely on anterior epithelial cells to receive all nutrition and to eliminate wastes [3].

2.1.3 Transport properties

The ocular lens has been described as a syncytium, due to the intricate cellular connections between epithelial cells as well as between epithelial and fiber cells. Research has shown that this vast network of communication, achieved by individual channels called gap junctions, creates a current flux that directs ions and nutrients inward at both poles (anterior and posterior) and outward at the equator [11-13] (**Fig. 2.3**). This flow through the lens appears to be largely due to the transport of sodium ions, which

draws water flow simultaneously [14]. The relationship between gap junctions and lens physiology is discussed in section 2.4.

2.2 CATARACTOGENESIS

2.2.1 Definition, Biochemistry and Development

Cataract is defined as an opacification of all or part of the lens [1,3]. Cataract typically results from alteration of the biochemistry of lens macromolecules, leading to changes in the regular spacing and volume of cells in the lens. Typical biochemical changes in the lens during cataractogenesis include large, high molecular weight insoluble protein aggregates, blue fluorescence and disintegration of lens fiber plasma membranes and peroxidation of lipids.

Cataract development is a multifactorial process, with various etiologies including inherited and age-related. In addition, cataract may develop as a consequence of disease, injury or exposure to toxins [15]. Both low income and low educational level are associated with an increased rate of cataract development, suggesting a role of socioeconomic or lifestyle factors in cataract development. Approximately two thirds of global blindness is represented by the combined countries of India, China and Africa [16], emphasizing a disproportionate incidence of vision loss in developing countries. In India, cataract prevalence is five times higher by 60 years of age than in the USA [17]. In addition to humans, other species are affected by cataract. Cataract is one of the most frequently diagnosed ocular diseases and a leading cause of blindness in dogs, with age-related cataract prevalence in dogs being similar to that in humans [18]. Cataract development is also observed in farmed fish, with serious economic consequences in European markets [19]. Age-related cataract is the most common cause of visual

impairment and the leading cause of preventable blindness worldwide [20]. Age-related cataracts are classified on the region of the lens affected, with nuclear, cortical and posterior subcapsular (PSC) being the most common [1]. In a survey of current cataract patients, it was confirmed that blindness is a highly feared aging ailment [21]. The aging population is expected to increase dramatically in the next decade in the United States, making cataract development a major disease and health concern [22].

Phacoemulsification cataract extraction followed by implantation of an intraocular lens (IOL) is the only current treatment option for cataract and is the most common surgery performed in the United States. The cost of this treatment and its complications represents an annual cost of billions of dollars in health care. In the United States, this procedure is the most frequent surgery performed in people aged ≥ 65 years, with an estimated annual cost of billions of dollars [23]. Although 1.35 million cataract surgeries are performed each year, cataract represents an important cause of low vision for Black Americans [24], who are twice as likely to be blind or visually impaired as white Americans [25]. With the growing aging population, it is estimated that a delay in the necessity of this surgery by only 10 years would reduce health care costs and the prevalence of disabling vision loss [23]. Although a relatively safe and successful procedure, cataract surgery is not readily available in all areas of the world [26] and is not without complications. The most common long term complication of cataract surgery in humans and animals is posterior capsular opacification (PCO). PCO is caused by the extensive migration of residual, transformed LEC to the posterior capsule. PCO disrupts visual acuity and can occur in up to 50% of post-operative patients, especially in younger individuals [27, 28]. Both pharmacologic and surgical treatments have been developed

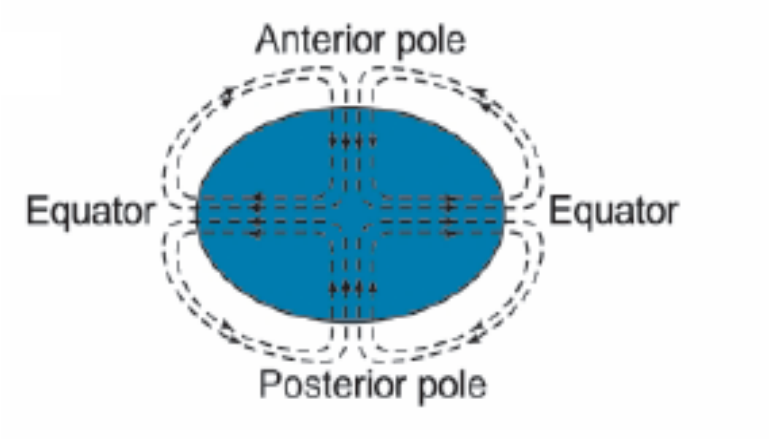

Figure 2.3: Lens Internal Circulation
Lens ion and waste transport is directed inwards at the poles (anterior and posterior) and outward at the equator [13].

to resolve this condition.

In light of the basis of cataract development and the consequences and challenges in availability of cataract surgery worldwide, intervention strategies from both biomedical and public health disciplines are warranted to deter cataract progression. The identification of modifiable risk factors that delay the development of cataract would not only lengthen the quality of life for many elderly and underrepresented populations worldwide, but also reduce health care costs associated with this condition and its complications.

2.2.2 Risk Factors

2.2.2.1 Age

Aging is a leading risk factor for cataract development. In the lens, aging is associated with changes in membrane composition, transport properties and ion permeability, as well as protein modifications [29]. These changes are discussed further.

The lens barrier. The "lens barrier" represents a critical change in lens transport dynamics with age [30]. This barrier refers to the physiological isolation of the lens nucleus from the rest of the lens, resulting in decreased nutrient transport and potential sequestration of oxidizing species in the lens center [31]. Age-related alteration in lens transport was first established by reports that older human lenses incubated with radiolabeled cysteine exhibited a decreased ability of the label to penetrate as compared to younger lenses [32]. Molecular bases for the formation of the lens barrier include alterations in cation permeability [33], changes in fiber cell membrane composition [34] and changes in gap junction properties [35].

Changes in ion permeability. The membrane potential of the lens declines with age and is linked to an increase in permeability of selective ions, such as calcium and sodium [29]. In support of this finding, dysregulation of calcium transport is a consistent finding in cataract. The calcium content of clear human lenses increases with age, and parallels the decreased activity of membrane-associated Ca-ATPase. In human lenses with cortical cataract, the total calcium content is four times higher than in clear lenses. The consequences of elevated calcium levels include damage to both protein and lipids, resulting in increased light scattering [36]. Elevated calcium activates the cysteine protease calpain, which has been shown to cleave cytostructural proteins in the lens, promoting cataractogenesis [37]. Transglutaminase, a calcium-dependent enzyme involved in cross-linking of peptides, is implicated in cataractogenesis [38]. Increased calcium levels are also associated with a blockage of gap junction intercellular communication in the lens [39].

Changes in membrane composition. The membrane composition of cells within the lens is unique compared to other tissues. Phosphatidylethanolamine (PE)-related phospholipids and a rare, saturated lipid, dihydrosphingomyelin, compose approximately 80% of lens membranes [40]. The high sphingolipid content of human lenses may confer a species-specific advantage and resistance to oxidation that allows human lenses to remain transparent in some cases until the eighth decade of life [41]. With age, the sphingolipid concentration of the lens increases, with a concurrent decrease in glycerolipid content. These changes are exacerbated in cataract. Huang and colleagues [42] reported that PE- related phospholipid content of lens membranes decreased from 16% to approximately 2% at 10 and 80 years of age, respectively, and was undetectable

in cataractous lenses. Alternatively, the amount of sphingolipids increased from 48% to 57% at 22 and 69 years of age, respectively, and reached 78% in cataractous lenses. The authors suggest these changes lead to a more ordered lipid membrane and increased lens stiffness, which subsequently alters lipid rafts, cell signaling, ion permeability, Ca-ATPase pumps and other key functions of the lens membrane.

Protein modification. A loss of glutathione and modification of lens proteins is a consistent finding in cataract. Post-translational changes in lens proteins, including glycation, carbamylation and racemization, lead to protein aggregation, light scattering and cataract. The post-translational protein changes evident in the lens have recently been reviewed and considered a model for other age- related changes in tissues [43].

2.2.2.2 Oxidative Stress

Oxidative stress refers to an imbalance in the rate of production of oxidant species versus the rate of their degradation [44]. Oxidative stress has been linked to cataract development in both humans and animals [45]. While aging is associated with a decreased ability to withstand oxidative stress, exposure of the lens to both environmental and chemical stress can exacerbate production of reactive oxygen species (ROS) which can damage lens proteins and other macromolecules.

It has been argued that a final common pathway for all of the variables associated with cataract development (UV light, diabetes, diet, dehydration) is the photo-oxidation of lens proteins and peroxidation of lipids [46]. Oxidative damage to lens crystallin proteins is particularly deleterious, since the metabolically sluggish fiber cells have limited repair mechanisms and modifications to these proteins are likely to accumulate over time [47]. Specifically, oxidative damage to lens crystallins causes protein

aggregation and a resultant increase in light scattering that can accumulate with age, leading to cataract. Furthermore, oxidative damage to lens-membrane lipids promotes the initiation and progression of age-related cataract [48, 49], and increased amounts of lipid peroxidation products are detected in both human and animal lenses with cataracts [50]. In vitro, cultured human LEC exposed to hyperoxic conditions for 48 hr undergo increased oxidation of unsaturated membrane phospholipids and changes in membrane composition that are similar to those seen in aging [51].

Oxygen tension is tightly regulated in the lens. The levels of oxygen tension in the lens cortex have been reported to be around 30mmHg, with decreasing levels closer to the nucleus [30]. In his article "Is the lens canned" John Eaton argues that low oxygen tension in the lens, similar to canned food, is designed to preserve the lens from oxidation and maintain clarity [52]. Increases in oxygen tension resulting from hyperbaric oxygen therapy lead to nuclear cataract in both humans and animals [53, 54].

Cigarette smoking, hyperglycemia and exposure to ultraviolet light increase oxidative stress and cataract risk [48]. These factors are discussed independently below.

Cigarette Smoking. Current cigarette smoking has been identified as an independent, modifiable risk factor in the development of nuclear cataract [55]. Cigarette smoking increases oxidative stress and the risk for development of incident nuclear cataract by 3-fold [56]. In a recent study of risk estimates for cataract in Australia, ocular UVB exposure explained 10% of the cortical cataract incidence in the study sample, while smoking accounted for 17% of nuclear cataract [57].

Hyperglycemia. Hyperglycemia is a risk factor for the development of age-related cataract [58]. Possible mechanisms for hyperglycemia-induced cataract include polyol

pathway disruption, lipid peroxidation, and glycation, leading to increased oxidative stress and opacification of the lens [59]. High dietary carbohydrate intake increases the incidence of cortical lens opacity in women [60], although there is no support of an association between high glycemic index and increased risk of cataract [61]. McCarty and colleagues report that risk factors for cortical cataract included duration of diabetes and lower intakes of β carotene and vitamin E, while risk factors for nuclear cataract were similar to risk of cortical cataract with the addition of lower vitamin C intakes [57].

Ultraviolet light. The skin and eyes are the only surfaces to be exposed to ultraviolet radiation (UVR). UVR includes UVA (400-320nm), UVB (320-280), and UVC (<280nm) radiation. UVC and some UVB radiation may be blocked by the ozone layer, although the continual depletion of this protective barrier may result in increased exposure to these rays [62]. High lifetime exposure to UVR is associated with cortical cataracts in humans [63]. Living in proximity to the equator [64] or at higher elevations [65] increases the risk of cataract. Cataract is generally associated with the shorter, more energetic wavelengths (UVB) as opposed to the longer wave rays [63].

Epidemiological evidence suggests that UVB exposure is a potential risk factor for cortical cataract [66]. The relationship between chronic UVB exposure and increased cataract risk was demonstrated by the Chesapeake Bay Waterman Study, an epidemiological study of individual exposure levels to UVB. In this study, UVB but not UVA, blue light, or visible light exposure was significantly linked to cortical cataract [67]. UVB radiation (280-320nm) is a potent biological source of stress to the lens epithelium and can lead to increased oxidative stress and cataract formation [68]. It is

estimated that 60% of the radiation at 320 nm is transmitted through the cornea and is absorbed by the lens, with highest toxicity at 297nm [20].

Exposure of the lens epithelium to UVB results in DNA damage, impairment of membrane pumps, lowered glutathione levels and inactivation of metabolic enzymes [68-70]. The major constituents of the human lens such as crystallin proteins, enzymes, membrane proteins, peptides, amino acids and DNA and RNA, all have their absorption maxima in the UVB and UVC regions [71]. The mechanism of UVB-induced damage to the lens is not well established, although reactive oxygen species (ROS) have been implicated as a mediator of this cytotoxicity. UVB-induced damage is enhanced by increased oxygen tension, as observed in cultured rat lenses [69]. In vitro, Andley and colleagues have shown that UVB irradiation of human crystallins leads to tryptophan degradation and the generation of hydrogen peroxide (H_2O_2) [72]. Elevated levels of H_2O_2 are reported in human cataractous lenses and in the surrounding aqueous humor, emphasizing the role of this oxidant in the progression of cataract [73]. Hydrogen peroxide induces DNA damage and leads to cataract in bovine lens epithelial cells [74].

Antioxidant defense against ROS. Reactive oxygen species can be generated both exogeneously and endogeneously within the lens, and ROS-induced damage can lead to protein modification, lipid peroxidation, and DNA damage, all of which can lead to cataract development [75]. Endogeneous sources of ROS within the lens include mitochondria, peroxisomes, lipooxygenases, NADPH oxidase and cytochrome P450. Exogenous sources include ultraviolet light, ionizing radiation, chemotherapeutics, inflammatory cytokines and environmental toxins [76]. The lens, similar to other tissues, is equipped with several lines of defense to combat ROS-induced biomolecule damage.

Major antioxidant defenses in the lens include non-enzymatic species such as vitamin C, vitamin E, lutein and zeaxanthin [77, 78, 79], as well as enzymes such as superoxide dismutase, glutathione peroxidase and catalase. In addition, the lens relies on intrinsic repair enzymes that can dethiolate protein-thiol mixed disulfides induced by oxidative stress. Two such protein/enzyme repair systems, the GSH-dependent thioltranferase and NADPH-dependent thioredoxin/thioredoxin reductase systems, have been identified in the lens and may be upregulated in response to oxidative stress [76].

Reduced glutathione (GSH) maintains lens proteins in their reduced state to preserve lens transparency [10]. GSH is found in high concentrations within the lens, although aging is associated with a decrease in GSH and a subsequent increase in protein oxidation. It has been reported that, with age, more that 60% of GSH is depleted [45] and over 50% of methionine and nearly all cysteine moieties are oxidized leading to disulfide crosslinking in the water insoluble protein fraction of the lens [80].

2.2.3: Role of LEC

Proper growth and differentiation of the lens is necessary for lens homeostasis, as indicated by reports that birth weight at one year is a predictor of cataract development later in life [81]. In support of this finding, Harocopos and colleagues found that cataract is associated with areas of higher cell density in the lens epithelium [82]. Dysregulation of LEC proliferation by induction of cell death is likely to disrupt the normal differentiation process and lead to changes in lens homeostasis.

Lens epithelial cell death. Damage to LEC is a precursor to protein/membrane damage to underlying fiber cells [83]. The mechanism of LEC death has significant effects on lens physiology. LEC apoptosis has been identified as an initiating factor in the

development of cataract [84]. Li and colleagues reported a greater percentage of apoptotic cells in cataractous capsular epithelial surgical samples as compared to cells from normal donors [85]. Various cell stressors have been shown to result in lens epithelial cell apoptosis, including UV light and hydrogen peroxide. The response of LEC to cell stress is mediated by cell signaling pathways.

Stress Signaling Pathways. Intracellular signaling pathways play a key role in mediating a variety of cellular responses, including the response to stress stimuli. The induction of apoptosis by ROS is mediated, in part, by the mitogen-activated protein kinase (MAPK) pathway [86]. MAPK's are a family of serine/threonine kinases that respond to a diverse group of stimuli and regulate key cellular processes such as growth, migration, differentiation and death. Three major MAPK pathways have been identified: extracellular signal regulated kinase (ERK 44/42), c-Jun N-terminal kinase (JNK) and p38 MAP kinase. The lens epithelium possesses all three of these signaling cascades [87], emphasizing its ability to respond to a variety of stressors. Of the three cascades, JNK activation has been shown to be a prerequisite for apoptosis in several cell lines [88]. Activation of JNK leads to the phosphorylation of transcription factors, including c-Jun, which regulates genes involved in cell proliferation and growth. The cell signaling pathways activated by LEC in response to stress are dependent upon the identity of the stressor. The environmental stressors, UVB and UVC radiation, activate both JNK and p38 pathways in HLE, whereas H_2O_2 does not activate these pathways [89]. Hydrogen peroxide has been shown, however, to activate MAPK in other cell lines [90]. Dietary compounds can modify stress signaling in HLE, as both xanthophylls and vitamin E have

been shown to prevent UVB-induced JNK activation, with xanthophylls being more potent [91].

2.2.4 Cellular Models

Immortalized and primary lens epithelial cell lines, as well as whole lens models, have been utilized to study lens physiology and cataractogenesis. While each has been useful in elucidating data on lens biology, there are clear advantages and disadvantages to the use of each model, depending on the experimental condition and research question. Some of the issues involved in using immortalized and primary LEC, as well as animal models, are discussed below.

Immortalized lens epithelial cells. Immortalized lens epithelial cell lines are a valuable tool to study lens physiology and overcome the growth restriction and limited tissue acquisition characteristic of primary cell lines. Although the first cell lines generated from the lens were from animals, human lens cell lines have recently been developed. The first such line was achieved by infection of primary cultures of infant lens epithelium with the SV-40 T antigen. This cell line retained the expression of typical lens proteins, such as α_A and β_{B2} crystallin [92]. Recently, a similar human LEC cell line has been developed by direct transfection with the SV-40 large T-antigen gene [93].

Immortalization of LEC may or may not be representative of the native lens epithelium. Certain transformed cell lines display multiple morphological and biochemical phenotypes [94]. The conditions and manipulations of cell culture may alter lens-specific metabolism and molecular markers [2]. Immortalized cell lines are also limited because they are not grown on their endogenous capsule and require non-physiological conditions for optimal culture [4].

Primary LEC. Primary LEC exhibit a programmed growth restriction and are therefore difficult to maintain in culture indefinitely. Primary cell lines, however, preserve the native characteristics of a cell, without genetic manipulation by immortalization. Cell cultures which have not been altered by transformation or immortalization, including the chicken embryonic lens system and the rat explant cultures have provided valuable study of lens differentiation and metabolism [2]. Primary canine LEC have been utilized in a variety of studies examining iron metabolism [95, 96]. In our laboratory, primary canine LEC are cultured from the endogenous lens capsule and can be maintained as epithelial cultures for up to passage 5 (**Fig 2.4**). Prior reports indicate that primary cultures of infant and fetal human lens epithelial cells can be cultured for up to 3 passages [97].

Optimal growth and differentiation. LEC appear to grow better on surfaces mimicking their endogenous lens capsule and when cultured on lens capsule retain the ability to synthesize alphaB-crystallin [98]. Coating cell culture vessels with extracellular matrix components of the lens capsule such as laminin and fibronectin has been shown to enhance cell attachment and migration [99]. Lens epithelial cell differentiation can be monitored by the gain of fiber-cell specific proteins or by the loss of epithelial-cell specific proteins. Previous evidence has indicated that differentiation of isolated epithelia in calf lenses is marked by an increase in the alpha A_2 crystallin isoform and an increase in the formation of the β crystallin chains β_{1a}, β_5 and β_p [100]. Gamma crystallin is

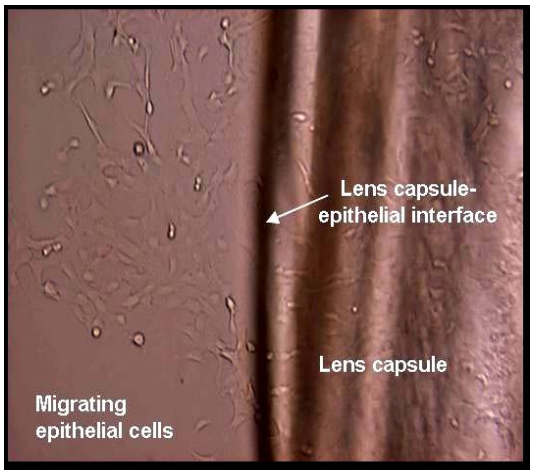

Figure 2.4: Photograph of primary canine lens epithelial cell culture
Location of lens capsule/epithelial interface is indicated by arrow. Migrating epithelial cells are visible from the interface at seven days post initial capsule culture. Representative sample is shown. Magnification 100X.

considered a marker of fiber cells [101]. Alpha crystallin expression may be a suitable marker of LEC, however some non-lens cells have been shown to express this protein [102]. LEC are characterized by a variety of structural proteins, including actin, tubulin, spectrin and vimentin. The formation of mature lens fibers in associated with a decrease in vimentin expression [103]. The presence of cytokeratins in the lens epithelium is debated, with expression considered to be absent in adult human lens epithelial cells [104]. Cytokeratins 8 and 18, however, may be found in cultured cells [105]. The optimal conditions for growth and maintenance of primary canine LEC as determined in our laboratory is discussed in Chapter 4.

Advantages and disadvantages. Several important issues in the use of animal models to study human cataract have been reviewed [31]. First, most laboratory animals such as rats, rabbits and mice, contain no UV filters. Second, the protein content of the lens can vary from as much as 65% in rat to 16% in the chick. Third, there is variability in concentrations of crystallin proteins, ascorbate and antioxidant enzymes amongst species. These species- specific differences in lens physiology may make cultured LEC a more reliable and meaningful model than *in vivo* studies [4].

2.3 DIETARY MODULATION OF OCULAR DISEASE

2.3.1 Retinoids and Carotenoids

2.3.1.1 Properties, food sources, bioavailability

Retinoids. The term retinoid refers to both the naturally occurring ring forms of vitamin A, as well as the synthetic analogs of retinol, with or without biological activity [106]. The structural formulas of some retinoids and their similary to β carotene are given in Fig. 2.5. Vitamin A and its related compounds are essential for vision. The vitamin A

metabolite, 11-cis retinal, serves as a chromophore of the visual pigments (rhodopsins). Light activation of these G-protein coupled receptors initiates phototransduction, whereby light energy is converted into electrical energy by the photoreceptor. The vitamin A derivative, retinoic acid, is important in the development of many vertebrate tissues and organs, including the lens [107]. Retinoic acid is a major signaling molecule, serving as a ligand whose binding to nuclear receptors controls gene transcription that regulates a wide range of biological responses. The primary nuclear receptors for retinoic acid are RAR (retinoic acid receptor) and RXR (retinoid X receptor). The active RAR/RXR heterodimer binds to DNA regulatory sequences and regulates gene transcription in response to binding by retinoic acid.

In addition to its role in vision, vitamin A is also important for the maintenance of epithelial surfaces, immune function, embryonic growth and development and reproduction [106]. Animals and humans consume vitamin A either directly as pre-formed vitamin A or indirectly as pro-vitamin A. Retinyl esters, the form most often found in foods, are contained in fish, avian and mammalian livers and are added via fortification to milk, margarine and breakfast cereals. Dietary retinyl esters are converted enzymatically to retinol in the intestinal lumen, prior to uptake by enterocytes. Unesterified retinol is then taken up by a saturable, carrier-mediated process [108]. Retinoids are found within the lens and are transported there bound to albumin in the aqueous humor [109]. On a per gram wet weight basis, human lenses contain 31 to 50 ng retinol and 21 to 25 ng retinyl palmitate [78]. The exact role of retinoids in the lens is not established, although regulation of growth and differentiation of LEC is plausible. Retinoids have also been found in the terrestrial gecko lens where they bind to crystallin

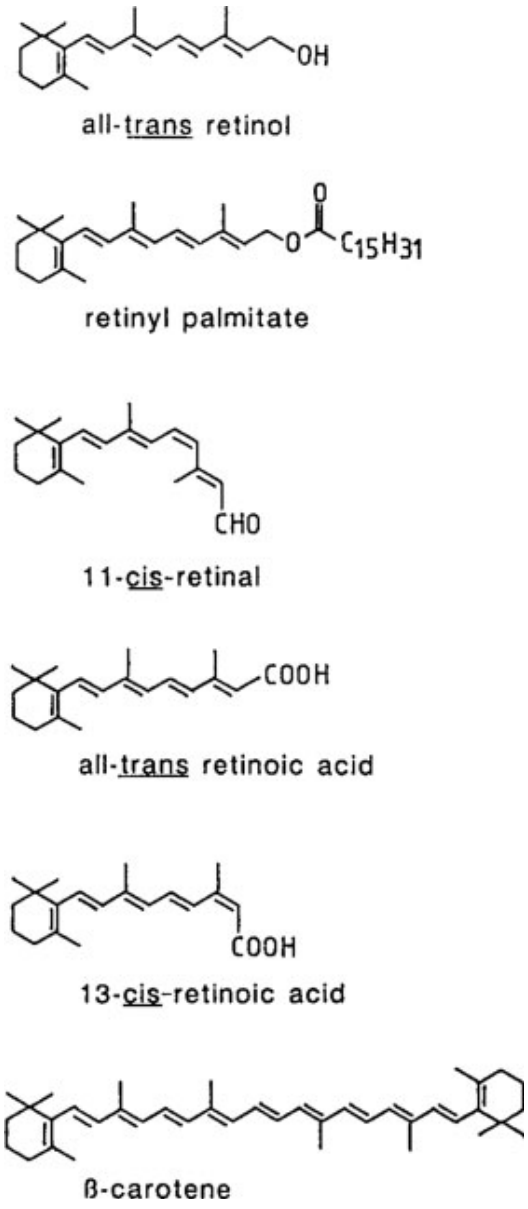

Figure 2.5: Structural formulas of vitamin A and β carotene [106]

proteins, perhaps eluding to a regulation of fiber cell differentiation in this species [110].

Carotenoids. Carotenoids are an isoprenoid family of over 600 identified compounds that are ubiquitous in nature. Carotenoids are present in all photosynthetic organisms, as well as some bacteria and fungi. In plants, carotenoids function as accessory light harvesting compounds and have a role in thermal energy dissipation. Carotenoids are responsible for the wide array of colors we see in nature, from the vibrant colors of leaves in the fall to the brilliant colors of fruits, flowers, birds, insects and marine invertebrates. Of the identified carotenoids, only about 60 are present in foods routinely consumed by humans. The primary dietary carotenoids relevant to humans include alpha carotene, beta carotene, beta cryptoxanthin, lycopene and lutein. These compounds are structurally similar, yet have varying degrees of vitamin A and antioxidant activity (**Fig 2.6**). Commercially, carotenoids are used as food colorants and are added to nutritional supplements. Carotenoids exhibit preferential accumulation in select tissues of the human body. For example, lycopene tends to be deposited in the adrenals, testes and prostate, whereas lutein and zeaxanthin are selectively accumulated in the lens and retina [111]. Carotenoid absorption from food is a sequential process involving mechanical and enzymatic disruption of the food matrix, release of the carotenoid and its incorporation into lipid droplets. Carotenoids are transferred from the lipid droplet into a mixed micelle, composed largely of bile salts and phospholipids that aid in its solubilization. Post solubilization, carotenoids are absorbed by intestinal epithelial cells, packaged into chylomirons and secreted into the lymph. Recent studies suggest that carotenoid absorption occurs by both facilitated diffusion and a receptor-mediated process involving the scavenger receptor B1 (SR-BI) [112]. The absorption of carotenoids from meals can

depend on many variables, including processing, speciation and other components present in the meal such as fat. Absorption is also affected by the nutritional status and the genetic background of the subject [113].

Lutein and zeaxanthin. Lutein and zeaxanthin are oxygenated carotenoids, or xanthophylls, and are structurally characterized by the presence of hydroxyl groups attached to each of the two terminal β ionone rings in the molecule (**Fig 2.6**). The presence of these functional groups increases the polarity and hydrophilicity of these compounds, facilitating reaction with singlet oxygen more readily than nonpolar carotenoids [114]. Many commonly consumed fruits and vegetables contain lutein and zeaxanthin, with lutein content usually exceeding zeaxanthin [115]. Lutein and zeaxanthin are present in a wide array of dark-green leafy vegetables such as kale, spinach, turnip greens and collards. Some studies suggest that bioavailability of lutein and zeaxanthin from these foods is higher than that of β carotene [116]. Lutein and zeaxanthin are highly bioavailable from egg yolks, perhaps due to the lipid matrix of this food [117]. A recent study examining the uptake and transport of zeaxanthin in Caco-2 intestinal cells showed that free zeaxanthin is preferentially taken up by intestinal epithelial cells, and that zeaxanthin esters ingested in food are processed similarly to cholesterol esters in the small intestine lumen, via the action of carboxyl ester lipase [118]. Lutein and zeaxanthin have purported roles in modulating many systemic diseases including cancer, heart disease and stroke, as well as two eye-related disorders, age-related macular degeneration and cataract [119]. Of all dietary carotenoids, only lutein and zeaxanthin accumulate within the lens [78, 120, 121], and are preferentially

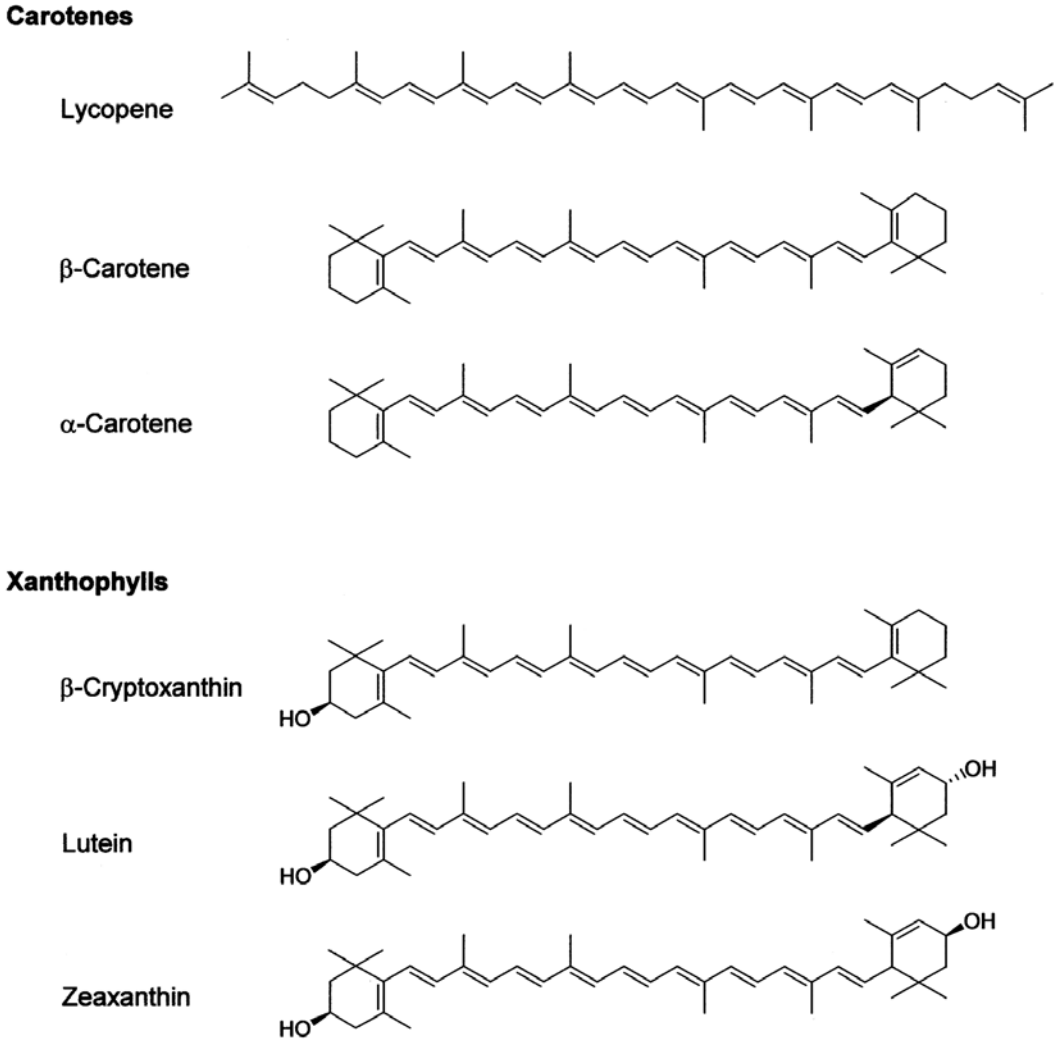

Figure 2.6: Common carotenoids in human plasma[112]

concentrated in the epithelium/cortex region [79]. Binding proteins for lutein and zeaxanthin in the eye have not been well characterized, although both zeaxanthin and meso-zeaxanthin were recently reported to bind to glutathione S-transferase (GSTP1) in the retina [122]. Similar proteins in the lens have not been identified. There are several plausible mechanisms by which lutein and zeaxanthin may protect against ocular diseases. Lutein and zeaxanthin have been shown to prevent oxidative damage to lipid membranes [123] and orient themselves in membranes such that they maximize interactions to reduce oxidative stress and lend stability to membranes [124]. They have also been shown to quench singlet oxygen in lens fibers [125] and to bind tubulin within the lens [126]. An important property of lutein and zeaxanthin independent of their antioxidant role, is their ability to increase gap junction communication [127]. These diverse effects have prompted the suggestion that lutein and zeaxanthin may be considered conditionally essential nutrients for eye health [128].

Studies in primates indicate that xanthophylls can be depleted by dietary restriction and this leads to structural abnormalities [129]. The ability of supplemental lutein and zeaxanthin to accumulate in ocular tissues is a key question in prevention of ocular diseases. Bone and colleagues have reported that humans supplemented with lutein and zeaxanthin respond with an increase in both serum concentration and macular pigment density [130]. A positive and statistically significant association between macular pigment optical density, serum lutein and zeaxanthin concentration, and dietary lutein and zeaxanthin intake has been reported in healthy Irish subjects aged 20 to 60 years [131]. In addition, older subjects consuming high-dose lutein supplements were found to have higher levels of both lenticular and macular carotenoids [132].

2.3.1.2 Risk of ocular disease: epidemiological studies

Epidemiological evidence suggests that dietary intake and/or plasma levels of retinoids and cartotenoids may reduce the risk of ocular disease, although results from these studies have not been consistent. These studies differ in methodology, length of study, study variables and populations. Select epidemiological reports concerning the use of retinoids and carotenoids and the prevention of ocular diseases are discussed below.

Retinoids. Delcourt and colleagues found that higher plasma retinol levels were associated with a decreased risk for nuclear, mixed cataract and cataract extraction in the POLA study [133]. An increased intake of vitamin A, as assessed by food frequency questionnaire, was associated with reduced prevalence of nuclear cataract [134].

Carotenoids. Alpha carotene, beta carotene and lycopene are not present within the human lens [78], although epidemiological studies have linked these carotenoids to a decreased risk of cataract development. A cross-sectional study of American women aged 53-73 years enrolled in the Nurses Health Study reported a reduced incidence of PSC opacities with an increased intake of alpha carotene, beta carotene and total carotenoids [135]. A reduced risk for cortical and nuclear cataract has been associated with intake of lycopene, alpha or beta carotene [136]. Lycopene has been shown to protect against the development of experimental cataract induced by both galactose and selenite in rats [137]. Smokers who supplement their diet with β carotene benefit from a reduction in cataract risk [138]. A mixture of β carotene and vitamin E can slow the increase in density of cataractous lenses in Americans, however, no association was found in English subjects [139]. In contrast, a similar antioxidant combination of β

carotene and vitamin E had no effect on the risk of cortical, nuclear and posterior subcapsular cataract in Finnish smokers in the ATBC study [140].

Lutein and zeaxanthin. Reports on prevention of eye diseases by carotenoids may be conflicting; however, an increasing number of Americans take lutein supplements for eye health [141]. It has been reported that lutein supplements can improve visual function in patients with retinal degeneration [142]. The relationship between lutein and zeaxanthin intake and risk of cataract and/or macular degeneration are discussed briefly.

Cataract. Dietary intake of lutein and zeaxanthin is associated with a decreased risk of nuclear cataract in the both the Nutrition and Vision Project (NVP) [143] and Beaver Dam Eye Study [144]. Lutein and zeaxanthin intake is also associated with a 19% decreased risk of cataract extraction in US men [145], and a 32% reduced rate of PSC cataract in women [146]. In a retrospective study of 1919 women, the rate of nuclear cataract was reduced in women with the highest versus lowest lutein intake [147]. High plasma lutein concentrations are linked to a decreased risk of PSC cataract in English subjects [136]. Several other studies have examined the relationship between plasma lutein (as a marker of intake) and the risk of cataract [144,148,149]. However, it should be noted that these types of studies often have a very poor correlation between dietary intake and concentrations of lutein and zeaxanthin in the serum [150]. In the POLA study, the highest quintile of zeaxanthin in the plasma was associated with a reduced risk of nuclear cataract or any cataract [151]. In contrast, plasma lutein was not associated with risk of any type of cataract A high dietary intake of lutein/zeaxanthin was inversely associated with the risk of nuclear cataract, but not of cortical or PSC cataract in the Melbourne visual impairment project [152]. In a recent review of epidemiological

studies, Moeller and colleagues report that spinach and other dark leafy greens, both concentrated lutein sources, were most consistently associated with protection against cataract [153]. Lutein supplementation as a treatment has been shown to improve visual function in patients with age-related cataract in a double blind intervention trial [154].

Age-related macular degeneration. Lutein and zeaxanthin supplementation has previously been associated with a reduced risk of development of age-related macular degeneration (AMD). Xanthophylls may improve visual performance in retina [154, 155]. In the Dietary Ancillary study of the Eye Disease Case Control Study (EDCCS) the effect of nutritional status on development of AMD was evaluated. The two carotenoids, lutein and zeaxanthin, were associated with a decreased risk of AMD, whereas no association was found with β carotene consumption [156]. Subjects with high plasma levels of lutein and zeaxanthin had a 79% reduced risk of developing age-related maculopathy (ARM) compared to subjects with lower intakes in the POLA study [151].

In summary, the conflicting results in some of these studies make it difficult to recommend dietary supplements for eye health. The synthesis of all these studies have resulted in the Food and Drug Administration basis for finding no evidence to support a health claim for lutein and zeaxanthin in relationship to eye health [157].

2.3.2 Antioxidants

In the United States the prevalence of age-related cataract will increase from approximately 5% at 65 yrs of age to 50% for persons older than 75 years [158]. The influence of nutritional antioxidants on this disease has been extensively and recently reviewed [159]. Some of those most important studies are highlighted here.

2.3.2.1 Clinical Interventions

Perhaps the largest and most powerful study examining the relationship between antioxidant status and ocular disease was the Age-related Eye Disease Study (AREDS), a randomized controlled clinical trial initiated in 1990 by the National Eye Institute. This study evaluated the risk of AMD and cataract development with and without supplementation with vitamin C (500mg), vitamin E (400IU), β carotene (15mg) and zinc oxide (80mg) with cupric oxide (2mg). Results from this study showed that the antioxidant-zinc combination decreased the risk of AMD over 5 years, with a 19% reduction in the loss of vision. There was no reported relationship, however, between antioxidant supplementation and cataract risk. Few other randomized trials have been performed to examine the effect of antioxidant supplements on cataract progression. A reduced risk of nuclear cataracts has been reported in a Chinese population given a multivitamin-multimineral supplement and in those given riboflavin/niacin supplements [160]. Supplementation with β carotene, vitamin C and vitamin E modestly decreased cataract progression in the REACT study [139]. The Women's Health Study [138] reported a decreased risk for cataract extraction in women with antioxidant supplementation. Long-term supplementation with antioxidants appears to provide the most benefit [161].

2.3.2.2 Antioxidant combinations: studies on dietary intake

Combinations of antioxidant intake via a balanced diet may provide greater benefit than supplementation with single nutrients. A study looking at the role of diet in cataract development reported a significant five fold decrease in relative risk for cataract between persons consuming greater than or equal to 1.5 servings of fruit, vegetables, or

both. The adjusted prevalence of all types of cataract was 40% and 80% lower for persons with moderate and high antioxidant index scores than those with low scores. This index score was based on combined plasma vitamin C, vitamin E and carotenoid concentrations [143]. Leske and colleagues, utilizing a similar antioxidant intake score for vitamin C, vitamin E, carotene and riboflavin, determined that persons with higher index scores for these vitamins had a 60% lower prevalence of cortical cataract compared with those who had lower scores [162]. Hankinson and colleagues calculated an antioxidant score based on intake of carotenes, vitamin C, vitamin E and riboflavin and reported a 24% reduction in adjusted rate of cataract surgery in women with high antioxidant scores relative to women with low scores [163]. Increased intake of vitamin A, thiamin, riboflavin and nicotinic acid has been associated with reduced prevalence of nuclear cataract [134]. In contrast, Vitale and colleagues reported no association between antioxidant scores based on plasma concentration of vitamin C, vitamin E and β carotene and cataract prevalence [164]. In addition, no significant association between nuclear cataract and vitamin C and E intake has been reported [165].

2.3.2.3. Individual Antioxidants

Epidemiological reports indicate that several key antioxidants consistently provide protection against cataract development. These are discussed briefly below.

Vitamin C. Vitamin C is a water soluble antioxidant present in millimolar concentrations in the aqueous humor and lens. Jacques and Chylack observed that high vitamin C intake (> 490 mg/d) reduced cataract prevalence by 25% as compared to those with lower intakes (< 125mg/d) [166]. Leske and colleagues reported that subjects with vitamin C

intake in the highest range had a lower prevalence for nuclear cataract compared with those whose intakes were in the lowest range [162].

Vitamin E. The prevalence of PSC cataract has been reported as 67% lower in persons with plasma vitamin E concentrations greater than 35μM relative to persons with concentrations less that 21μM [166]. Robertson and colleagues found that the prevalence of cataract was 56% lower in persons who consumed a vitamin E supplement (greater than 400 IU/day) than in persons not consuming supplements [167].

Other Antioxidants and Nutrients. Consumption of several other antioxidants and functional foods has been found to affect cataract development both epidemiologically and experimentally. In a prospective study of the relationship between dietary fat and risk of cataract extraction, a higher intake of long chain omega 3 fatty acids as well as fish consumption, was found to modestly reduce the risk of cataract [168]. A recent study has shown that supplementation with grape seed extract proanthocyanidin suppresses cataract formation induced by selenite in rats. This result was associated with an increase in GSH levels and a decrease in MDA levels [169]. Lipoic acid is a potent antioxidant and can both directly scavenge radicals, as well as recycle oxidized ascorbic acid, vitamin E and glutathione. In experimentally induced diabetic rats, pretreatment with lipoic acid prevented light scattering in lenses, as compared to rats not receiving the supplement [170].

2.3.2.4 Multivitamin and mineral supplements and cataract

Two population based studies have reported that regular use of a multi-vitamin supplement lowers the risk for cataract [171, 172]. In support of these studies, Mares-Perlman and colleagues (2000) found that the five year risk of any cataract was

diminished by 60% among persons who reported using a multivitamin or any supplement containing vitamin C or E for more than 10 years [172]. Regular use of a multivitamin supplement was associated with a decreased prevalence for PSC, cortical, nuclear and mixed cataract [162]. An impressive 90% reduced rate of nuclear cataract is reported in subjects who did versus did not use a multivitamin supplement [173]. In contrast, Hankinson and colleagues found no relationship between multivitamin use and risk for cataract extraction [163].

2.3.2.5 Plasma levels

Plasma levels of several vitamins and minerals are associated both negatively and positively with cataract risk. Elevated blood levels of vitamin C are reportedly associated with reduced risk of cataract [174, 143, 175], while others find no association [136]. Jacques and colleagues found that the risk of cortical cataract decreased for subjects with the highest quintile of Vitamin D and total carotenoids, but the risk increased for those with low vitamin C and increased levels of B6 and selenium [176]. High plasma levels of alpha carotene, lycopene and lutein but not vitamin E, zeaxanthin and beta cryptoxanthin are protective against nuclear, cortical and PSC cataract, respectively [136]. The Italian-American Trial of Nutritional Supplements and age-related cataract reported that high vitamin C levels were associated with a protective effect on nuclear and PSC cataract, whereas high vitamin E levels were associated with an increased prevalence of cortical cataract, PSC and of any cataract [177].

2.4 ROLE OF GAP JUNCTIONS IN CATARACT DEVELOPMENT

2.4.1 Biology of Gap Junctions

Gap junctions are intercellular membrane channels that are found in a diverse group of organisms, ranging from invertebrate nematodes to vertebrate humans [178]. Gap junctions are formed by the synthesis of two membrane protein complexes termed connexons **(Fig. 2.7)**. Each connexon is formed by a hexamer of connexin proteins, a highly conserved and related gene family, with nearly 20 different members. The major connexins in the lens are connexins 43 (Cx43), 46 (Cx46), and 50 (Cx50) [179, 180]. Connexin 43 (Cx43) is found between LEC, whereas connexins 46 (Cx46) and 50 (Cx50) are found between lens fiber cells and at the epithelial/fiber cell interface [3]. Connexins are made and assembled as normal proteins, yet their turnover is relatively high, with an average of 1.5-5 hours for Cx43 [181]. Connexin stability and function is regulated on several levels, including post-translational modification. Phosphorylation of Cx43 in the lens has been linked to the disassembly, degradation and removal of gap junctions from the cell membrane [182]. LEC have one of the highest rates of gap junction communication of any cell [182]. Gap junctions in the lens maintain communication between mitotically active epithelial cells and underlying quiescent fiber cells. The latter, due to the absence of organelles, are not capable of metabolism, yet must continue to grow throughout the lifetime of the host. Lens fiber cell viability is dependent on transfer of ions, secondary messengers and small metabolites through what has been termed the lens internal circulation [13] (**Fig 2.1**). This circulation involves water and ion transport through the anterior and posterior poles, with removal of wastes at the lens

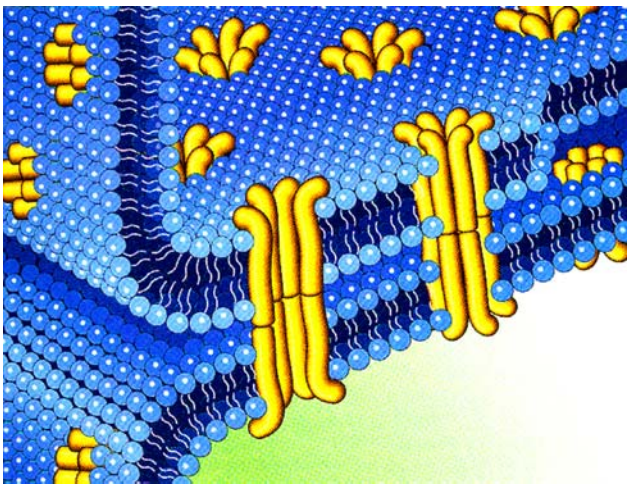

Figure 2.7: Representation of Gap Junction Channels [183]

equator. The role of gap junctions in lens physiology is exemplified by evidence that disruption in gap junction function is associated with cataract development. Cx46 and/or Cx50 knockout mice display a severe nuclear cataract. While Cx43 knockout mice die shortly after birth, lenticular examination reveals changes associated with early cataract development [184]. The mechanism of connexin disruption and cataract has not been fully elucidated, although disturbance in cellular communication likely causes osmotic imbalances and structural modifications that lead to loss of transparency.

2.4.2 Regulation of Cell Communication

2.4.2.1. *Cell Stress*

The expression and/or post-translational processing of Cx43, along with subsequent gap junction intercellular communication, are sensitive to both oxidative and environmental stress. Heat stress, t-butyl hydroperoxide and UVC have all been shown to phosphorylate Cx43 in WB-F344 rat liver epithelial cells, with impairment of gap junctional intercellular communication (GJIC) in the latter [185].

UVB exposure has been shown to elevate levels of H_2O_2 and calcium in the lens [186], both of which can decrease gap junction intercellular communication. Hydrogen peroxide has been shown to be a potent inhibitor of gap junction communication in several cell lines [187]. High levels of glucose have previously been shown to alter gap junction intercellular communication in the lens and retina. Exposure of bovine retinal endothelial cells to high glucose levels (25mM) resulted in decreased connexin 43 protein levels, increased phosphorylation of connexin 43, and a 40% reduction in GJIC compared to controls [188]. Closure of gap junction channels in response to oxidative and

environmental stressors may constituent a means in which to prevent cell death signals from reaching healthy cells [189].

2.4.2.2 *Cell Signaling*

Phosphorylation of connexin proteins by phorbol esters and growth factors is preceded by activation of MAPK and/or protein kinase C (PKC)-dependent signaling pathways [190]. The phorbol ester, TPA, induces Cx43 phosphorylation and decreases GJIC by downstream activation of MAPK (ERK) activation, independent of PKC involvement [191-193]. In Chapter 5, we discuss how TPA-induced activation of the PKC pathway alters GJIC in primary canine LEC.

Several isoforms of PKC exist, including conventional, unconventional and atypical forms. PKC α and γ (both conventional isoforms) are predominantly expressed in chicken lens epithelial cultures, and are modulated by TPA [194]. PKC α has been identified as a TPA-sensitive isoform within the lens epithelium [195], and PKC γ has recently been linked to Cx43 phosphorylation in the cytoplasm of LEC [196]. It should be noted that PKC may have varying effects on Cx43 phosphorylation. Rivedal and colleagues have shown that the selective PKC inhibitor (GF109203X) displayed a strong effect on gap junction communication, but had little effect on TPA-induced Cx43 phosphorylation in rat liver epithelial cells [193]. PKC γ has recently been shown to be present in lens epithelial cells and is the primary sensor of changes in diacylglycerol (DAG) at low levels [196]. Oxidative stress, H_2O_2 (100μM), activates PKC γ in lens epithelial cells with subsequent phosphorylation of Cx43 on ser368 [197].

2.4.2.3 *Dietary Modulation of GJIC*

Several important dietary constituents have been shown to modulate GJIC, such as retinoids, carotenoids, vitamin D, thyroid hormones, and others. The mechanism for stimulation of GJIC by these compounds is not fully understood, although several studies have pointed to the regulation of connexin expression as a plausible mechanism. A focus on modulation of GJIC by select dietary compounds is discussed below.

Retinoids. The ability of retinoids to modulate cell growth through gap junctions was previously established [198]. All-trans retinoic acid has been shown to enhance or inhibit GJIC in different cell types and concentrations. Treatment with retinoic acid increases Cx43 expression by a post-translational mechanism by which the mRNA is stabilized [199]. All-trans retinoic acid has been shown to enhance GJIC in renal epithelial cells through upregulation of Cx43 expression [200]. Retinoids are able to upregulate connexin 43 mRNA within about 6 hr of treatment, but carotenoids require about three times longer to produce the same response [127, 201, 202, 183]. Both Cx43 levels and GJIC are upregulated in human osteoblastic cells exposed to low levels of RA [203].

Carotenoids. Carotenoids, in addition to antioxidant potential, have important non-antioxidant roles in disease. The stimulation of GJIC by carotenoids is not related to their antioxidant ability [204] or their pro-vitamin A ability [127]. In fact, there seems to be little correlation between the effects of carotenoids on GJIC and their ability to quench singlet oxygen [205]. Metabolites of carotenoids often have similar or increased activity as the parent compound. Recently, decomposition products of canthaxanthin, such as all-trans and 13-cis 4-oxo retinoic acid have been shown to enhance GJIC, with a concomitant increase in Cx43 mRNA [206]. Similarly, lycopene and its major

metabolite, acyclo-retinoic acid, have been shown to increase GJIC in human fetal skin fibroblasts [207]. Recently the keto-oxy carotenoids, astaxanthin and canthaxanthin, have been shown to have similar functions in the upregulation of Cx43 [206, 208]. Lutein and zeaxanthin associate with the cell membrane, exhibiting structural orientations which lend stability to bilayer structure [124]. It is possible that these xanthophylls, in addition to scavenging radicals, may serve a separate function in regulation of membrane stability and integrity of gap junction communication in lens epithelial cells.

Other dietary compounds. Several other dietary antioxidants and bioactive food components such as green tea and soy compounds, as well as vitamin D, have been shown to regulate GJIC in a variety of cell lines**.** Treatment with the major flavonoid in cocoa and tea, (-)-epicatechin, was found to stimulate GJIC in WB-F344 rat liver epithelial cells after 24 hr. The presence of (-)-epicatechin counteracted the inhibitory effect of TPA on GJIC in these cells [209]. Low concentrations of vitamin D (0.01 to 1.0μM) have been shown to increase GJIC in C3H/10T/1/2 cells after 72 hr [210]. Genistein (15μg/ml), the major isoflavone in soy, has been shown to increase Cx43 levels and reduce proliferation in a breast cancer cell line [211].

CHAPTER 3

APOPTOTIC AND NECROTIC MECHANISMS OF STRESS-INDUCED HUMAN LENS EPITHELIAL CELL DEATH

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3.1 Abstract

Exposure to ultraviolet radiation (UVR) and reactive oxygen species (ROS) can damage the human lens and contribute to cataract formation. Recent evidence suggests that apoptosis in lens epithelial cells (LEC) is an initiating event in noncongenital cataract formation in humans and animals. The present study examines the cellular and molecular mechanisms by which environmental (ultraviolet B [UVB]) and chemical (hydrogen peroxide [H_2O_2], t-butyl hydroperoxide [TBHP]) stress induces cell death in an SV-40 immortalized human lens epithelial (HLE) cell line. Treatment of HLE cells with UVB, H_2O_2, and TBHP significantly decreased cell density with LD_{50} values of 350 J/m^2, 500 μM, and 200μM, respectively. Cellular morphology, DNA fragmentation, and annexin/propidium iodide staining consistent with apoptosis was observed only in UVB-treated cells, whereas lactate dehydrogenase (LDH) release was significantly higher in H_2O_2- and TBHP-treated cells. In addition, activation of apoptotic stress-signaling proteins, including c-Jun NH2-terminal kinase (JNK), caspase-3, and DNA fragmentation factor 45 (DFF45) was observed only in UVB-treated cells. Inhibition of JNK activity

increased UVB-induced cell death, suggesting that this pathway may serve a prosurvival role in HLE cells. These findings suggest UVB predominantly induces apoptosis in HLE cells, whereas H_2O_2 and TBHP induce necrosis.

3.2 Introduction

The human lens epithelium comprises the most metabolically active cell layer of the lens and is the initial cell layer exposed to environmental and oxidative insult. Ultraviolet B (UVB) radiation, a biologically relevant source of environmental stress, can initiate deleterious changes in lens epithelial cells (LEC) including chromatin and nuclear condensation, inhibition of cell growth, and disruption of ionic homeostasis [71, 212]. Both human and animal studies suggest that UVB exposure initiates the development of cortical and posterior subcapsularcataract [63, 213, 214]. The molecular basis of UVB damage has been linked to the generation of reactive oxygen species (ROS), although the exact basis of this damage is unknown [215]. In addition to environmental stress, chemical stressors such as oxidant radicals derived from H_2O_2 or mitochondrial respiration may damage LEC [216]. Elevated levels of H_2O_2 are reported in the aqueous humor of cataract patients [73] and can cause opacification of the lens in vitro [217]. Hydrogen peroxide and t-butyl hydroperoxide (TBHP) have recently been found to deplete glutathione and damage ion pump activity in LEC [218]. These changes can disrupt epithelial integrity and cause a loss of lens transparency. Recent data suggest that LEC apoptosis is an initiating factor in noncongenital cataract formation [84, 85]. LEC apoptosis occurs normally during embryological lens development [219], although its importance in the adult lens is not well-characterized. Dysregulation of LEC apoptosis, however, is associated with opacification of the rat lens and can be stimulated by both

oxidative stress and UVB radiation [45, 84, 85, 219]. Recent evidence suggests that mitogen-activated protein kinases (MAPKs) are important in regulating lens apoptosis [220, 221] as well as cataractogenesis [89]. Mitogen-activated protein kinases mediate signal transduction in response to mitogenic and environmental stress and regulate cellular events such as proliferation, differentiation, migration, and death [86, 222, 223]. Three major MAPK cascades have been identified, including the extracellular signal-regulated kinase 44/42 cascade, which preferentially regulates cell growth and differentiation, and the c-Jun NH2-terminal kinase (JNK) and p38 cascades that mediate cellular stress responses. Lens epithelial cells possess all three major MAPK signaling cascades, of which JNK and p38 are strongly activated by ultraviolet radiation (UVR; Refs. [221, 224-227]. Recently, the lens epithelium has been identified as the major site of the lenticular stress response, with activities of JNK and p38 highest in this cell layer when compared with other sections of the lens in human, bovine, and rat [87]. Ultraviolet radiation–induced apoptosis is regulated by a number of molecular processes, including the activation of cysteine-containing aspartate-specific proteases (caspases;[228]). Caspase-3 is an effector caspase that, when cleaved, activates DFF45, a transcription factor involved in intranucleosomal DNA fragmentation and apoptosis [229, 230]. Phosphorylation and activation of JNK is reported to occur prior to caspase-3 cleavage [231, 232]. In addition, inhibition of caspase activity can block nuclear degeneration in differentiating LEC cultures [233] and suppress UVB-induced apoptosis of cultured human keratinocytes [234]. The present study examined the mechanism(s) by which environmental (UVB) and chemical (H_2O_2 and TBHP) stress induce human lens epithelial (HLE) cell death. We report that UVB predominantly induces apoptosis in HLE

cells, whereas H_2O_2 and TBHP predominantly induce necrosis. This work lends support to the growing body of evidence that UVB exposure causes deleterious changes in the lens that may cumulatively lead to cataractogenesis.

3.3 Materials and Methods

3.3.1 Materials

Antibodies that recognize the active, phosphorylated form of JNK (designated P-JNK 54 and PJNK46) and c-Jun (designated P-c-Jun), as well as total JNK (JNK), total c-Jun (c-Jun), caspase-3 (pro and active forms), and cleaved DFF45, were purchased from Cell Signaling Technology (Beverly, MA). Hydrogen peroxide and TBHP were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture supplies were from Gibco Life Technologies (Valley Stream, NY). Annexin V–fluorescein isothiocyanate (FITC) Apoptosis Detection Kit was purchased from Oncogene Research Products (La Jolla, CA).Lactate dehydrogenase (LDH) cytotoxicity assay was purchased from Roche Applied Sciences (Indianapolis, IN). Suicide Track DNA ladder isolation kit and JNK inhibitor (SP600125) were from Calbiochem (La Jolla, CA).

3.3.2 Cell Culture

HLE cells (SRA 01-04, a SV-40 Tantigen transformed cell line) were provided by Dr. Venkat Reddy (Kellogg Eye Institute, University of Michigan, Ann Arbor, MI) and cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 378C in a humidified atmosphere containing 5% CO_2. Cells were incubated for 24 hrs prior to all experiments, unless otherwise noted. Cells were seeded and grown to 80% confluence prior to experimental procedures.

3.3.3 UVB Exposure and Oxidant Treatment

Ultraviolet B irradiation of HLE cells was performed using a 3UV transilluminator (UVP, Upland, CA). Prior to irradiation, growth medium was removed and tissue culture plates were inverted on a support at a fixed distance above the transilluminator. Following irradiation, FBS-containing growth medium was replaced and cells were further incubated for indicated times. Irradiance measurements were monitored with a UVX radiometer (UVP, Upland, CA) fitted with calibrated wavelength sensors. Stock solutions of H_2O_2 (9.8 M) and TBHP (7.2 M) were prepared immediately before use in phosphate buffered saline (PBS) and administered for the appropriate times and concentrations.

3.3.4 Cell Density

Cell density was assessed using a crystal violet staining assay 24 hrs following incubation with UVB (0–1000 J/m^2), H_2O_2 (0–800 μM), or TBHP (0–600 μM; [235]. Briefly, test medium was removed and 200 μl of crystal violet dye (0.2% in 2% ethanol) was added to each test well for 10 mins. Cells were washed with distilled water, and dye was reconstituted by SDS buffer (0.5% in 50% ethanol) and incubated at 37°C for 1 hr. Cell density was quantified by spectrophotometric detection at 610 nm. For time-course experiments, cells were treated with UVB (500 J/m^2), H_2O_2 (500 μM), or TBHP (200 μM) and stained with crystal violet 6, 12, 24, and 48 hrs posttreatment. Control cells receiving no treatment were included at each time point. Bromodeoxyuridine (BrdU assay) incorporation was also utilized to assess cell proliferation according to manufacturer's instructions (Oncogene Research Products, Cambridge, MA). All

experiments assessing cell density were performed in triplicate with at least n = 3/experiment.

3.3.5 Annexin V/PI Staining

Quantification of stress-induced apoptotic and/or necrotic HLE was determined by FITC-conjugated annexin V and PI (Apoptosis Detection Kit; Oncogene) staining 4 hrs following treatment with UVB (500 J/m^2), H_2O_2 (500 μM), or TBHP (200 μM). After treatment, cells were washed in PBS and centrifuged at 200 g for 5 mins. Cellular pellets were resuspended in annexin V labeling solution and incubated according to manufacturer's instructions. Analysis was performed using an Olympus IX50 inverted fluorescent microscope fitted with appropriate filter cubes. A minimum of 300 cells were counted for each treatment group (n = 4).

3.3.6 Transmission Electron Microscopy

HLE cells were treated with UVB (400 J/m^2), H_2O_2 (400 μM), or TBHP (300 μM) and incubated for 12 hrs. After treatments, cells were prepared and transmission electron microscopy was performed as previously described [236].

3.3.7 Lactate Dehydrogenase Release Assay

Release of LDH from cellular cytosolic pools was quantified using the cytotoxicity detection kit (Roche Applied Sciences, Indianapolis, IN). Briefly, 96-well plates were seeded (2.0 X 10^4 cells per well) and allowed to incubate overnight at 37°C. Cells were treated with UVB (500 J/m^2), H_2O_2 (500 μM), or TBHP (200 μM) and incubated for 12 hrs. The supernatant was carefully removed, centrifuged, and transferred to a separate 96-well plate. Reaction mixture consisting of catalyst/dye combination was prepared and 100

μl was added directly to the cell supernatant. After incubation at 15–25°C for 30 mins, absorbance was read using a spectrophotometer at 490 nm.

3.3.8 Analysis of DNA Fragmentation

Human lens epithelial cells were treated with UVB (400 J/m^2), H_2O_2 (500 μM), or TBHP (200 μM) and incubated for 6 or 24 hrs at 37°C. DNA fragmentation was performed using Suicide Track DNA ladder isolation kit (CalBiochem). Briefly, after appropriate treatments, cells (2.0 x 10^6) were harvested in extraction buffer, and the cell lysate was centrifuged at 15,000 g for 5 mins. The supernatant was carefully removed and prepared for DNA precipitation. After resuspension of the pellet, DNA was quantified and equal amounts (2 μg) were separated on an agarose gel (1.5%). DNA fragmentation was visualized under UV light after staining with ethidium bromide (5 μg/ml). All experiments assessing DNA fragmentation were performed in duplicate with n = 3 for each experiment.

3.3.9 Stress Signal Protein Immunoblotting

Activation of caspase-3 and DFF45 was assessed in HLE cells 6 hrs following treatment with UVB (500 J/m^2), H_2O_2 (500 μM), or TBHP (200 μM). Concentration-dependent activation of caspase-3 was determined 6 hrs following treatment with UVB (0–1200 J/m^2). Time-dependent activation of caspase-3 was determined 0–6 hrs following UVB (500 J/m^2) treatment. Treatment times were based on previous data demonstrating maximal DNA fragmentation and DFF45 cleavage 4–8 hrs following stress treatment [228]. Activation of JNK was determined in HLE cells 30 min and 4 hrs following treatment (n = 3) with UVB (600 J/m^2), H_2O_2 (100 and 500 μM), or TBHP (100 and 500

μM). Treatment times were chosen based on previous data from this laboratory and others [89, 237]. After appropriate treatments and incubations, cells were washed in ice-cold PBS (pH 7.4) and collected in cell lysis buffer. Crude proteins were extracted, quantified, and separated by electrophoresis (180 V, 1 hr) as previously described [89]. After appropriate primary and secondary antibody incubations, protein signals were developed using chemiluminescence detection reagents (Bio-Rad, Hercules, CA). Membranes were exposed to Kodak X-OMAT AR film (Rochester, NY) for an appropriate length of time and developed according to manufacturer's recommendations.

3.3.10 Inhibition of JNK Activity

To inhibit JNK signaling, HLE cells were pretreated (1 hr) with the specific JNK inhibitor, SP600125 (0–50 μM) followed by UVB (500 J/m^2) irradiation. Human lens epithelial cells were harvested either 30 min or 3 hrs following irradiation, and JNK and c-Jun activities, respectively, were determined by immunoblotting. To determine the effect of JNK inhibition on UVB-induced cell death, HLE cells were incubated (1 hr) in the presence or absence of SP600125 (50 μM) followed by irradiation with UVB (0–600 J/m^2). Cell density was assessed 24 hrs following irradiation by crystal violet staining.

3.3.11 Data Analysis/Statistics

Data are expressed as mean ± SEM. Statistical differences between treatments were determined by analysis of variance (ANOVA) using SPSS (Chicago, IL) statistical analysis software ($P < 0.05$).

3.4 Results

3.4.1 Environmental and Chemical Stress Reduce HLE Cell Density

Treatment of HLE cells with UVB (0–1000 J/m^2), H_2O_2 (0–800 μM), and TBHP (0–600 μM) resulted in a decrease in cell density (Fig. 1A–C) as assessed by crystal violet staining. Treatment with 200, 400, and 600 J/m^2 UVB resulted in a 25%, 50%, and 70% reduction in cell density, respectively. Treatment with 200, 400, and 600 μM H_2O_2 resulted in a 10%, 50%, and 80% reduction in cell density, respectively. Treatment with 200, 400 and 600μM TBHP resulted in a 10%, 80% and 78% reduction in cell density, respectively. Similar decreases in cell density were obtained using a BrdU cell proliferation assay (data not shown). Average doses of stress required to reduce HLE cell proliferation by 50% (LD_{50}) obtained from crystal violet and BrdU assays for UVB, H_2O_2, and TBHP were 400J/m^2, 500 μM, and 250 μM, respectively. In order to allow for meaningful comparisons between the three stress treatments, concentrations that resulted in similar reductions in cell density were chosen for all subsequent experiments.

3.4.2 Stress-Induced Reductions in HLE Cell Density Are Time-Dependent.

Figure 3D–F establishes the effect of time on HLE cell density after initial exposure to UVB, H_2O_2, or TBHP. After 6, 12, 24, and 48 hrs, treatment with UVB (500 J/m^2) resulted in a 10%, 10%, 50%, and 70% decrease in cell density, respectively. After 6-, 12-, 24-, and 48-hr exposure, treatment with H_2O_2 (500 μM) resulted in a 10%, 30%, 50%, and 35% decrease in cell density, respectively. After 6-, 12-, 24-, and 48-hr exposure, TBHP (200 μM) resulted in a 30%, 60%, 55%, and 75% decrease in cell density, respectively.

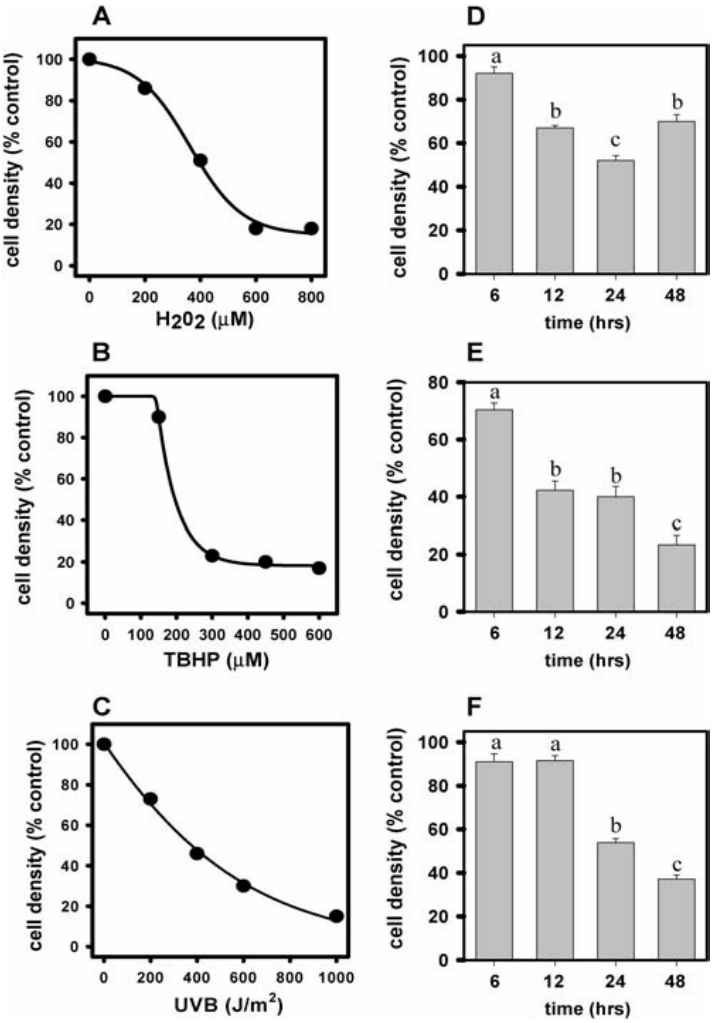

Figure 3.1: Concentration and time dependent effects of environmental and chemical stress on cell density

Concentration dependence (left panel): human lens epithelial (HLE) cells were treated with H_2O_2 (0-800μM), t-butyl hydroperoxide (TBHP; 0-600μM), and ultraviolet B (UVB; 0-1000 J/m^2), and cell density was determined 24 hrs post-exposure using crystal violet staining as described in Materials and Methods. Values expressed as percent survival of untreated controls (given as 100%). Time dependence (right panel): Cells were treated with H_2O_2 (500μM), TBHP (200μM) and UVB (500J/m^2) and cell density was determined at 6, 12, 24, 48 hrs after exposure using the crystal violet assay. Values are expressed as percent control (given as 100%). Means without a common letter differ, $P<0.05$.

3.4.3 UVB Increases Annexin V/PI Staining.

Differentiation of necrotic cell death from apoptotic death was achieved via dual staining with annexin V-FITC and propidium iodide (PI) (Fig. 3.2). A significant increase in the percentage of cells (12.6%) staining positive for annexin V, compared with controls, was observed in HLE cells treated with UVB (400 J/m^2, $P<0.05$). Hydrogen peroxide (400 μM) and TBHP (300 μM) treatment did not significantly increase annexin V staining. Treatment of HLE cells with UVB, H_2O_2, and TBHP significantly increased the percentage of cells staining positive for PI by 8.2%, 5.7%, and 17%, respectively, compared with controls ($P<0.05$).

3.4.4 Stress-Induced Changes in Cell Morphology and Membrane Integrity. Representative electron micrographs of cells treated with UVB (500 J/m^2), H_2O_2 (500 μM) and TBHP (200 μM) are shown in Figure 3.3. Treatment with UVB resulted in morphological changes including cell shrinkage, membrane blebbing, and the formation of apoptotic bodies (Fig. 3.3B; [238]). Treatment with H_2O_2 (Fig. 3.3C) and TBHP (Fig. 3.3D) resulted in cellular enlargement, vacuole formation, and organelle disorganization. Normal HLE cell morphology is shown in untreated controls (Fig. 3.3A; [93]). Potential loss of membrane integrity associated with treatment of HLE cells with UVB, H_2O_2, and TBHP was monitored by LDH release. Treatment of HLE cells with H_2O_2 (500 μM) and TBHP (200 μM) for 6 hrs significantly increased LDH release by 70% and 130%, respectively, compared with those receiving UVB (500 J/m^2, data not shown).

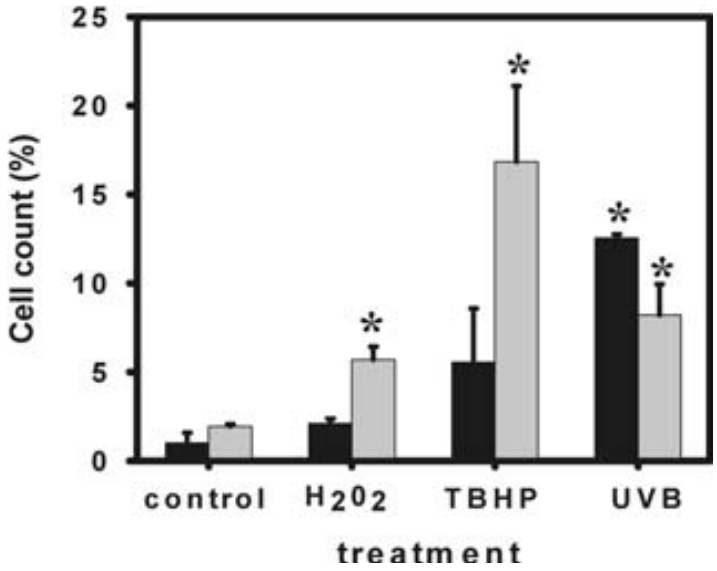

Figure 3.2: Ultraviolet B (UVB) increases annexin V/PI staining
Human lens epithelial (HLE) cells were treated with H_2O_2 (400μM), t-butyl hydroperoxide (TBHP; 300μM), or UVB (400 J/m^2) for 4 hrs followed by staining with annexin V and PI. Values are expressed as percent of annexin-positive (black bars) and PI positive (gray bars) stained cells. A minimum of 300 cells were counted per group (n=3). Data are expressed as mean ± SEM. Statistically significant increases in staining versus untreated controls is indicated (*P<0.05).

3.4.5 UVB-Induced DNA Fragmentation.

DNA fragmentation is visualized as small, fragmented bands that create a characteristic laddering or smearing of DNA on ethidium bromide–stained agarose gels. Electrophoretic analysis of DNA for UVB (500 J/m^2), H_2O_2 (500 μM), and TBHP (200 μM) treated cells at both early (6 hrs) and late (24 hrs) times is shown in Figure 3.4. DNA fragmentation is evident only in cells treated with UVB (lanes 2 and 6) at both early and late times, with DNA fragmentation at 6 hrs more prominent than 24 hrs. DNA fragmentation was not evident at 6 or 24 hrs after treatment with TBHP (lanes 3 and 7) or H_2O_2 (lanes 4 and 8).

3.4.6 UVB Activates Caspase-3: Concentration and Time Dependence

To further characterize the mechanism(s) by which UVB induces apoptosis in HLE cells, concentration and time-dependent activation of caspase-3 was determined (Fig. 3). Caspase-3 activation is marked by the cleavage of its precursor, procaspase-3. Cleavage of procaspase-3 to activated caspase-3 was evident 6 hrs posttreatment with UVB (600 J/m^2; Fig. 3.5A), whereas neither H_2O_2 (500 μM) nor TBHP (200 μM) activated caspase-3. Stress-induced activation of DFF45, a downstream target of caspase-3, was also examined. Treatment with UVB (600 J/m^2) resulted in activation of DFF45, whereas neither H_2O_2 (500 μM) nor TBHP (200 μM) activated this protein in HLE cells (data not shown).To further examine the effects of UVB on caspase-3 activation, concentration and time dependency was examined (Fig. 3.5B). Caspase-3 activation generally increased from (0–1200 J/m^2) with maximal activation at 600 J/m^2. Figure 3.5C shows the time-dependent changes in caspase-3 activation after UVB exposure (600 J/m^2). Activation of caspase-3 was observed as early as 3 hrs with maximal activation occurring at 6 hrs.

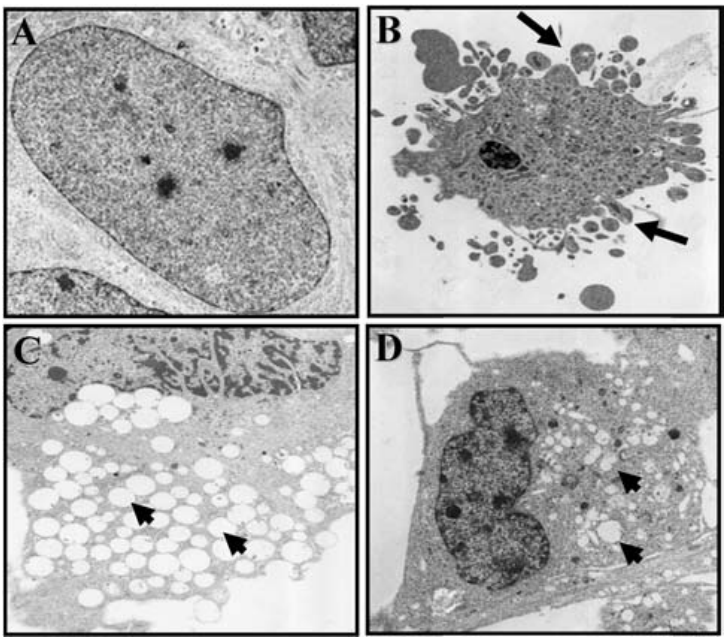

Figure 3.3: Stress-induced changes to human lens epithelial (HLE) cell morphology
Representative transmission electron micrographs of cell morphology 12 hrs after treatment with (A) control, (B) ultraviolet B (UVB; 400J/m^2), (C) H_2O_2 (400μM), and (D) t-butyl hydroperoxide (TBHP; 300μM). Arrows, apoptotic bodies; arrow-heads, vacuoles. Original magnification, x7500.

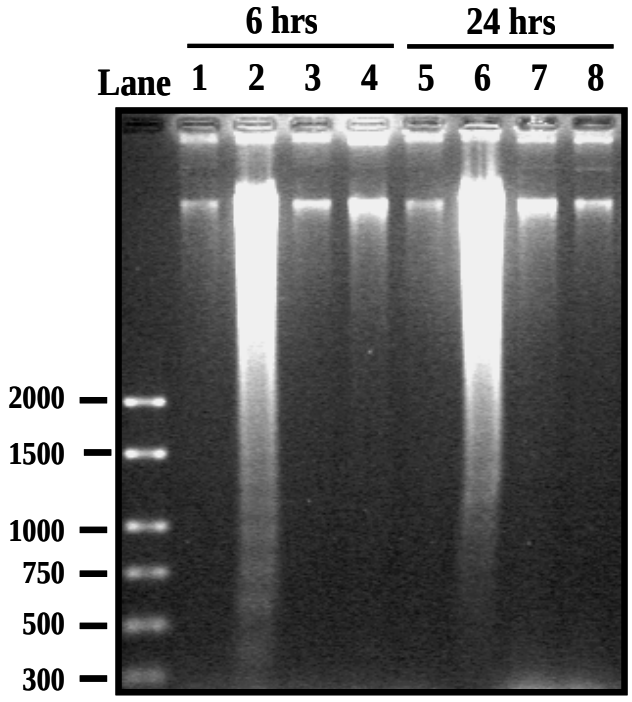

Figure 3.4: Ultraviolet B (UVB) induced-DNA Fragmentation
Cells were treated with UVB (500 J/m^2, lanes 2, 6); H_2O_2 (500μM, lanes 4, 8); and t-butyl hydroperoxide (TBHP; 200μM, lanes 3,7) for 6 hrs (lanes 1-4) and 24 hrs (lanes 5-8), and intranucleosomal DNA fragmentation was visualized by ethidium bromide staining after agarose gel electrophoresis. Standard DNA ladders and control cells (lanes 1, 5) are included for reference. DNA fragmentation patterns are representative of results obtained from at least three independent experiments.

3.4.7 Stress-Induced JNK Activation and Role in UVB-Induced Cell Death.

Stress-induced JNK activation in HLE at 30 min and 4 hrs is shown in Figure 3.6A. UVB treatment (600 J/m^2) induced JNK activation rapidly at 30 min, with decreased activation by 4 hrs. In contrast, H_2O_2 and TBHP (100 and 500 μM) treated cells did not activate JNK at either time regardless of dosage. Total JNK protein levels remained constant for all test conditions. SP600125 (SP), a specific JNK inhibitor, was used to inhibit JNK signaling in HLE cells. Pretreatment of cells (1hr) with increasing concentrations of SP (2–50 μM) result in decreasing JNK activation in response to UVB (Fig. 3.6B). Total JNK levels were independent of treatment. The effect of SP on UVB-induced c-Jun activation is given in Figure 3.6C. Treatment of HLE cells with UVB (500 J/m^2) increased c-Jun activity (p-c-Jun). Pretreatment of HLE cells with SP (50 μM, 1hr) modestly decreased UVB-induced c-Jun activation. Treatment with SP alone did not alter c-Jun activation. Total levels of c-Jun did not change under these conditions. The role of JNK inhibition on UVB-induced cell death was examined by pre-treating cells with SP (0 or 50 μM) 1 hr prior to exposure to UVB (0–600 J/m^2). Preliminary experiments with this compound indicate that it is not toxic to HLE cells at doses as high as 100 μM (data not shown). Pretreatment with SP increased UVB-induced cell death by approximately 50%, 80%, and 40% at 100, 300, and 600 J/m^2, respectively, compared with those cells receiving UVB alone (Fig. 3.6D). Ultraviolet B–induced cell death was not altered by pretreatment with SB203580, a specific inhibitor of p38 activity (data not shown).

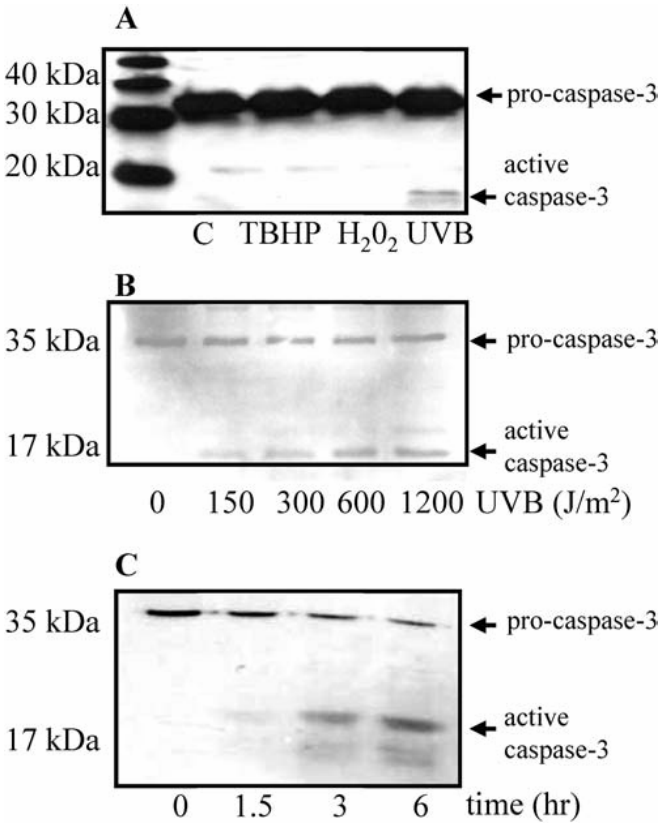

Figure 3.5: Stress-induced activation of caspase 3: concentration and time dependency

Human lens epithelial (HLE) cells were treated with ultraviolet B (UVB; 600J/m^2), H_2O_2 (500μM) or t-butyl hydroperoxide (TBHP; 200μM) harvested after 6 hrs and probed with caspase-3 antibody (A). Concentration-dependent activation of caspase-3, 6 hrs following treatment with UVB (0-1200 J/m^2) is given (B). Time-dependent activation of caspase-3, 1.5, 3 and 6 hrs following treatment with UVB (600 J/m^2) is given (C). Immunoblots are representative of results obtained from at least three independent experiments (n=3/experiment).

3.5 Discussion

The present study investigated the mechanisms by which environmental (UVB) and chemical (H_2O_2 and TBHP) stress induces HLE cell death. We present several lines of experimental evidence to suggest that UVB predominantly induces apoptosis in HLE cells, whereas H_2O_2 and TBHP treatment primarily result in necrosis. Although all three stressors induced HLE cell death, H_2O_2 and TBHP-induced cell death was much more rapid than that observed with UVB. Rapid cell death is associated with acute cellular injury characteristic of necrosis, whereas prolonged cell death is more characteristic of apoptosis. Hydrogen peroxide concentrations as high as 0.6mM have been reported in the aqueous humor of the lens in persons with cataracts [73]. This concentration compares with the LD_{50} of H_2O_2 used in this study. Necrosis and apoptosis induce distinct morphological changes that clearly distinguish between these two modes of cell death. In this study, UVB treatment induced morphological changes (i.e., formation of apoptotic bodies) consistent with apoptosis, whereas H_2O_2- and TBHP-treated cells displayed morphological changes (i.e., formation of vacuoles) consistent with necrosis [239, 240]. Our results contrast those of Wickert et al. [241], who report that UVB (1.5 J/cm^2) induces morphological evidence of necrosis in rat LEC. This discrepancy may be due to the higher UVR dose used in their study. Morphological observations in studies using a human lens model show that low doses of UVR (2 mJ/cm^2) induce apoptosis, whereas higher doses (10 mJ/cm^2) induce both apoptosis and necrosis [242]. The observance in this study of some necrotic cell death in UVB-treated HLE cells is in agreement with this latter report. In addition to morphological evidence, release of LDH from cytosolic pools

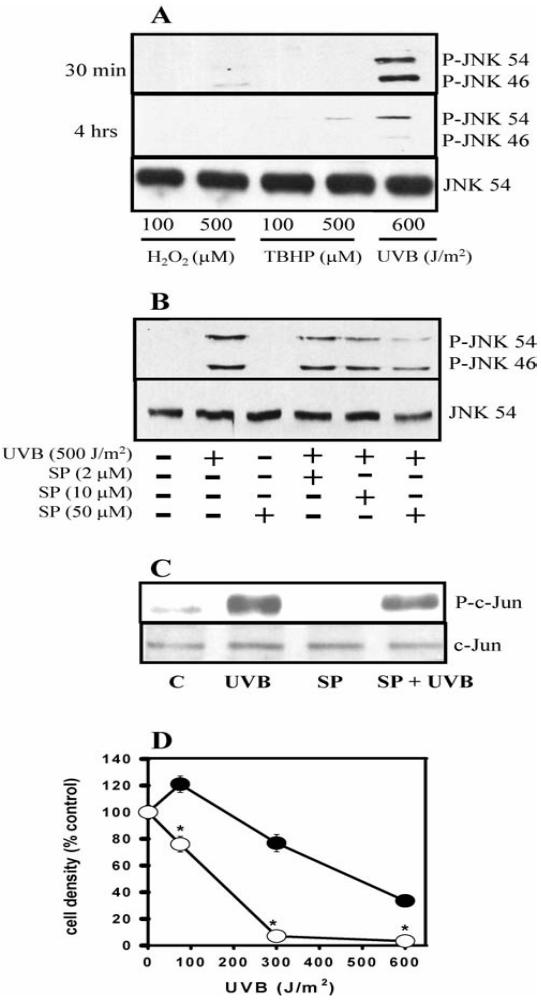

Figure 3.6: Stress induced JNK activation and role in ultraviolet B (UVB)-induced cell death

(A) Human lens epithelial (HLE) cells were treated with UVB, H_2O_2, or t-butyl hydroperoxide (TBHP); harvested after 30 min or 4 hrs; and probed with active (P-JNK 54/46) or total (JNK 54) c-Jun NH_2-terminal kinase (JNK). (B) Human lens epithelial cells were pretreated (1 hr) in the absence or presence of the JNK inhibitor, SP600125 (SP), and harvested 30 min following UVB treatment. Active and total JNK was determined by immunoblotting. (C) Representative immunoblot (n=3) of active c-Jun (P-c-Jun) and total c-Jun 3 hrs following treatment with UVB (500J/m^2) or SP (50μM) alone and in combination. (D) Cell density (% control) in the presence (open circles) and absence (closed circles) of SP (50μM) 24 hrs following treatment with UVB (0-600J/m^2) as assessed by crystal violet staining. Significant reductions in cell density between SP-treated (open circles) and untreated (closed circles) cells is given (*P<0.05).

is an indicator of loss of membrane integrity associated with necrotic cell death [243]. Our results indicate that LDH release is significantly higher in those cells treated with chemical stressors compared with UVB.

The modest increase in LDH activity observed in UVB treated HLE cells may be due to a late apoptosis/necrosis stage of cell death. DNA fragmentation is an early marker of apoptosis characterized by the presence of bands or ‘‘ladders’’ upon electrophoresis [244]. In the present study, UVB treatment at both early and late timepoints induced DNA fragmentation patterns consistent with apoptosis, whereas treatment with H_2O_2 and TBHP did not result in DNA fragmentation, a finding consistent with necrosis [245]. The decrease in UVB-induced DNA fragmentation observed at the 24-hr timepoint may represent a late stage of apoptosis. Another early marker of apoptosis is the translocation of phosphatidylserine from the interior of the cell membrane to the exterior, detected by binding of FITC–labeled annexin V [246]. This process is thought to occur prior to DNA fragmentation [245]. In contrast, PI breaches leaky membranes, a characteristic event of late apoptosis and/or necrosis [247]. In support of our morphological findings, UVB treatment increased annexin V staining relative to controls, suggesting apoptosis as the predominant mode of cell death. In contrast, H_2O_2 and TBHP treatment increased PI staining relative to controls, suggestive of necrosis as the predominant mode of cell death. Our study contrasts previous reports that indicate that H_2O_2 causes apoptotic cell death in rat LEC [85] and rat whole lens [248] models. This discrepancy may be due to differences in cellular models, the concentration of peroxide, or the time exposure used to treat cells. Choudhary et al. [249] report H_2O_2-induced apoptosis in cultured rat lenses at concentrations as high as 200 μM, with higher concentrations favoring necrosis. In

contrast, we observed that lower concentrations of H_2O_2 did not induce apoptosis, and higher concentrations favored necrotic HLE cell death. Our finding that TBHP induced necrosis in HLE cells is consistent with observations of necrotic cell death in TBHP-treated PC12 cells [250]. The activation of MAPK signaling cascades by UVR has emerged as an important consideration in cataractogenesis [225, 251, 252]. Our results suggest that JNK activation is an early event in UVB-induced apoptosis in HLE cells and precedes the activation of caspase-3 and DFF45. The activation of JNK prior to UV-induced apoptosis is in agreement with others who show that JNK activation is required for UV-induced apoptosis in murine fibroblasts [88]. Ultraviolet B–induced JNK activation preceded DNA fragmentation in HLE cells. This observation is in agreement with others who show that JNK activation precedes DNA fragmentation in UVC-treated Jurkat T-cells [253]. Treatment with H_2O_2 and TBHP, at high and low concentrations, did not activate JNK or result in DNA fragmentation in HLE cells. This suggests that the response of HLE cells to UVB stress is distinct and utilizes existing stress-induced signaling pathways to execute cell death.

Although UVR-induced JNK activation has been observed in numerous cell lines including keratinocytes [254-256], Jurkat T-cells [253], and rat PC-12 pheochromocytoma cells [257], its exact role in initiating apoptosis appears to be cell and context-specific [258-260]. To further elucidate the mechanism by which UVB induced HLE cell death, the activation of two stress proteins associated with apoptosis, caspase-3, and DFF 45 was examined. These proteins mediate DNA fragmentation, and their activation is therefore critical to the apoptotic process [228]. Caspase-3 cleavage has previously been shown to mediate JNK activation in human leukemia cells [261]. We

report an increase in activated/cleaved forms of caspase-3 and DFF45 in UVB-treated HLE cells. Furthermore, UVB-induced caspase-3 activation was dependent on both time and dose. In contrast, neither H_2O_2 nor TBHP activated caspase-3 or DFF45 in the present study. The importance of caspase-3 activation in apoptosis is supported by studies demonstrating that inhibition of this protein suppressed UVB-induced apoptosis in cultured human keratinocytes [234]. The induction of apoptosis post–UV exposure can be protective in nature, aimed at removing damaged cells and preventing neoplastic damage [262]. Activation of JNK has recently been reported to be essential for the IL-3–mediated survival of FL5.12 cells [263]. In order to explore the influence of UVB-induced JNK activation on HLE cell death, we investigated the response of HLE cells to the specific JNK inhibitor, SP600125. We report an increase in UVB-induced HLE cell death with JNK inhibition. This suggests that JNK activation in response to UVB may serve as a prosurvival (e.g., antiapoptotic) signal in HLE cells. Interestingly, activation of c-Jun, a downstream target of JNK, was only modestly reduced with SP600125. This suggests that the effects of SP600125 on UVB-induced cell death may be mediated by other JNK-activated transcription factors such as ATF-2, ELK-1, or p53 [264]. It is of interest to note that inhibition of p38 activity did not alter UVB induced HLE cell death (data not shown). This contrasts a recent report demonstrating the involvement of p38 activity, but not JNK, in cataract formation [221]. The role of JNK and p38 signaling in UVB-induced lens apoptosis and cataractogenesis merits further consideration. Data presented in this study establishes a preliminary sequence of cellular and molecular events leading to UVB-induced apoptosis in HLE cells. We report JNK activation as an early event in UVB-induced apoptosis, which occurs within 30 min after UVB irradiation. Subsequent

activation of caspase-3 occurs as early as 1 hr and peaks 6 hrs post-UVB. Ultraviolet B–induced JNK activation occurs prior to morphological changes, nuclear DNA fragmentation, and annexin V staining and may serve as a prosurvival mechanism in HLE cells. Further clarification of the molecular events involved in HLE cell apoptosis may lead to the development of therapeutic strategies to prevent and/or delay cataractogenesis in both humans and animals.

3.6 Acknowledgments

We thank Dr. Venkat Reddy of the Kellogg Eye Center (University of Michigan, Ann Arbor, MI) for providing the HLE cell line, and Robert King, Aaron Downs, and Linda Burianek for their expert technical assistance. Special thanks to Dr. Mark Failla for helpful discussions and critical input related to this manuscript.

CHAPTER 4

ISOLATION AND CHARACTERIZATION OF PRIMARY CANINE LENS EPITHELIAL CELLS

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4.1 Abstract

The purpose of this study was to characterize the proliferation and differentiation of primary canine lens epithelial cells (LEC) under standard culture conditions. Canine LEC were isolated by mechanical dissection of the canine globe and enzymatic digestion of the lens capsule from fresh lenses. Isolated capsules and cell suspensions were seeded in laminin-coated culture flasks. Canine LEC proliferated and formed monolayers which could be passaged and maintained for several weeks. Cells were characterized morphologically and cell lysates examined for expression of protein markers of epithelial origin and differentiation. Canine LEC exhibit morphological characteristics of epithelial cells when cultured on laminin/lysine coated flasks. Expression of the lens epithelial marker, vimentin, was highest at passage 2 and diminished subsequently (passages 3-6). Expression of γ-crystallin, a protein found only in differentiated lens fiber cells, increased

at passage 6. A laminin/lysine coated surface supported optimal proliferation of canine LEC. Both an initial seeding density of 1.0 x 10^5cells/cm^2 and culture in Dulbecco's Modified Essential Media (DMEM) supplemented with 10% FBS supported a doubling time of less than 48 hr in canine LEC. This study demonstrates that primary canine LEC retain the characteristics of lens epithelial cells for several passages under the described culture conditions and represent a suitable *in vitro* model for investigating lens physiology and cataractogenesis.

4.2 Introduction

The vertebrate lens is composed of two major cell types viz., epithelial and fiber cells. A single monolayer of cuboidal epithelial cells lines the anterior surface of the lens, which is divided into central, germinative and transitional zones of epithelial cells. Upon migrating to the lens equator, cells in the transitional zone differentiate to give rise to lens fiber cells, which are terminally differentiated cells devoid of cellular organelles. Lens fiber cells accumulate large amounts of crystallin proteins that are essential for the transparency of the lens. Both epithelial and fiber cells are encased within the lens capsule, a proteinaceous basement membrane composed of structural proteins such as laminin, collagen and fibronectin. The lens epithelium plays an important role in the regulation of nutrient and ion transport to all cells in the lens, and actively participates in adaptive responses to environmental and chemical stressors[11, 12]. Damage to the lens epithelium caused by ultraviolet radiation or oxidative stress represents an initiating event in cataract formation, emphasizing the importance of maintaining the integrity of the epithelial layer[20, 68]. Both immortalized and primary lens epithelial cell models have previously been utilized in studies of lens physiology and cataractogenesis. Studies with

rodent and chicken embryonic cell lines, as well as human lens epithelial cell lines, have been useful in elucidating the characteristics of the lens epithelium[265-267]. Primary cultures, due to intact cell signaling pathways that regulate development, may have several advantages in studying lens epithelial cell growth as compared to immortalized cell lines[268]. Disadvantages of primary cultures include difficulty in obtaining tissues, limited lifespan in culture, and variability among different tissue donors. Primary cultures of canine lens epithelial cells have previously been utilized to examine iron metabolism[95, 96, 269] and cataract development[8]. However, information regarding optimal growth conditions and markers of epithelial origin and differentiation has not been previously reported. Primary canine LEC may be a useful model for studies on the development of human cataract, due to the similar incidence of age-related cataract in humans and dogs[18]. The purpose of this study was to examine the isolation, growth, and expression of differentiation markers of primary canine LEC using standard conditions. We conclude that primary canine LEC are epithelial in origin, proliferate for up to 5 passages under optimal growth conditions, and retain the ability to differentiate in culture. Thus, primary canine LEC provide a suitable *in vitro* model for investigation of the lens epithelium environment.

4.3 Materials and Methods

4.3.1 Establishment of canine LEC culture

Whole canine globes were collected from medium to large mixed-breed dogs euthanized for population control purposes at a local animal shelter. An estimated age of each dog was achieved by dental plaque examination and selection was restricted to dogs between 1 and 4 years of age. Eyes were transported in 2% betadine on ice to the

laboratory and all dissections completed within 1 hr of enucleation. Excess conjunctiva was removed and access to the anterior chamber achieved by a circumferential scalpel cut 1mm below the limbus, and along the scleral/venus plexus. For LEC culture, the anterior lens capsule was excised from the underlying fiber cell mass and two capsules representing one dog were placed in a 1.5ml centrifuge tube containing 0.5ml of 0.05% trypsin- EDTA (Invitrogen). Capsules were incubated for 15 min at 37°C, and centrifuged at 2000 rpm for 3 min at room temperature. Trypsin was neutralized by the addition of 1.0 ml of Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) with penicillin/streptomycin (0.4%) and tubes were centrifuged as above. Supernatant (0.5 ml) was removed and remaining medium with capsules transferred to T25 cell culture flasks coated with laminin/poly-D-lysine-coated (Lam/Lys, 100μg/ml) and containing 2.0 ml of medium at 37˚C in a humidified atmosphere of 95% air: 5% CO_2. Outgrowth of cells from the capsules was observed after 5-7 days. Fresh medium replaced spent medium every 2-3 days thereafter. The first passage was performed on approximately day 12 when large areas of confluent cells were evident. The monolayer was washed with PBS before the addition of 3.0 ml trypsin-EDTA. After 5-10 min at 37°C, trypsin was neutralized with a 2X volume of complete medium and the cell suspension was centrifuged at 2000 rpm for 7 min at 9°C in a swinging-bucket centrifuge. The supernatant was aspirated and the pellet was resuspended in fresh complete medium and 1.0 ml transferred to a Lam/Lys coated T25 flasks containing 2.0 ml of complete medium. Cells were subcultured 1:3 to 1:12 upon reaching 90% confluency. Capsules were generally discarded at first passage and not included in experimental cultures.

4.3.2 Morphology

Canine LEC from passage 2 were seeded on chamber slides ($1.0x10^3$ cells per well) and maintained until the monlayer was confluent. Cells were fixed with 4.7% formalin, stained with 0.5% crystal violet and examined under phase contrast microscopy (Olympus IX50) fitted with a digital camera. Images were captured from three independent slides at 40X magnification.

4.3.3 Expression of epithelial and differentiation protein markers

Cell Harvest and Immunoblotting: Canine LEC lysates from passages 1-6 were collected for immunoblot analysis as previously described [89]. Proteins were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) and electrophoresed at 180 V for approximately 1 hr using the Mini-PROTEAN III system (Bio-Rad, Hercules, CA). Separated proteins were transferred to a nitrocellulose membrane at 100 V for 1 hr. The membrane was blocked for 1 hr at room temperature in 5% non-fat dry milk (NFDM) in Tris-buffered saline (TBS, pH 7.4) with 1% Tween-20 (TTBS). Membranes were incubated at 4˚C overnight with appropriate primary antibodies to vimentin (RV202, Abcam, Cambridge, MA) and γ crystallin, (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:250 in 5% NFDM with TTBS. Membranes were washed in TTBS for 30 min and incubated with secondary antibody (HRP-linked anti-mouse or anti-rabbit, 1:2000, (Cell Signaling Technology (Danvers, MA)). Membranes were washed for 30 min in TTBS and protein signals detected using Enhanced Chemilumenscence Plus (ECL plus) (GE Healthcare, Piscataway, NJ). Membranes were exposed to autoradiography film for appropriate times.

4.3.4 Determination of cell number and doubling time

Cells were collected by trypsinization at the indicated passage number and visually counted using a hemocytometer (Fisher Scientific, Pittsburgh PA). For experiments to optimize cell culture conditions and determine doubling time, numbers of cells present in flasks seeded with lens epithelial cells from a minimum of three separate sets of lens capsules were counted. Doubling time was calculated using the following equation: [$DT=t \log 2/\log(N_t/N_0]$, where N_0= initial cell number and N_t=cell number at culture period, t. Cell counts were averaged and the standard deviation was calculated.

4.3.5 Optimization of cell adherence and growth

Adherence: The effect of Lam/Lys coating on cell adherence was determined by seeding coated and non-coated 12-well dishes with 5.0×10^5 cells per well. Following incubation (37°C, 5% CO_2) at 30, 60 and 120 min, unattached cells were collected and counted. The percent of adhered cells was calculated based on initial seeding density. G*rowth*: The effect of Lam/Lys coating on cell proliferation was determined by seeding coated and non-coated 12-well plates with 1.0×10^4 cells per well. Dishes were incubated at 37°C, 5% CO_2 for 2 d at which time maximum density was approximately 90% confluency. Monolayers were exposed to trypsin-EDTA, cell number was determined with the hemocytometer, and doubling times calculated for each treatment.

Seeding Density: The effect of initial seeding density on subsequent cell proliferation was determined by seeding Lam/Lys coated 12-well dishes with cell densities ranging from $1.0\text{-}10.0 \times 10^4$ cells/cm^2. After 2 d, cells were collected after trypsinization to detach adhered cells and counted.

Serum Concentration

The effect of serum concentration in medium on canine LEC proliferation was determined by seeding first passage cells in DMEM containing 2.5, 5, or 10% FBS. Cells in these three separate experimental conditions were passaged regularly with cell counts performed and doubling times calculated, until doubling times exceeded 14 days.

4.3.5 Statistics

Data are expressed as means ± SEM. Statistical differences between treatments were determined by analysis of variance (ANOVA) with Tukey's posthoc comparisons or students T-test using Minitab (Chicago IL) statistical analysis software ($p<0.05$).

4.4 Results

4.4.1 Cell Morphology

Representative morphology of canine LEC (40X, passage 2) is shown in Fig. 4.1A. Canine LEC display typical morphological characteristics of epithelial cells, including cuboidal shape and tight packing[93].

4.4.2 Expression of epithelial and differentiation protein markers in canine LEC

The lens-specific epithelial cell protein marker, vimentin, was examined by immunoblotting to confirm the epithelial origin of LEC cultures. Vimentin expression was observed in canine LEC at passage 2 (P2) with minimal to no expression observed in passages 3 through 6 (P3-P6) (Fig. 4.1B). Gamma crystallin expression was also monitored to examine whether canine LEC differentiate under the given culture conditions. Expression of γ crystallin was observed in P6 cultures of canine LEC, (Fig. 4.1C).

4.4.3 Optimization of growth and adherence of canine LEC Laminin/Poly-Lysine Coating

The percentage of cells adhered to Lam/Lys coated plates at 30, 60, and 120 min after plating was 79, 98, and 99%, and 53, 62, and 80% for non-coated plates, respectively (Fig. 4.2A). At all times tested, cell adherence was significantly greater in Lam/Lys coated versus non-coated tissue culture plates ($p<0.05$). The mean doubling time for LEC seeded into flasks with and without Lam/Lys coating was 6.5 and11 d, respectively (Fig 4.2B).

The typical cell yield of one T25 flask containing two capsules was approximately $1.0x10^6$ cells. The subsequent experiments were performed to examine the effect of initial seeding density and serum concentration on the proliferation of canine LEC. Initial seeding densities of $1.0 x10^4$, $5.0 x10^4$ and $10.0 x10^4$ resulted in mean doubling times of 6.8, 3.4 and 2.3 days, respectively (Fig 4.3). Doubling time for canine LEC cultured in medium with 10% FBS was significantly shorter ($p<0.05$) than when cells were incubated in media containing 2.5% or 5% FBS at passages 3, 4, and 5. The proliferative capacity of LEC cultures was maintained through passage 5 when cells were cultured in medium with 10% FBS. Doubling times of LEC cultures increased with passage number regardless of FBS concentration present in growth medium. A second incubation of capsules via transfer to a new Lam/Lys coated plate generally yielded another $1.0x10^6$ cells, however, capsules could not reliably withstand continued incubation after this point. The use of canine serum in place of FBS did not affect cell growth or attachment (data not shown).

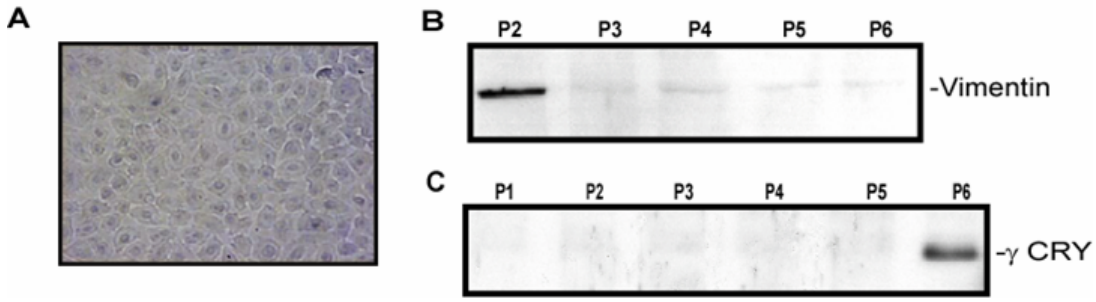

Figure 4.1: Epithelial morphology and protein marker expression in primary cultures of canine LEC

A. Cells were seeded in chamber slides as described in Materials and Methods and morphology examined by phase contrast microscopy at 40x magnification. A representative image (P2) is shown.

B. Expression of vimentin was analyzed by immunoblotting lysates of canine LEC passages 2-6 (P2-P6). A representative immunoblot is shown.

C. Expression of gamma crystalline (γ-CRY) was analyzed by immunoblotting lysates of canine LEC passages 1-6 (P1-P6), as described in Materials and Methods. A representative immunoblot is shown.

4.5 Discussion

The present study describes the isolation, optimal growth conditions, and cellular differentiation markers of primary cultures of canine LEC. The first objective of this study was to confirm the epithelial origin of LEC cultures by examining the expression of vimentin. Cells of epithelial origin in the lens express a variety of cell type specific proteins, including vimentin [270], some cytokeratins [271], Pax6, and FOXE3 [272]. Vimentin is a major component of the intermediate filament network and has been associated with epithelial cell migration *in vitro* [270, 273]. In the present study, expression of vimentin was observed in early (P2), but not subsequent passages of LEC cultures. This loss of vimentin expression from epithelial cells in later passages has been reported in bovine lenses and is commonly observed as epithelial cells differentiate into mature fiber cells [274].

The second objective of this study was to determine the ability of primary canine LEC to differentiate in culture. Epithelial cell differentiation in the lens is marked by the expression of several proteins including β and γ crystallins, membrane intrinsic protein (MIP) and membrane protein 20 (MP20) [275]. The crystallins are a family of lens-specific heat shock proteins important in maintaining lens transparency. Alpha crystallin is located in both epithelial and fiber cells, whereas both β and γ crystallins are found exclusively in lens fiber cells [276-278]. Differentiation of LEC can be induced *in vitro* by introduction of certain growth factors to the medium, such as fibroblast growth factor (FGF) and bone morphogenic protein (BMP) [279]. In the present study, γ crystallin expression was highest in late passage LEC cultures (P6) when growth had slowed to a doubling time of over one week. Because γ crystallin is fiber cell-specific, expression of

this protein in late passage LEC suggests the initiation of their transition to fiber cells and that this protein may be a good indicator of cell differentiation *in vitro*.

The third objective of this study was to investigate the effect of cell surface coating, seeding density and serum concentration on canine LEC proliferation. Culture of canine LEC on a Lam/Lys coated surface promoted greater attachment and proliferation as compared to a noncoated surface. Laminin is a major component of the lens capsule and binds to some integrin subunits, which are differentially regulated during lens fiber differentiation [280]. Growth of canine LEC also was supported on a non-coated surface, although doubling times were significantly increased. Both an initial seeding density of 10.0 x 10^4cells/cm^2 and culture in DMEM supplemented with 10% FBS resulted in a doubling time of approximately 48 hr. Canine LEC, under the conditions outlined in this study, appear to be reliable for experimentation from passages 2-5.

As primary human cell cultures are expensive and limited, it is beneficial to identify other species for investigation of the LEC biology and pathology. Because some human and canine proteins exhibit similar sequences, canine LEC may represent a better model for human LEC than mouse, rat, or bovine [275]. Our data support the use of primary cultures of canine LEC as a suitable model for research on lens biology based on the maintenance of epithelial characteristics through early passages with the optimization of several cell culture conditions.

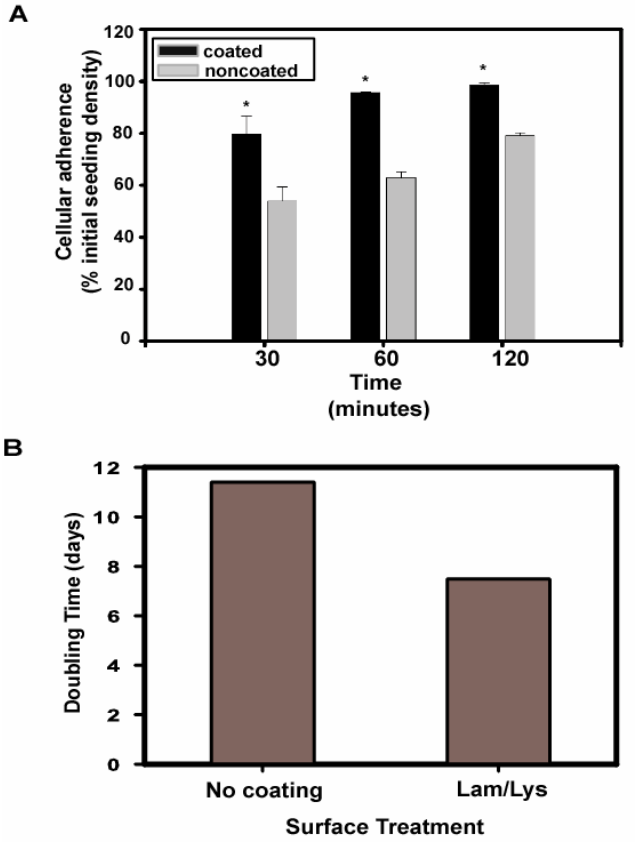

Figure 4.2: Effect of culture surface coating on canine LEC attachment and proliferation.

A. Canine LEC (P2) were added to non-coated or laminin/poly-D lysine (100μg/ml) coated 12-well dishes at an initial seeding concentration of $5.0x10^5$ cells per well. The percentage of cells attached to culture surface was determined by counting cells at 30, 60 and 120 min. Data represent the percentage of cells attached at each time as a percentage of initial seeding density. Data are means ± SEM. Asterisk indicates significant differences between coated and noncoated dishes at each time (*, Student's T-test, $p<0.05$).

B. Canine LEC (P2) were seeded in non-coated or laminin/poly-D lysine (100μg/ml) coated 12-well dishes at an initial seeding concentration of $1.0x10^4$ cells per well. Cell population doubling times were estimated by direct counting of cells released from the dish with trypsin-EDTA as described in Methods.

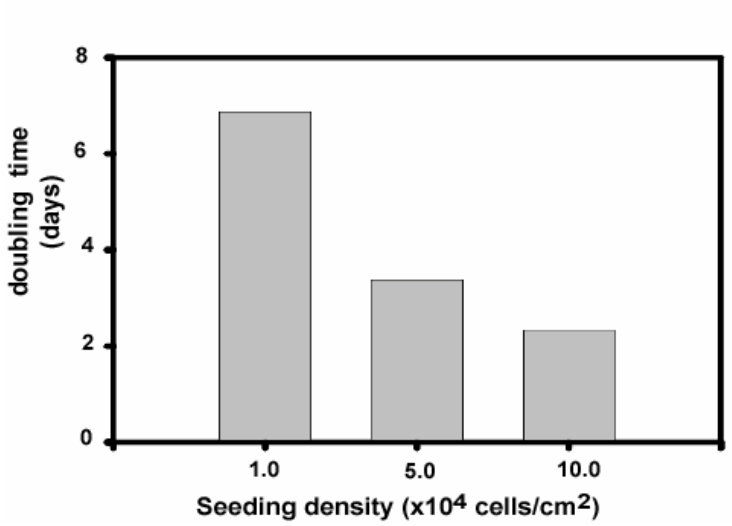

Figure 4.3: Effect of initial seeding density on proliferation of canine LEC
Indicated densities of canine LEC (P2) were seeded in laminin/poly-D-lysine (100μg/ml) coated 12-well dishes and doubling times were calculated after monolayers in the most confluent wells were approximately 90% confluent (approximately 2 days).

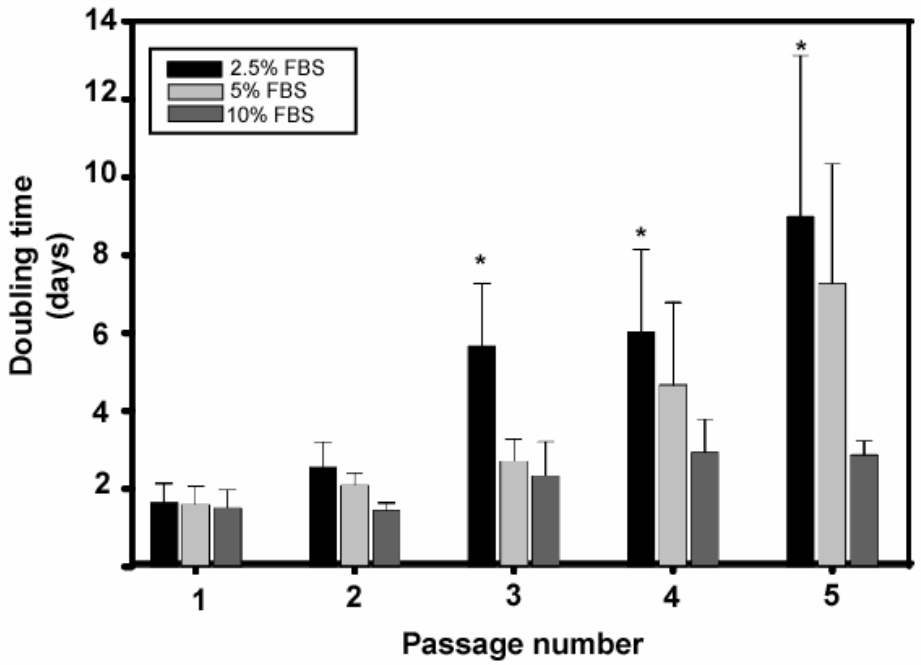

Figure 4.4: Effect of serum concentration on canine LEC proliferation
Canine LEC were suspended in DMEM containing 2.5, 5 or 10% FBS and seeded in T25 cell culture flasks. Doubling time (days) was calculated for passages 1-5. Data are presented as means ± SEM. Significant differences are noted by presence of asterisks at passages 3-5 for cultures grown in medium with 10% as compared to 2.5% FBS (ANOVA, $p<0.05$).

CHAPTER 5

REGULATION OF GAP JUNCTION INTERCELLULAR COMMUNICATION IN PRIMARY CANINE LENS EPITHELIAL CELLS: ROLE OF PROTEIN KINASE C

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5.1 Abstract

Purpose: Gap junction intercellular communication (GJIC) is important in maintaining lens epithelial cell homeostasis and reductions in GJIC may be associated with the development of cataract. Protein kinase C (PKC) activation can disrupt gap junction communication via phosphorylation of connexin 43 (Cx43) proteins that compose gap junction channels. This study examined the role of PKC activation in modulating GJIC in a primary canine lens epithelial cell (LEC) line. *Methods*: TPA (12-O-tetradecanoyl-phorbol-acetate), a potent PKC activator and inhibitor of GJIC, was utilized in the present study. Primary cultures of canine LEC were treated with TPA (0-1000ng/ml) for 0.5 hr and GJIC was assessed by scrape loading/dye transfer (SL/DT), and immunoblotting to detect phosphorylation of Cx43 protein. Inhibition of general and calcium-dependent PKC activity was achieved by pretreatment of cells with GF109203X and Gö6976,

respectively. *Results*: Treatment with TPA (1-1000ng/ml) significantly decreased GJIC in canine LEC as assessed by SL/DT. Pretreatment with 10 and 100 ng/ml TPA decreased GJIC by 80% as compared to controls and increased Cx43 phosphorylation as assessed by immunoblotting. Pretreatment of cells with GF109203X and Gö6976, partially restored TPA-inhibited GJIC by 40% and 60%, respectively, and reduced Cx43 phosphorylation. Expression of calcium dependent PKC isoforms was detected in canine whole lens and LEC. *Conclusions*: Treatment with TPA significantly reduces GJIC in canine LEC. These effects are mediated, in part, by activation of calcium-dependent PKC isoforms. Primary canine LEC are a useful model in the study of the molecular mechanisms involved in GJIC and cataractogenesis.

5.2 Introduction

The lens is an avascular tissue containing an extensive network of gap junction intercellular communication (GJIC) that enables the regulation of nutrient and waste transport [179]. Lens homeostasis is maintained by circulating currents of ions directed inward at the anterior and posterior poles, while removal of wastes is directed outward at the equator. The basis for this ion flux is established by regional differences in membrane ion permeability and by specialized cell-to-cell channels, termed gap junctions [11, 13]. Metabolic cooperation established by GJIC allows mitotically active lens epithelial cells (LEC) to communicate with quiescent fiber cells, maintaining lens transparency [11-13]. Gap junction channels are composed of proteins called connexins, a highly conserved family of proteins that have been identified in a variety of mammalian species [180, 281]. Hexameric configurations of connexins, termed connexons, associate in adjoining cells, forming a pore which connects the cytosolic compartments of

adjacent cells and enables the transfer of molecules <1kD in size [180]. Three connexin proteins have been identified in the vertebrate lens: connexins 43, 46 and 50 [282]. Connexin 43 (Cx43)-containing gap junction channels have been localized to the LEC monolayer whereas connexins 46 (Cx46) and 50 (Cx50) are found in fiber cells [282]. Mutations in either Cx46 or Cx50 are linked to congenital cataracts in humans [283] and in mouse knockout models [284], suggesting the importance of these connexins in cataractogenesis.

Communication through gap junctions regulates basic cell processes such as cell growth and differentiation [285] and can be modulated by physiologic stimuli such as pH and calcium concentration [286], as well as by carcinogens, including the tumor promoter, 12-O-tetradecanoyl phorbol 13-acetate (TPA) [194]. TPA inhibits GJIC through the activation of protein kinase C (PKC) [287-289] resulting in hyperphosphorylation of Cx43 and internalization of Cx43 containing-plaques [290]. The regulation of gap junction communication by PKC is mediated by phosphorylation of serine 368 residues in the C terminus of Cx43 protein [195, 291].

PKC is a family of serine/threonine kinases containing twelve different isoforms which regulate a variety of physiological cell processes [292]. Classical or conventional PKC's, which include α, β_I, β_{II}, and γ isoforms, are calcium-dependent, and activated by both diacylglyerol and TPA [293, 294]. Both PKC α and γ isoforms are present within LEC [295], however PKC γ is the primary isoform which is thought to regulate GJIC in LEC [296-298].

The present study examines the regulation of GJIC by PKC in primary cultures of canine LEC treated with the phorbol ester, TPA. We provide evidence that TPA

treatment results in a significant reduction in GJIC in canine LEC, which corresponds with phosphorylation of Cx43 protein. Furthermore, our results suggest that TPA-induced reduction in GJIC is regulated by activation of calcium-dependent PKC isoforms in canine LEC. GJIC is altered in cataractogenesis [14], therefore elucidating the signaling mechanisms which regulate GJIC in the lens may lead to potential strategies for preventing or delaying cataract development.

5.3 Materials and Methods

5.3.1 Cell Culture

Whole canine globes were collected from medium to large breed dogs euthanized for population control purposes at a local animal shelter. An estimated age of each dog was achieved by dental plaque examination and selection was restricted to dogs between approximately 1 and 5 years of age with no obvious signs of cataract or other ocular disease. Eyes were transported in 2% betadine on ice to the laboratory and all dissections completed within 1 hr of enucleation. Excess conjunctiva was removed and access to the anterior chamber achieved by a circumferential scalpel cut along the sceral/venus plexus. For LEC culture, the anterior lens capsule was excised away from the underlying fiber cell mass and two capsules representing one dog were placed in a 1.5ml centrifuge tube containing 0.5ml trypsin EDTA, incubated for 15 min at 37°C, and centrifuged for 3min at 2000rpm. Trypsin was neutralized with the addition of 1.0ml of Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) with penicillin/streptomycin (0.4%) and centrifuged again at 2000rpm for 3 min. Supernatant (0.5ml) was removed and remaining medium with capsules transferred to laminin/polylysine-coated (100μg/ml) T25 cell culture flasks with 2.0 ml of medium at

37˚C in a humidified atmosphere containing 5% CO_2. Growth of primary canine LEC from the capsules was observed approximately 5-7 days following dissection, and medium was changed every 2-3 days after this point. Cells of passage number 1-3 were used for all subsequent experiments. For experiments using whole canine lens, lenses were removed and immediately wrapped in foil and placed in liquid nitrogen. Whole lenses (including capsule and fiber cell mass) were homogenized in lysis buffer (50mM TrisCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1%Triton X-100, 0.1% SDS, 50mM sodium fluoride, 10mM sodium pyrophosphate, protease cocktail) and were centrifuged for 12,000 x g for 15 min. Supernatants representing whole lens lysates were stored at -80°C until later use.

5.3.2 Materials

TPA was obtained from Cell Signaling Technology (Beverly, MA) at a concentration of 200μm in dimethyl sulfoxide (DMSO) and diluted to the indicated concentrations in serum-free medium for all experiments. The final concentration of DMSO as solvent was less than 0.1%. The PKC inhibitors, Gö6976 and GF109203X, were obtained from Calbiochem (La Jolla, CA). Each inhibitor was diluted in DMSO to a stock solution of 500μM and 2mM, respectively. Cells were pre-treated with PKC inhibitors for the indicated times and doses as described in Results. MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), sorbitol and hydrogen peroxide were obtained from Sigma Aldrich (St Louis, Mo). Monoclonal connexin 43 and polyclonal phospho-connexin 43 (Ser368) antibodies were obtained from Chemicon International (Temecula, CA). Antibodies for PKC α, β_I, and β_{II} isoforms were obtained from Santa Cruz

Biotechnology (Santa Cruz, CA). PKC γ antibody was obtained from Transduction Laboratories (San Jose, CA).

5.3.3 Gap Junction Communication Assay

GJIC was assessed by scrape loading/dye transfer (SL/DT) assay, as previously described [299], with some modifications. Cells were cultured in 12-well laminin-coated plates at a density of 2.0 x 10^5 cells/ml. Following appropriate treatments, medium was removed and replaced by 1.0ml PBS containing 0.05% Lucifer yellow (Sigma Aldrich, St. Louis, MO) and three scrapes were made per well, using a steel surgical blade. After 5 min, cells were washed three times with PBS and fixed in 3.7% paraformaldehyde, pH 7.0. Distance of dye transfer was determined using a Zeiss 510 META Laser Scanning confocal microscope. Using Scion Image Software (available for download at www.scioncorp.com), three sites per scrape were randomly chosen as points for the measurement of distance of dye transfer to neighboring cells, using a line perpendicular to the scrape line. Effect of test compound on gap junction communication is presented as distance of dye travel as a percent of control.

5.3.4 Immunoblotting

Cells were washed in PBS (pH 7.4) and scraped into cell extraction buffer (50mM TrisCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1%Triton X-100, 0.1% SDS, 50mM sodium fluoride, 10mM sodium pyrophosphate, protease cocktail). Extracts were centrifuged for 13,000 x g for 5 min. Protein concentration of the supernatant was measured by bicinchoninic acid (BCA) protein analysis (Pierce Chemical Company, Rockford IL).

After appropriate treatments, cellular protein samples were diluted into modified Laemmli sample loading buffer (100mM TrisCl (pH 6.8), 5% BME, 2% SDS, 25%

glycerol, 0.1% bromophenol blue) and heated at 95˚C for 5 min. Equal amounts of protein (20 μg for p-Cx43, β-actin and 50μg for PKC isoforms) were loaded onto a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) and electrophoresed at 180 V for approximately 1 hr using the Mini-PROTEAN III system (Bio-Rad, Hercules, CA). After electrophoresis, protein was transferred to a nitrocellulose membrane at 100 V for 1 hr. The membrane was blocked for 1 hr at room temperature in a Tris-buffered saline (TBS, pH 7.4) solution containing 5% nonfat dry milk. After blocking, membranes were incubated at 4˚C overnight with appropriate primary antibodies (1:1000) in TBS containing 0.1% Tween-20 (TTBS) and 5% bovine serum albumin (BSA). Membranes were washed extensively with TTBS and incubated with secondary antibodies (anti-rabbit or anti-mouse 1:2000, Cell Signaling Technology), conjugated with horseradish peroxidase in TTBS for 1 hr. Membranes were washed again and protein signals were developed using ECL chemiluminescence detection reagents (Amersham Biosciences, Piscataway, NJ). Membranes were exposed to Kodak X-OMAT AR film (Rochester, NY) for an appropriate length of time and developed according to manufacturer's recommendations.

5.3.5 Immunofluorescence

Primary canine LEC were fixed in methanol at -20˚C for 20 minutes. Cells were rinsed three times in PBS and permeabilized with 0.5% Triton X-100-PBS for 30 min at room temperature. Cells were blocked with 5% w/v BSA in PBS (Sigma Aldrich) and incubated with phospho-connexin 43 (Chemicon, 1:50) in 5% BSA (w/v) overnight at 4˚C. Cells were rinsed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR, 1:50) in 5%

BSA in PBS for 2 hr at room temperature. After incubation, cells were rinsed with PBS and mounted on Superfrost slides (Fisher, Rockville, MD) with 5µl Prolong Gold antifade solution (Molecular Probes) and observed by fluorescent microscopy using a Zeiss Axioscope equipped with a CCD digital camera.

5.3.6 Cytotoxicity

Cytotoxicity of treatments was assessed using the MTT reduction assay. Results are expressed in absorbance at 610nm as percent of control. The data were obtained from 3 independent experiments.

5.3.7 Statistical Analysis

Data are expressed as means ± SEM. Statistical differences between treatments were determined by analysis of variance (ANOVA) with Tukey's posthoc comparisons or students T-test using Minitab (State College, PA) statistical analysis software ($p<0.05$).

5.4 Results

5.4.1 TPA treatment decreases GJIC in canine LEC

Changes in GJIC upon exposure to varying concentrations of TPA were examined using the SL/DT assay. As shown in Figure 5.1B (left panel), treatment with TPA (100 ng/ml, 0.5hr) decreased dye transfer in canine LEC as compared to control (Fig. 5.1A, left panel). Treatment with 1, 10, and 100 ng/ml TPA for 0.5hr resulted in a statistically significant reduction in GJIC by 30, 80 and 87%, respectively, as compared to control, ($p<0.05$) (Fig. 5.1C).

5.4.2 TPA treatment increases Cx43 phosphorylation in canine LEC

Previous research suggests an association between increased Cx43 phosphorylation and decreased GJIC in a variety of cell lines treated with TPA [190, 300-305]. In agreement

with observations in other cell lines, primary canine LEC treated with TPA (0-1000 ng/ml) exhibited a dose-dependent increase in Cx43 phosphorylation (Fig. 5.2A, top panel). Levels of β-actin remained constant and served as loading control. Increased phosphorylation of Cx43 was evident at 10, 100 and 1000ng/ml TPA, and was statistically different from controls at concentrations of 100ng/ml and 1000ng/ml ($p<0.05$, Fig. 5.2A, bottom panel). The time course of Cx43 phosphorylation after TPA treatment (100ng/ml) is established in Figure 5.2B. Phosphorylation of Cx43 was observed within 30 min, and up to 4 hr post TPA treatment (Fig. 5.2B, top panel). β-actin levels were similar for all treatments. TPA-induced Cx43 phosphorylation was significantly different from control at 0.5 and 1 hr timepoints ($p<0.05$, Fig. 5.2B, bottom panel). TPA-induced phosphorylation of Cx43 has been linked to ubiquitination and subsequent proteosomal and/or lysosomal degradation of the protein [302]. We investigated the cellular localization of phospho-Cx43 in response to TPA treatment by immunofluorescence microscopy. TPA treatment (100ng/ml, 0.5 hr) increased cytoplasmic staining for Cx43 as compared to untreated controls (data not shown).

5.4.3 TPA treatment reduces cell viability in canine LEC

To evaluate cytotoxicity and the effects of reduced GJIC on subsequent cell proliferation, we examined the cell viability of TPA-treated canine LEC at early (0.5 hr) and later (24 hr) timepoints via the MTT assay [306]. TPA treatment (0- 1000ng/ml) did not significantly affect canine LEC cell viability after 0.5 hr, however treatment with TPA (100ng/ml, 0.5hr) resulted in a 23% decrease in viability after 24 hr (data not shown). This result was statistically significant from control ($p<0.05$).

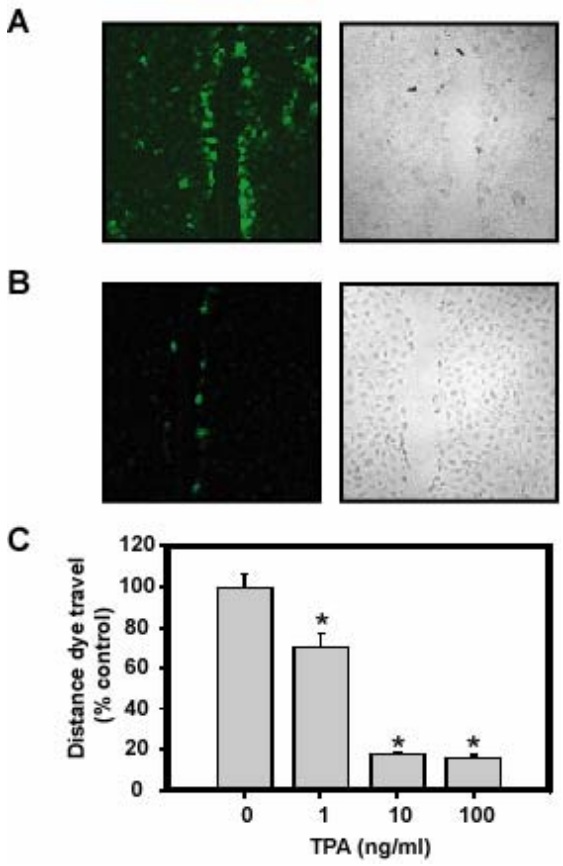

Figure 5.1: TPA reduces GJIC in primary canine LEC
A. Untreated canine LEC were examined for degree of dye transfer as monitored by confocal microscopy. Representative pictures of n=3 experiments are shown. Left panel: confocal image (10X); right panel: bright field image (10X) showing scrape line. Gap junction communication was assessed via SL/DT as described in Materials and Methods. **B**. Canine LEC were treated with TPA (100ng/ml) in serum-free DMEM for 0.5hr and degree of dye transfer monitored by confocal microscopy. Representative pictures of n=3 experiments are shown. Left panel: confocal image (10X); Right panel: bright field image (10X) showing orientation of scrape line. Gap junction communication was assessed via SL/DT as described in Materials and Methods. **C**. Quantification of GJIC represented as distance of dye travel (as percent of untreated controls (given as 100%)) using Scion Image Software. Data are presented as means ± SEM with significant differences from control denoted (*, ANOVA, $P<0.05$).

5.4.4 PKC inhibition partially restores TPA-inhibited GJIC in canine LEC

Protein kinase C (PKC) is one of the signaling pathways involved in regulation of Cx43 phosphorylation [293, 307-309]. We examined the role of PKC in mediating the effects of TPA on GJIC and Cx43 phosphorylation, using both a general (GF109203X) [310] and selective (Gö6976) PKC inhibitor [311]. Changes in GJIC, as determined by dye transfer, were observed in canine LEC following treatment with TPA, alone or in combination with the PKC inhibitor, GF 109203X (Figure 5.3A; left panel). TPA treatment of canine LEC (100 ng/ml, 0.5hr), reduced dye transfer by 75%, compared to untreated controls ($p<0.05$). Pretreatment of canine LEC with GF109203X (1μM, 1hr), prior to TPA, resulted in a 40% decrease in dye transfer. Treatment of cells with GF 109203X alone did not significantly inhibit dye transfer, compared to controls. Changes in the phosphorylation state of Cx43 (p-Cx43) upon treatment of canine LEC with TPA, alone or in combination with the PKC inhibitor, GF 109203X, are given in Figure 5.3A (right panel). Treatment of canine LEC with TPA (100ng/ml, 0.5hr) increased Cx43 phosphorylation. Pretreatment of cells with GF109203X (1μM, 1hr) prevents TPA-induced Cx43 phosphorylation. β-actin levels remained unchanged, regardless of cellular treatment. To evaluate the role of calcium-dependent PKC isoforms (α, βl, βll, γ) in the regulation of GJIC, changes in dye transfer were determined in canine LEC following treatment with TPA, alone or in combination with the isoform-specific PKC inhibitor, Gö6976 (Figure 5.3B; left panel). TPA treatment of canine LEC (100 ng/ml, 0.5hr), reduced dye transfer by 70%, compared to untreated controls ($p<0.05$). Pretreatment of canine LEC with Gö6976 (500nM, 1hr), prior to TPA, resulted in a 60%

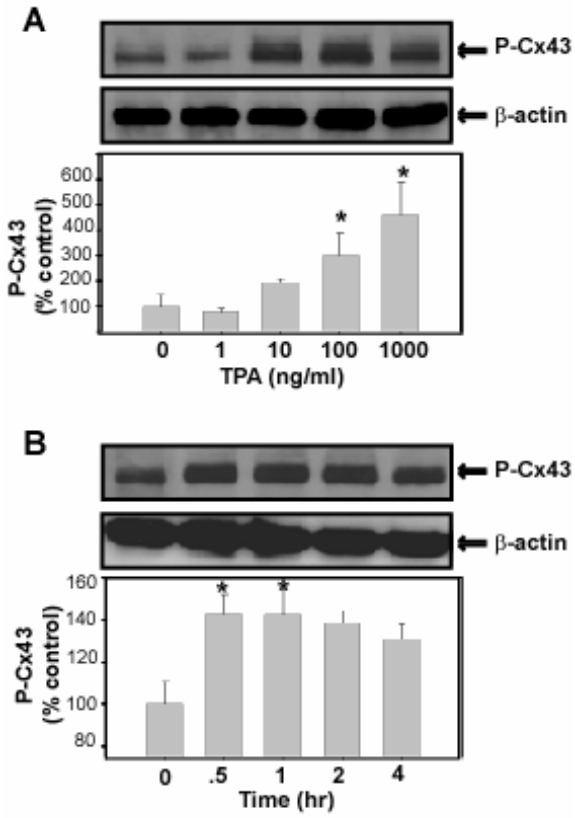

Figure 5.2: TPA phosphorylates connexin 43 (Cx43) in canine LEC
A. Canine LEC were treated with TPA (1, 10, 100 and 1000 ng/ml) in serum-free DMEM for 0.5hr. Cx43 phosphorylation was assessed via immunoblotting using a phospho-Cx43 (Ser 368) antibody as described in Materials and Methods. A representative immunoblot is shown with quantification (n=3). Asterisk indicates significant differences from control ($p<0.05$). β actin was included as a loading control. Bottom panel: Densitometry of (n=3) immunoblots. **B**. Canine LEC were treated with TPA (100ng/ml) for 0.5, 1.0, 2.0 and 4.0 hr in serum-free DMEM. Cx43 phosphorylation was assessed via immunoblotting using a phospho-Cx43 (Ser 368) antibody as described in Materials and Methods. A representative immunoblot is shown with quantification (n=3). Asterisk indicates significant differences from control ($p<0.05$). β actin was included as loading control.

decrease in dye transfer. Treatment of cells with Gö6976 alone did not significantly inhibit dye transfer, compared to controls. Changes in the phosphorylation state of Cx43 (p-Cx43) upon treatment of canine LEC with TPA, alone or in combination with the PKC inhibitor, Gö6976, are given in Figure 5.3B (right panel). Treatment of canine LEC with TPA (100ng/ml, 0.5hr) increased Cx43 phosphorylation. Pretreatment of cells with Gö6976 (500nM, 1hr) prevents TPA-induced Cx43 phosphorylation. β-actin levels remained unchanged, regardless of cellular treatment.

5.4.5 PKC isoform expression in canine LEC

Protein expression of calcium-dependent PKC isoforms (α, β_I, β_{II}, and γ) was examined in primary canine LEC cultures and canine whole lens. In canine LEC, PKC α and β_I isoforms are expressed (Fig. 5.4A, top panel). β-actin levels were similar for all treatment groups (Fig. 5.4A, bottom panel). In canine whole lens, PKC α and β_I isoforms are expressed, and, in contrast to LEC cultures, PKC γ was also detected (Fig. 5.4B, top panel). β-actin levels were similar for all treatment groups (Fig. 5.4B, bottom panel). PKC β_{II} was not detected in either canine LEC or whole lens.

5.4.6 Sorbitol and H_2O_2 increase Cx43 phosphorylation in canine LEC

Sorbitol and hydrogen peroxide are both physiological stressors to the lens. The effect of these stressors on Cx43 phosphorylation in canine LEC is established in Figure 5.5. Primary canine LEC treated with TPA (100 ng/ml) exhibited an increase in Cx43 phosphorylation (Fig. 5.5A) as compared to control. Increased phosphorylation of Cx43 was evident at both doses of sorbital tested (50mM and 25mM), although dimished phosphorylation was evident at the lower dose. The effect of H_2O_2 treatment (0-

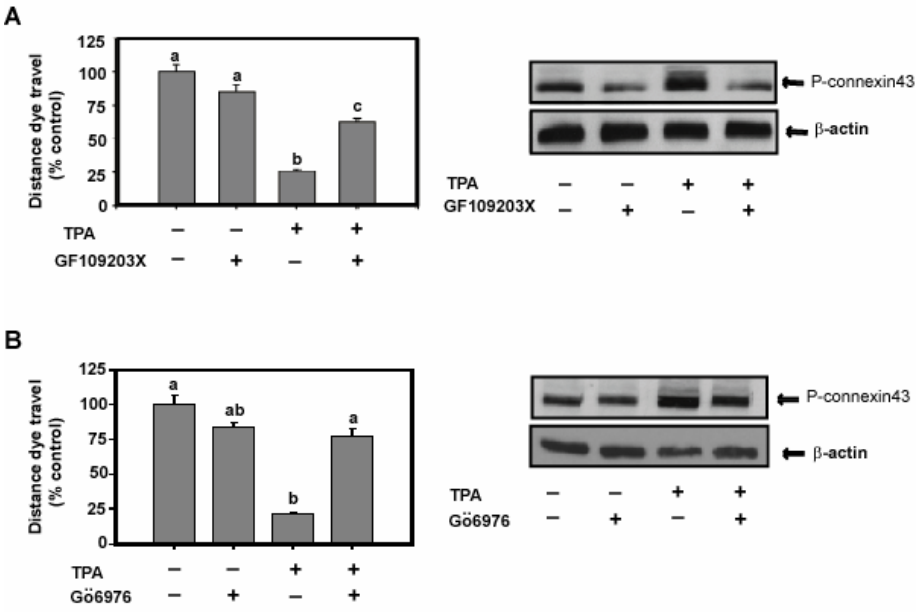

Figure 5.3: PKC inhibition blocks TPA-induced changes in GJIC and Cx43 phosphorylation in canine LEC

A. Left panel: Canine LEC were untreated, treated with GF109203X (1μM, 1 hr) or treated with TPA (100ng/ml, 0.5hr) alone or in combination with GF109203X. Gap junction communication was assessed by SL/DT and quantified as described in Materials and Methods. Data presented as distance of dye transfer (as percent control). Means without a common letter differ (ANOVA, $P<0.05$). Right panel: Phosphorylated Cx43 (p-Cx43) was assessed by immunoblotting using a phospho-Cx43 (Ser 368) antibody as described in Materials and Methods. Representative immunoblot is shown (n=3). β-actin was included as loading control. **B**: Left panel: Canine LEC were untreated, treated with Gö6976 (500nM, 1 hr), or treated with TPA (100ng/ml, 0.5hr) alone or in combination with Gö6976. Gap junction communication was assessed by SL/DT and quantified as described in Materials and Methods. Data presented as distance of dye transfer (as percent control). Means without a common letter differ (ANOVA, $P<0.05$). Right panel: Phosphorylated Cx43 (p-Cx43) was assessed via immunoblotting using a phospho-Cx43 (Ser368) antibody as described in Materials and Methods. Representative immunoblot is shown (n=3). β-actin was included as loading control.

500μM) on Cx43 phosphorylation is established in Figure 5.5B. Increased phosphorylation of Cx43 was observed at 500μM H_2O_2. TPA (100ng/ml) treatment was included as a positive control. Total levels of Cx43 did not change (bottom panel).

5.5 Discussion

In this study, we demonstrate that TPA treatment reduced GJIC and increased Cx43 phosphorylation in primary cultures of canine LEC and these effects were dependent upon activation of calcium-dependent PKC isoforms. To our knowledge, this is the first study to examine the role of PKC in modulating the effects of TPA in primary cultures of canine LEC. Intercellular communication mediated by connexins influences cellular proliferation and differentiation in a variety of diseases, including cataractogenesis [178]. Primary LEC cultures afford several advantages in the study of cataractogenesis including ease and accessibility of tissue acquisition [269] and the maintenance of epithelial characteristics in culture (unpublished data). In addition, the use of primary LEC cultures for investigating GJIC is advantageous over immortalized cells, due to reduced GJIC [312] and altered sensitivity to TPA in the latter [313]. TPA treatment has been shown to both increase Cx43 phosphorylation and inhibit GJIC in a variety of cell lines [190, 300-304, 314]. In agreement with these reports, the present study demonstrates that TPA treatment increased Cx43 phosphorylation and reduced GJIC in a concentration and time dependent manner in primary canine LEC. TPA treatment increased cytoplasmic phospho-Cx43 staining in canine LEC, a result consistent with internalization and degradation of Cx43-containing gap junctions (data not shown). We report that treatment with TPA at the doses and times used in this study

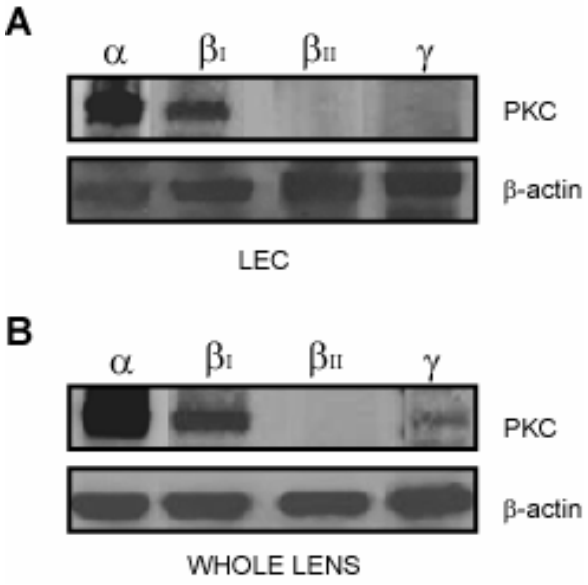

Figure 5.4: Comparative expression of calcium-dependent PKC isoforms in canine LEC and whole lens

A. Top panel: Untreated canine LEC were harvested and collected for immunoblotting (50μg protein) using anti-PKC isoform specific antibodies (PKC α, PKC β_I, PKC β_{II}, and PKCγ) as described in Materials and Methods. Representative immunoblot is shown (n=3). Bottom panel: β actin was included as loading control. **B**. Top panel: Canine whole lenses were homogenized and cell lysates (50μg protein) collected for immunoblotting using anti-PKC isoform specific antibodies (PKC α, PKC β_I, PKC β_{II}, and PKCγ) as described in Materials and Methods. Representative immunoblot is shown (n=3). Bottom panel: β actin was included as loading control.

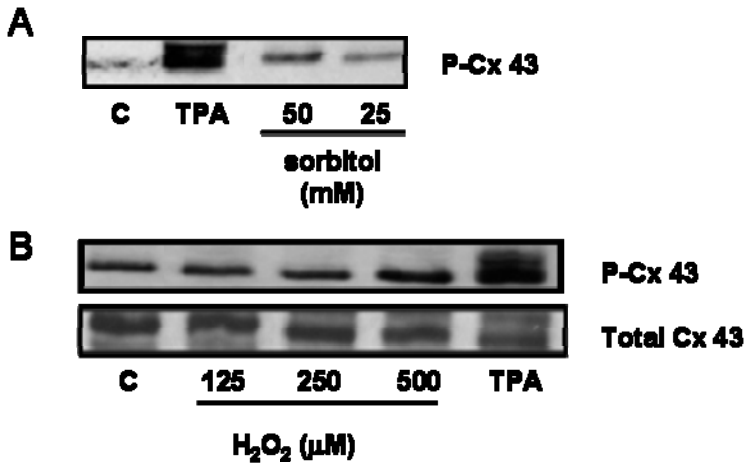

Figure 5.5: Sorbitol and H_2O_2 increase Cx43 phosphorylation in canine LEC
A. Untreated (control, C), TPA treated (100ng/ml) and sorbitol treated (25, 50mM) canine LEC were harvested and collected for immunoblotting using p-Cx43 antibody as described in Materials and Methods. Representative immunoblot is shown (n=3). **B**. Top panel: Canine LEC were treated with H_2O_2 (0-500μM) and cell lysates collected for immunoblotting using p-Cx43 antibody as described in Materials and Methods. Representative immunoblot is shown (n=3). Bottom panel: Total Cx43 was included as loading control.

was not cytotoxic to canine LEC but that treatment with a moderate dose of TPA resulted in lowered cell viability 24 hr after exposure (data not shown). Our data suggest that GJIC in canine LEC is regulated, in part, via activation of calcium-dependent PKC isoforms. We utilized both a general and calcium-dependent isoform-specific PKC inhibitor to elucidate the role of this signaling pathway in modulating GJIC in primary canine LEC.

In the present study, Gö6976, a potent PKCα, β_I and β_{II} inhibitor, significantly reduced TPA-induced inhibition of GJIC. When compared to the general PKC inhibitor, GF109203X, Gö6976 was more effective at reducing TPA-induced inhibition of GJIC, suggesting involvement of calcium-dependent PKC isoforms in regulating GJIC in canine LEC. Both inhibitors prevented TPA-induced phosphorylation of Cx43 in canine LEC. Previous reports indicate that activation of the calcium-dependent isoform, PKCγ, phosphorylates Cx43 and decreases GJIC in cells treated with a variety of stimuli, including TPA and growth factors [297, 298, 315]. Both PKC α and γ are detected in cultured bovine LEC [295]and in embryonic chicken lenses [194]. While both PKC α and γ are involved in LEC differentiation [316], PKC γ is primarily responsible for modulating GJIC in LEC [296-298]. PKC γ has previously been detected in whole canine lenses [317], however no studies have examined PKC γ expression in cultured canine LEC.

We report that the calcium-dependent isoforms PKC α and β_I were expressed in canine LEC. Interestingly, PKC γ expression was detected in canine whole lens, but not LEC cultures. This suggests that PKC γ expression in LEC may be reduced under culture conditions or is expressed predominantly in canine lens fiber cells. The expression of

PKC γ in canine lens fiber cells and its role in modulating GJIC in this cell type is currently under investigation.

5.6 Conclusion

The findings of the present study suggest a role for calcium-dependent PKC activation in regulating TPA-induced Cx43 phosphorylation and decreased GJIC in primary canine LEC. Canine LEC are an appropriate model for investigating cataractogenesis due to the similar prevalence of age-related cataract in dog and man [18]. Clarification of those signaling pathways involved in regulating GJIC in the lens epithelium may elucidate new strategies in the prevention and/or delay of cataractogenesis in both humans and animals.

5.7 Acknowledgments

Funding for this project was provided by state and federal funds appropriated to The Ohio State University, Ohio Agriculture Research and Development Center. We thank Ms. Mandy Agler and the Campus Microscopy and Imaging Facility staff at The Ohio State University for their technical assistance.

CHAPTER 6

RETINOIC ACID INCREASES CX43-MEDIATED GAP JUNCTION COMMUNICATION AND PROMOTES DIFFERENTIATION IN PRIMARY CANINE LENS EPITHELIAL CELLS

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6.1 Abstract

Purpose: Gap junction intercellular communication (GJIC) is important in maintaining lens epithelial cell (LEC) homeostasis. GJIC mediates key cellular functions, including regulation of cell growth and differentiation. Dysregulation of lens growth may be associated with the development of cataract. Retinoic acid, a vitamin A derivative, regulates proliferation and differentiation in epithelial cells. Retinoic acid has previously been shown to increase GJIC by modulating connexin 43(Cx43), a structural protein of epithelial gap junctions. This study examined the effect of retinoic acid on modulating GJIC and Cx43 in primary canine LECs. In addition, the effect of retinoic acid on differentiation of primary canine LEC was examined. **Methods:** Primary canine LEC were treated with retinoic acid (0-10μM) for 24 hr and with 10μM for 0-24 hr. Cx43 protein level was assessed by immunoblotting. Levels of Cx43 mRNA were assessed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). GJIC was

assessed by scrape loading/dye transfer (SL/DT). Cytotoxicity of retinoic acid was determined using the MTT assay. The effect of retinoic acid on β crystallin expression, a marker of LEC differentiation, was assessed via immunoblotting. **Results:** Treatment with retinoic acid (10μM, 24 hr) significantly increased Cx43 protein and mRNA levels and increased GJIC in primary canine LEC. Treatment with retinoic acid was not cytotoxic to LEC. Retinoic acid treatment (10μM) for 3 and 5 days increased expression of β crystallin protein. **Conclusions:** Treatment with retinoic acid significantly increased GJIC, Cx43 protein and mRNA levels in canine LEC. These retinoic-acid induced changes in GJIC parameters were associated with increased LEC differentiation. The role of functional gap junctions in the modulation of cellular differentiation and the potential dietary regulation of these processes is currently under investigation.

6.2 Introduction

Gap junctions are small channels or pores that connect the cytoplasm of adjacent cells and enable the transfer of molecules <1kD in size [180]. These channels are composed of proteins called connexins, a highly conserved protein family identified in a variety of mammalian species [180, 281]. Three connexin proteins have been identified in the vertebrate lens: connexins 43, 46 and 50 [282]. Connexin 43 (Cx43)-containing gap junction channels have been localized to lens epithelial cells (LEC), whereas connexins 46 (Cx46) and 50 (Cx50) are found in fiber cells [282]. Gap junction intercellular communication (GJIC) regulates basic cell processes such as cell growth and differentiation [285, 318]. In addition, the lens, as an avascular tissue, relies on GJIC to regulate nutrient and waste transport [179]. Metabolic cooperation established by GJIC allows mitotically active LEC to communicate with quiescent fiber cells, maintaining

lens transparency [11-13]. Retinoids have diverse effects on a variety of organs and tissues. Retinoic acid (RA) is a potent derivative of vitamin A and regulates key cellular processes including growth and differentiation. Vitamin A deficiency causes severe metaplasia and transformation of epithelial cells. RA has been shown to prevent epithelial tumorigenesis by directing cells to differentiate [319]. Both retinoids and carotenoids have been shown to increase GJIC and control cellular growth in several cell lines [320, 321]. Metabolites of carotenoids with retinoid-like structures exhibit more potent activity than the parent compound [207]. The proposed mechanism of RA-induced GJIC involves regulation of connexin protein expression and/or modification. Increases in both Cx43 protein and mRNA have been reported with RA treatment in a variety of cell lines, an effect correlated to RA-induced differentiation [322, 323].

Lens epithelial cell differentiation is important in maintaining proper growth of the lens. Aberrant or disrupted lens growth can lead to cataract development. Differentiation of LEC can be monitored by the gain of fiber-cell specific proteins or by the loss of epithelial-cell specific proteins. Previous evidence has indicated that differentiation of isolated epithelia in calf lenses is marked by an increase in the alpha A_2 crystallin isoform and an increase in the formation of the β crystallin chains β_{1a}, β_5 and β_p [100]. The formation of mature lens fibers is associated with a decrease in vimentin expression [103].

The present study examines the effects of RA on connexin43 protein and mRNA levels and its correlation with GJIC in primary cultures of canine LEC. In addition the role of functional gap junctions in RA-induced cellular differentiation is explored. We provide evidence that RA treatment results in a significant induction of GJIC in canine

LEC, which corresponds with increases in total Cx43 protein and mRNA levels. Furthermore, our results suggest that RA-induced induction in GJIC corresponds to an increase in differentiation as established by expression of β crystallin protein. GJIC is altered in cataractogenesis [14], therefore elucidating the signaling mechanisms which regulate GJIC in the lens may lead to potential strategies for preventing or delaying cataract development.

6.3 Materials and Methods

6.3.1 Cell Culture

Whole canine globes were collected from medium to large breed dogs euthanized for population control purposes at a local animal shelter, as previously described (ref). For LEC culture, the anterior lens capsule was excised away from the underlying fiber cell mass and two capsules representing one dog were placed in a 1.5ml centrifuge tube containing 0.5ml trypsin EDTA, incubated for 15 min at 37°C, and centrifuged for 3min at 2000rpm. Trypsin was neutralized with the addition of 1.0ml of Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) containing penicillin/streptomycin (0.4%) and centrifuged again at 2000rpm for 3 min. Supernatant (0.5ml) was removed and remaining medium with capsules transferred to laminin/polylysine-coated (100μg/ml) T25 cell culture flasks with 2.0 ml of medium at 37˚C in a humidified atmosphere containing 5% CO_2. Growth of primary canine LEC from the capsules was observed approximately 5-7 days following dissection, and medium was changed every 2-3 days after this point. Cells of passage number 1-3 were used for all subsequent experiments.

6.3.2 Materials

Retinoic acid, MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), lutein and Lucifer Yellow were obtained from Sigma Aldrich (St Louis, Mo). Retinoic acid was dissolved in dimethyl sulfoxide (DMSO) under subdued light and nitrogen gas at a stock concentration of 50mM and stored in aliquots at -80°C. Stock solutions were diluted to the indicated concentrations in serum-free medium for all experiments. Lutein was dissolved in ethanol and prepared in 0.1% Tween-40 for cellular treatments. The final concentration of DMSO and ethanol as solvents was less than 0.1%. Lucifer yellow was dissolved in phosphate buffered saline (PBS) at a concentration of 0.05% w/v. Monoclonal, canine-specific connexin 43 and polyclonal phospho-connexin 43 (Ser368) antibodies were obtained from Chemicon International (Temecula, CA). Antibody to β crystallin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

6.3.3 Gap Junction Communication Assay

GJIC was assessed by scrape loading/dye transfer (SL/DT) assay, as previously described [299], with some modifications. Cells were cultured in 12-well laminin-coated (100ug/ml) plates at a density of 2.0 x 10^5 cells/ml. Following appropriate treatments, media was removed and replaced by 1.0ml PBS containing 0.05% Lucifer yellow (Sigma Aldrich, St. Louis, MO) and each well was scraped through the confluent monolayer, using a steel surgical blade. After 5 min, cells were washed three times with PBS and fixed in 3.7% paraformaldehyde, pH 7.0. Distance of dye transfer was determined using a Zeiss 510 META Laser Scanning confocal microscope. Using Scion Image Software (available for download at www.scioncorp.com), three sites per scrape were randomly chosen as points for the measurement of distance of dye transfer to neighboring cells,

using a line perpendicular to the scrape line. Effect of test compound on gap junction communication is presented as distance of dye travel as a percent of control.

6.3.4 Immunoblotting

Cells were washed in PBS (pH 7.4) and scraped into cell extraction buffer (50mM TrisCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1%Triton X-100, 0.1% SDS, 50mM sodium fluoride, 10mM sodium pyrophosphate, protease cocktail). Extracts were centrifuged for 13,000 x g for 5 min. Protein concentration of the supernatant was measured by bicinchoninic acid (BCA) protein analysis (Pierce Chemical Company, Rockford IL). After appropriate treatments, cellular protein samples were diluted into modified Laemmli sample loading buffer (100mM TrisCl (pH 6.8), 5% BME, 2% SDS, 25% glycerol, 0.1% bromophenol blue) and heated at 95˚C for 5 min. Equal amounts of protein (20 μg) were loaded onto a 10% or 12.5% (β crystallin) sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) and electrophoresed at 180 V for approximately 1 hr using the Mini-PROTEAN III system (Bio-Rad, Hercules, CA). After electrophoresis, protein was transferred to a nitrocellulose membrane at 100 V for 1 hr. The membrane was blocked for 1 hr at room temperature in a Tris-buffered saline (TBS, pH 7.4) solution containing 5% nonfat dry milk. After blocking, membranes were incubated at 4˚C overnight with appropriate primary antibodies (1:1000) in TBS containing 0.1% Tween-20 (TTBS) and 5% bovine serum albumin (BSA). Membranes were washed extensively with TTBS and incubated with secondary antibodies (anti-rabbit or anti-mouse 1:2000, Cell Signaling Technology), conjugated with horseradish peroxidase in TTBS for 1 hr. Membranes were washed again and protein signals were developed using ECL chemiluminescence detection reagents (Amersham Biosciences, Piscataway, NJ).

Membranes were exposed to Kodak X-OMAT AR film (Rochester, NY) for an appropriate length of time and developed according to manufacturer's recommendations.

6.3.5 Cytotoxicity

Cytotoxicity of treatments was assessed using the MTT reduction assay, according to the manufacturer's instructions. Results are expressed in absorbance at 610nm as percent of control. The data were obtained from 3 independent experiments.

6.3.6 Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using Absolutely RNA Microprep Kit (Strategene) according to the manufacturer's suggestions. The ImProm II Reverse Transcriptase kit (Promega; Madison WI) was used to synthesize the first strand of cDNA as per instructions. Quantitative RT-PCR was performed in the Mx3000p Multiplex Quantitation System as follows: 95°C for 15 minutes, then 40 cycles of 94°C fro 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, using the QuantiTect SYBR Green PCR kit (Strategene). Primer design was as follows: Forward: CGTTCTGGGTCCTGCAGAT and Reverse: TCTCTTCCTTGCGCATCACG. The relative amount of connexin 43 mRNA, normalized to GAPDH, was calculated according to the method described by Ramakers and colleagues [324]. Results were expressed as the ratio of the target gene to GAPDH housekeeping gene expression.

6.3.7 Statistical Analysis

Data are expressed as means ± SEM. Statistical differences between treatments were determined by analysis of variance (ANOVA) with Tukey's posthoc comparisons or students T-test using Minitab (State College, PA) statistical analysis software ($p<0.05$).

6. 4 Results

6.4.1 Retinoic acid treatment increases Cx43 protein in canine LEC

Previous research suggests both a stimulatory and inhibitory effect of retinoic acid on Cx43 protein and GJIC, depending on the concentration and cell type [199, 200, 325-329]. In the present study, primary canine LEC treated with retinoic acid (0-10 μM, 24 hr) exhibited a dose-dependent increase in Cx43 protein (Fig. 6.1A, top panel). Levels of β-actin remained constant and served as loading control. Increased Cx43 protein was noted at all concentrations and was statistically different from controls at a concentration of 10μM ($p<0.05$, Fig. 6.1A, bottom panel). The time course of Cx43 protein level after retinoic acid pre-treatment (10 μM) is established in Figure 6.1B. Increase in Cx43 protein was observed as early as 3 hr and was statistically significant by 12 hr post retinoic acid treatment ($p<0.05$, Fig. 6.1B, bottom panel). β-actin levels were similar for all treatments.

To evaluate RA-induced cytotoxicity, we examined the cell viability of retinoic acid-treated (0-100μM) canine LEC after 24 hr via the MTT assay. Retinoic acid treatment (0-10 μM) did not significantly affect canine LEC cell viability after 24 hr, however treatment with higher concentrations, 50 and 100μM, resulted in a 64 and 60% decrease in viability after 24 hr, respectively. This result was statistically significant from control ($p<0.05$, Fig. 6.1C, bottom panel).

6.4.2 Retinoic acid treatment increases Cx43 mRNA levels in canine LEC

The effect of RA on Cx43 mRNA levels is established in Fig. 6.2. Primary canine LEC treated with retinoic acid (0-10μM) exhibited a dose-dependent increase in Cx43 mRNA

levels after 18 hr. Increases in Cx43 were statistically different from control at both 1 and 10μM ($p<0.05$).

6.4.3 Retinoic acid increases GJIC in canine LEC

Changes in GJIC upon exposure to varying concentrations of retinoic acid were examined using the SL/DT assay. As shown in Figure 6.3A (right panel), treatment with retinoic acid (10 μM, 24hr) increased dye transfer in canine LEC as compared to control (Fig. 6.3A, left panel). Treatment with 0.1, 1.0 and 10.0 μM retinoic acid for 24 hr resulted in a statistically significant increase in GJIC by 75, 110 and 80%, respectively, as compared to control, ($p<0.05$) (Fig. 6.2B).

6.4.5 Retinoic acid increases β crystallin protein expression in canine LEC

The crystallin proteins are important proteins in the maintenance of lens transparency and β crystallin proteins have been shown to increase with differentiation of LEC to fiber cells. Protein expression of β crystallin was examined in primary canine LEC treated with retinoic acid for 1, 3, 5 and 7 days. Expression of β crystallin protein was increased over control levels at 3, 5 and 7 days post retinoic acid treatment. β actin served as loading control (Fig.6.4).

6.4.6 Lutein modestly increases Cx43 protein expression in canine LEC

The effect of lutein on Cx43 protein is established in Figure 6.5. As previously reported, primary canine LEC treated with retinoic acid (10 μM, 24 hr) exhibited an increase in Cx43 protein as compared to control (Fig. 6.5). Increased Cx43 protein was noted at all concentrations of lutein tested (2-8μM), although no dose effect was indicated. The time

course of Cx43 protein level after lutein treatment (4μM) demonstrates an increase in Cx43 protein at 12 and 24 hr, with diminished levels at 48 and 72 hr.

6.5 Discussion

In this study, we demonstrate that RA treatment increased GJIC and Cx43 total protein and mRNA levels in primary cultures of canine LEC and these effects were associated with an increase in expression of the differentiation marker, β crystallin. To our knowledge, this is the first study to examine the role of RA in modulating GJIC in primary cultures of canine LEC. Intercellular communication mediated by connexins influences cellular proliferation and differentiation in a variety of diseases, including cataractogenesis [178]. Primary LEC cultures afford several advantages in the study of cataractogenesis including ease and accessibility of tissue acquisition [269] and the maintenance of epithelial characteristics in culture (unpublished data). In addition, the use of primary LEC cultures for investigating GJIC is advantageous over immortalized cells, due to reduced GJIC in the latter [312]. Treatment with RA has been shown to both increase Cx43 protein and mRNA as well as modulate GJIC in a variety of cell lines [190, 300-304, 314]. In agreement with these reports, the present study demonstrates that RA treatment increased Cx43-mediated GJIC in a concentration and time dependent manner in primary canine LEC. We report that treatment with RA at the doses and times used in this study was not cytotoxic to canine LEC but that treatment with a higher (50μM) dose of RA resulted in lowered cell viability 24 hr after exposure. Our data suggest that RA-induced changes in GJIC in canine LEC is regulated, in part, via modulation of protein and mRNA levels of Cx43. Furthermore, RA-induced changes in GJIC were associated with an increase in expression of the lens differentiation marker, β

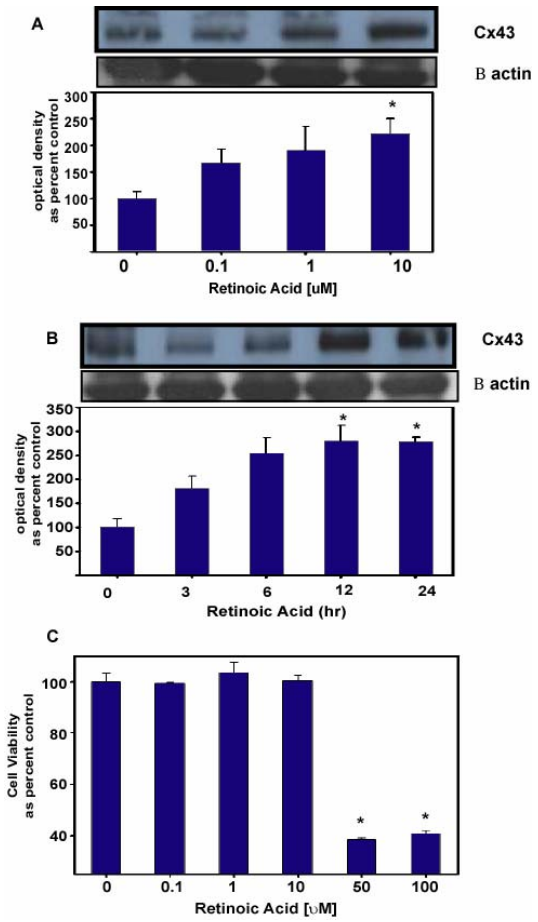

Figure 6.1: Retinoic acid increases Cx43 protein in canine LEC: dose and time dependence

A. Primary canine LECs were treated with retinoic acid (0-10μM) for 24 hr. Expression of total Cx43 protein was assessed via immunoblotting using a canine-specific antibody. A representative immunoblot is shown with quantification (n=3). Asterisk indicates significant difference from control (*ANOVA, $p<0.05$).

B. Canine LECs were treated with retinoic acid (10μM) for 0-24 hr. Expression of total Cx43 protein was assessed via immunoblotting. A representative immunoblot is shown with quantification (n=3). Asterisk indicates significant difference from control (*ANOVA, $p<0.05$).

C. Canine LEC were seeded at a density of $1.0x\ 10^4$ cells in a 24 well plate and treated with retinoic acid 0-100μM for 24 hr. Cell viability was assessed via the MTT assay. Values are expressed as cell viability as percent of untreated controls. Asterisk indicates significant difference from control (*ANOVA, $p<0.05$).

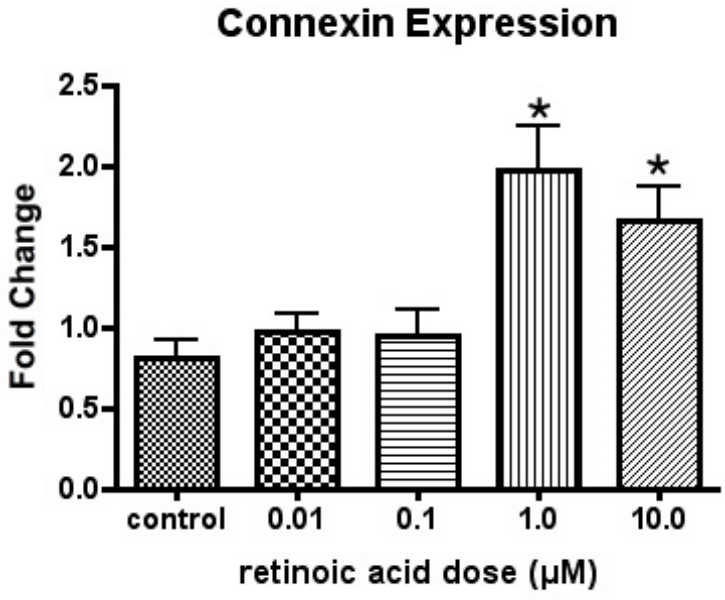

Figure 6.2: Retinoic acid increases Cx43 mRNA
Primary canine LECs were treated with retinoic acid (0-10μM) for 18hr and expression of Cx43 assessed via qRT-PCR. Expression of Cx43 is significantly increased from control at concentrations of 1.0 and 10.0μM. Data expressed as fold change in expression. Error bars indicate SEM and significant differences from control denoted (*ANOVA, $P<0.05$).

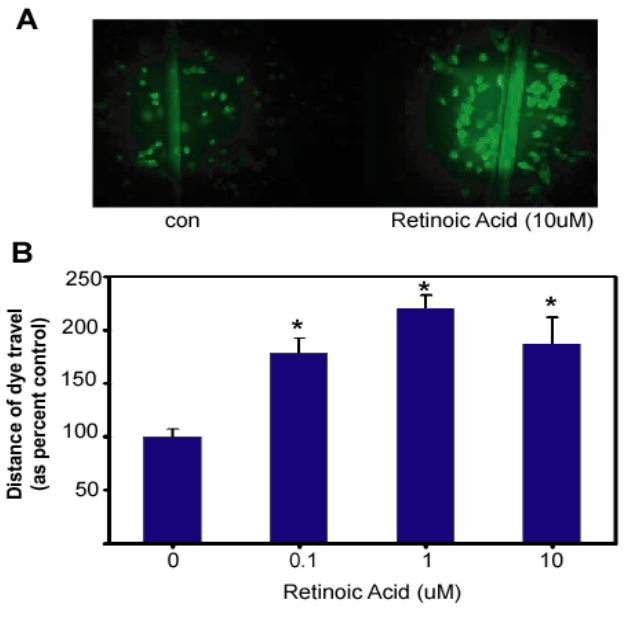

Figure 6.3: Retinoic acid increases GJIC in canine LEC
A. Primary canine LEC were untreated or treated with retinoic acid (0-10μM) for 24 hr and cell communication assessed by the scrape loading dye transfer assay. Degree of dye transfer was monitored by fluorescence microscopy. Representative pictures (40X) of n=3 experiments are shown.
B. Quantification of GJIC represented by the distance of dye transfer [as percent of untreated controls (given as 100%)] using Scion Image Software. Data represented as mean ± SEM with significant differences from control denoted (*ANOVA, $P<0.05$).

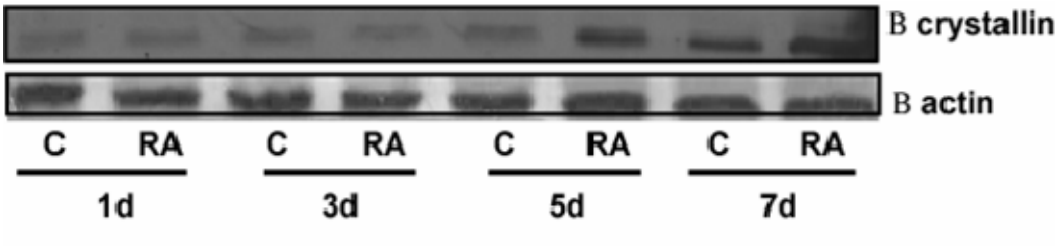

Figure 6.4: Retinoic acid increases β crystallin expression in canine LEC
Primary canine LECs were treated with retinoic acid (10μM) for 1, 3, 5 and 7 days, with replacement of fresh medium and treatment every two days. Expression of β crystallin protein was assessed via immunoblotting. A representative immunoblot is shown (n=3). β actin served as loading control.

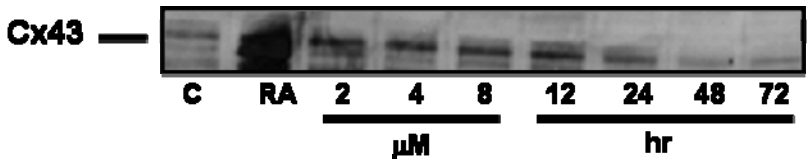

Figure 6.5: Effects of lutein on Cx43 protein expression in canine LEC
Primary canine LECs were treated with lutein (2-8μM) and 4μM (12-72 hr), with replacement of fresh medium and treatment every two days. Retinoic acid (RA, 10μM, 24hr) served as a positive control. Expression of Cx43 protein was assessed via immunoblotting. A representative immunoblot is shown.

crystallin. Preliminary evidence suggests that carotenoids such as lutein may also have modest effects on Cx43 protein expression in canine LEC. The effect of functional gap junctions in the regulation of LEC differentiation, as well as the effect of other dietary compounds on GJIC, is currently under investigation.

CHAPTER 7

EPILOGUE

The overall goal of this dissertation was to examine the influence of environmental and chemical factors on cellular signaling in lens epithelial cells (LEC). The first study was conducted to examine the mechanisms by which immortalized human LEC respond to environmental and chemical stress. The second study was conducted to characterize the isolation and optimal growth conditions of primary canine LEC. The third study was conducted to characterize gap junction intercellular communication (GJIC) in primary canine LEC and examine the role of the protein kinase C (PKC) cell signaling pathway in the modulation of GJIC. In the final study, the role of retinoids and carotenoids in the modulation of GJIC and cellular differentiation in canine LEC was examined.

There are several overall conclusions of the studies outlined here. First, lens epithelial cells utilize cell signaling pathways to coordinate responses to a variety of cell stressors, including environmental and chemical stress. Activation of selective signaling proteins belonging to the MAPK pathway leads to distinct cellular responses which dictate cell proliferation and cell death. The knowledge of the specificity of these pathways may lead to more targeted inhibition and prevention of the changes that lead to cataractogenesis. Second, primary canine LEC are a useful model for studying lens

physiology and retain the characteristics of LEC *in vitro* at early passages, with optimization of several basic cell culture conditions. Third, gap junction intercellular communication (GJIC) is an important mode of water and nutrient transport in lens epithelial cells. GJIC in primary canine LEC can be blocked by TPA, an activator of the PKC signaling pathway. Blockage of GJIC is associated with increased phosphorylation of connexin 43 (Cx43), a structural protein of epithelial gap junctions. PKC activation modulates GJIC in canine LEC, as inhibition of this pathway restores GJIC in TPA-treated cells. Finally, a variety of dietary compounds, including retinoids and carotenoids, have been shown to enhance GJIC in several cell lines, but not in LEC. Retinoic acid, a potent vitamin A metabolite, increased GJIC in primary canine LEC and this effect is associated with an increase in the amount of Cx43 protein and mRNA. In addition, retinoic acid promotes the differentiation of LEC, allowing for proper growth of the lens.

While these studies move us forward in understanding the complexity and specificity of cellular signaling in response to various stressors in the lens, several questions remain. We provided preliminary evidence that the c-Jun N-terminal kinase (JNK) pathway may be a pro-survival pathway in lens epithelial cells. The elucidation of the exact role of signaling pathways in modulating cell death needs exploration. Is stress-induced activation of JNK designed to eliminate damaged cells or both damaged and healthy cells? In addition, what role do gap junctions play in regulating the cellular signals that dictate cell death? We have shown that the PKC pathway regulates GJIC in primary canine LEC, but inhibition of this pathway does not completely restore GJIC. What other signaling pathways might be involved in the regulation of GJIC in lens

epithelial cells? Retinoic acid and other retinoid-like compounds have been shown to increase GJIC and Cx43 protein levels in various cell lines. We report that all-trans retinoic acid has similar effects in primary canine LEC. What is the effect of increased GJIC on cataract development? Can decreased GJIC caused by cell stress be prevented by supplementation with dietary retinoids or carotenoids? Dietary carotenoids have several metabolites with activities that are more potent than the parent compound. Are these metabolites formed within the lens and do they also regulate GJIC?

Answers to some of these questions will further define our knowledge of the various mechanisms by which lens epithelial cells respond to environmental stresses and may generate potential preventative strategies for cataract treatment.

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