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25-Hydroxycholesterol increases IL-8 production in retinal pigment epithelial cells by activation of PI3K and ERK pathways

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25-HYDROXYCHOLESTEROL INCREASES IL-8 PRODUCTION IN RETINAL PIGMENT EPITHELIAL CELLS BY ACTIVATION OF PI3K AND ERK PATHWAYS

Dissertação apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Engenharia Biomédica, realizada sob a orientação científica do Doutor Paulo Pereira (Universidade de Coimbra) e co-orientação do Doutor Alexandre Fernandes (Universidade de Coimbra).

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ABBREVIATIONS

$^1O_2$	Singlet oxygen
25-OH	25-hydroxycholesterol
7-KC	7-ketocholesterol
7β-OH	7-beta-hydroxycholesterol
A2E	Pyridinium bis-retinoid
AMD	Age-related macular degeneration
ANOVA	Analysis of variance
AP-1	Activator protein-1
ATCC	American Type Culture Collection
ATP	Adenosine-5'-triphosphate
BRB	Blood-retinal barrier
BSA	Bovine serum albumin
C3	Complement component 3
C5	Complement component 5
C9	Complement component 9
CCR-2	Chemokine (C-C motif) receptor-2
CFB	Complement factor B
CFH	Complement factor H
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CNV	Choroidal neovascularization
CRP	C-reactive protein
Ct	Cycle threshold
kDa	Kilodalton
DMEM-F12	Dulbecco's Modified Eagle's Medium/ Nutrient Mixture F-12 Ham
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
DTT	Dithiothreitol
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme

E3	Ubiquitin protein ligase
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular-regulated kinase
FBS	Fetal bovine serum
FR180204	5-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)-1*H*-pyrazolo[3,4-c]pyridazin-3-amine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSH	Reduced glutathione
H_2O_2	Hydrogen peroxide
HDL	High-density lipoprotein
HIF	Hypoxia-Inducible Factor
HNE	4-hydroxynonenal
HRP	Horseradish peroxidase
I-κB	Inhibitor of κB
IgG	Immunoglobulin G
IL-8	Interleukin-8
Itk	Interleukin-2-inducible T-cell kinase
JNK	c-Jun N-terminal kinase
LDL	Low-density lipoprotein
LLE-AMC	Benzyloxycarbonyl-Leu-Leu-Glu-amidomethylcoumarin
LLVY-AMC	Succinyl-Leu-Leu-Val-Tyr-amidomethylcoumarin
LSTR-AMC	*N-t*-butyloxycarbonyl-Leu-Ser-Thr-Arg-amidomethylcoumarin
LXR	Liver X receptor
LY294002	2-(4-Morpholino)-8-phenyl-4H-1-benzopyran-4-one
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MG-132	Carbobenzoxy-L-Leucil-Leucil-L-Leucinal
MKK	Mitogen-activated protein kinase kinase
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor κB

dNTP	Deoxyribonucleotide triphosphate
O^{-2}	Superoxide
$OH^{\cdot}$	Hydroxyl radical
OSBP	Oxysterol-binding protein
PBS	Phosphate-buffered saline
PEDF	Pigment epithelium-derived growth factor
PI3K	Phosphatidylinositol-3-kinase
POS	Photoreceptor outer segments
Pro	Proline
PUFA	Polyunsaturated fatty acids
RNA	Ribonucleic acid
mRNA	Messenger RNA
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RT	Reverse transcriptase
RT-PCR	RT-polymerase chain reaction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TMB	3,3',5,5'-tetramethylbenzidine
U0126	1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene
UPP	Ubiquitin-proteasome pathway
VEGF	Vascular endothelial growth factor
VHDL	Very high-density lipoprotein
VHL	von Hippel-Lindau

RESUMO

A degenerescência macular relacionada com a idade (DMRI) é a principal causa de cegueira na população com idade superior a 60 anos no mundo ocidental. Apesar dos mecanismos moleculares associados à DMRI não serem ainda bem conhecidos, o stress oxidativo parece desempenhar um papel fundamental no desenvolvimento e progressão da doença. Alguns estudos recentes sugerem ainda que esta patologia apresenta uma importante componente inflamatória. Recentemente, o nosso laboratório demonstrou que a inactivação oxidativa do proteassoma pode contribuir para alterações celulares e fenotípicas associadas à DMRI. Esta disfunção de componentes críticos da VUP (via da ubiquitina-proteassoma) poderá constituir um novo mecanismo que permite associar a lesão oxidativa da retina à expressão de características fenotípicas próprias da DMRI, nomeadamente o aumento da produção de mediadores inflamatórios, acumulação de drusen (devido à diminuição no recrutamento de macrófagos) e neovascularização da retina.

Estudos anteriores, incluindo estudos do nosso laboratório, demonstraram que a acumulação de produtos da oxidação do colesterol, ou oxiesteróis, em tecidos oculares, como o cristalino e a retina, está associada ao processo de envelhecimento. Mais, os oxiesteróis levam a um aumento na produção de citocinas pró-inflamatórias em vários tipos celulares, incluindo o epitélio pigmentado da retina (EPR). Este aumento poderá contribuir para a componente inflamatória de diversas doenças relacionadas com a idade, como a DMRI.

No entanto, os mecanismos moleculares que regulam a expressão de citocinas inflamatórias nestas condições não são ainda claros. Assim, o principal objectivo deste estudo foi esclarecer os mecanismos moleculares através dos quais os oxiesteróis aumentam a produção de IL-8 no EPR. Para isso, uma linha celular humana do EPR (ARPE-19) foi exposta a diferentes oxiesteróis.

A exposição das células ARPE-19 ao 25-hidroxicolesterol (25-OH) resultou no aumento da secreção de IL-8, ao passo que os outros oxiesteróis testados diminuíram os níveis de IL-8. Consistente com um efeito a nível da expressão genética da IL-8, o tratamento das células ARPE-19 com 25-OH aumentou os níveis de RNA para esta citocina. Deve ainda acrescentar-se que o 25-OH aumentou a produção de IL-8 no EPR por um mecanismo independente do stress oxidativo ou da inactivação do proteassoma.

O tratamento das células ARPE-19 com 25-OH resultou ainda na activação da ERK, mediada pela PI3K. Consistente com este mecanismo, a inibição destas vias de sinalização diminuiu significativamente a produção de IL-8 em resposta ao 25-OH.

No seu conjunto, os resultados aqui apresentados permitem propor um novo mecanismo molecular através do qual o 25-OH aumenta a produção de IL-8 em células do EPR.

Assim, este estudo pode constituir uma contribuição importante para esclarecer os mecanismos moleculares que conduzem à inflamação no RPE, que é uma componente importante para doenças degenerativas da retina associadas ao envelhecimento, incluindo a DMRI.

Palavras-Chave: Degenerescência macular relacionada com a idade, inflamação, interleucina-8, PI3K, ERK, 25-hidroxicolesterol.

ABSTRACT

Age-related macular degeneration (AMD) is the main cause of blindness in the western world in the population over 60 years of age. While the cause of AMD is currently not known, it has been suggested that multiple factors are involved including environmental, nutritional and genetic factors. Most of these risk factors appear to have oxidative stress as a common denominator. Recent findings further suggest a role for inflammation in the pathogenesis of AMD. We have recently demonstrated that the oxidative impairment of the proteasome is a mechanistic link between oxidative stress and inflammation. These observations provide a new association between retinal oxidative insult and AMD-like phenotypes, such as the increased production of inflammatory cytokines, like interleukin-8 (IL-8).

Several studies, including some from our laboratory, indicate that oxysterols, which result from cholesterol oxidation, accumulate in ocular tissues, such as the lens and retina, upon aging. Age-related oxysterols accumulation may account, at least in part, for the inflammatory component of several age-related diseases, as they increase the production of inflammatory cytokines, such as IL-8, in several cell types, including the retinal pigment epithelium (RPE).

Nevertheless, the molecular mechanisms underlying the increased IL-8 production by oxysterols in RPE are still unclear. Therefore, the main goal of this study was to elucidate the molecular mechanisms whereby cholesterol oxides increase the production of IL-8 in the RPE. For that purpose, a human RPE cell line (ARPE-19) was exposed to different oxysterols.

Exposure of ARPE-19 cells to 25-hydroxycholesterol (25-OH) resulted in an increase in IL-8 secretion, whereas the other oxysterols tested decreased IL-8 levels. Consistent with an effect of 25-OH on IL-8 gene expression, treatment of ARPE-19 cells with 25-OH increased the levels of IL-8 mRNA. Furthermore, 25-OH increased IL-8 production in RPE by a mechanism that does not involve oxidative stress nor inactivation of the proteasome.

Treatment of ARPE-19 cells with 25-OH also resulted in the PI3K-dependent ERK activation. Furthermore, blocking any of these signaling pathways prevented the IL-8 up-regulation induced by 25-OH.

Taken together, the results presented in this thesis elucidate a novel signaling pathway whereby 25-OH increases IL-8 production in RPE cells.

By understanding the molecular mechanisms underlying the inflammatory properties of oxysterols in the retina and its contribution to AMD, it might be possible to envision novel therapeutic targets for this pathology.

Keywords: Age-related macular degeneration, inflammation, interleukin-8, PI3K, ERK, 25-hydroxycholesterol.

1. INTRODUCTION

1.1. Anatomy and physiology of the eye

The ocular globe is constituted by six muscles involved in the ocular movements and three concentric layers working together to provide vision, nutrition and protection to the eye (1).

The exterior layer is constituted by the cornea and sclera. The medium or vascular layer is formed by the iris, choroid, corium and uvea. The interior layer is composed by the retina (Figure 1) (1; 2).

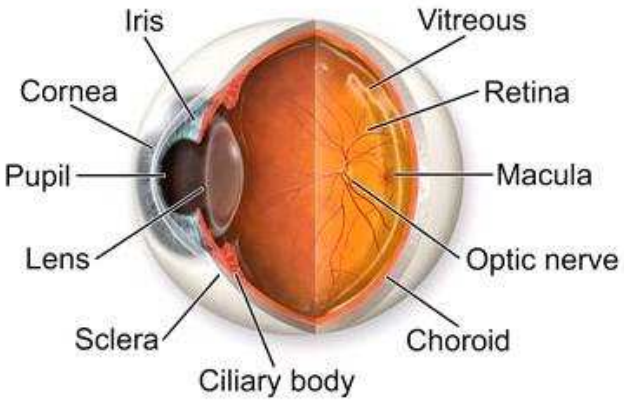

Figure 1: The structure of human eye (135).

The human retina is approximately 0.2 mm thick, and has an area of approximately 1100 mm^2. Each retina possesses about 200 million neurons. The optic disc, where neuronal cells merge to form the optic nerve, is the only area of the retina that is "blind" as it lacks photoreceptors. It appears as an oval white area of 3 mm^2. Temporal to this disc is the macula (3).

The macula is the central posterior portion of the retina and has the highest concentration of photoreceptors, which facilitate central vision and provides high-resolution visual acuity (4; 5; 6; 7). In the center of the macula lays the fovea, a depression with high concentration of cone cells, responsible for the central vision (5).

Although the retina is a complex multilayered structure, it can be functionally divided in two parts: the neuronal retina, composed by the photoreceptors (cones and rods) and their neuronal connections, is responsible for the phototransduction process; the retinal pigment epithelium (RPE) and its basal lamina known as Bruch's membrane maintain the integrity between retina and choroid (Figure 2) (5; 8).

The choroid is a vascular tunica lying between the sclera and retina and its function is to provide oxygen and nourishment to the outer layers of the retina (4; 8).

Bruch's membrane is a semi-permeable exchange barrier that separates the RPE from the choroid (4; 8; 9).

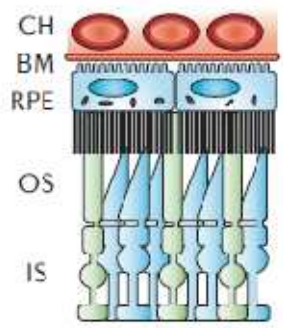

Figure 2: Schematic diagram of the retina and choroid (CH). The choroidal vasculature is at the top and the inner retina is at the bottom. CH, choroid; BM, Bruch's membrane; RPE, retinal pigment epithelium, OS, outer segments of retina; IS, inner segments of retina. Adapted from Rattner et al. (8).

The RPE is a monolayer of pigmented cells located between the choriocapillaris and the photoreceptors outer segments (POS) and forms part of the blood-retinal barrier (BRB) (8; 9; 10). RPE cells receive their name from the melanin pigment granules (melanosomes) located in the apical cytoplasm (9). These pigments are responsible for absorbing the light focused by the lens in the retina (10).

The RPE is essential for visual function, providing multiple functions that support normal photoreceptor function (Figure 3).

The RPE transports ions, water and metabolic end products from the subretinal space to the blood and takes up nutrients such as glucose, retinal and fatty acids from the blood, delivering them to the photoreceptors (9; 10).

The RPE also plays an important role in the visual cycle, by participating in the light-dependent cycling and reisomerization of retinal (the vitamin A derivative that serves as the chromophore for the visual pigments). Retinal is constantly exchanged between the photoreceptors and RPE. Photoreceptors are unable to reisomerize all-

trans-retinal, formed after photon absorption, back into 11-*cis*-retinal. To maintain the photoreceptor excitability, retinal is transported to the RPE, reisomerized to 11-*cis*-retinal and transported back to photoreceptors. This process is known as the visual cycle of retinal (8; 9; 10).

Another RPE function is the maintenance of photoreceptor excitability by the phagocytosis of the POS (10). Photoreceptors are exposed to intense levels of light. This leads to accumulation of photo-damaged proteins and lipids. Therefore, the concentration of light-induced toxic substances inside the photoreceptors increases over time (10). To maintain the excitability of photoreceptors, the POS are digested daily by the RPE and essential substances, such as retinal, are recycled and returned to photoreceptors to rebuild light-sensitive outer segments from the base of the photoreceptors (4; 8; 9; 10). The process of phagocytosis must be tightly coordinated between the RPE and the photoreceptors in order to maintain a proper photoreceptor activity (10).

In addition, the RPE is able to secrete a variety of growth factors and cytokines, which are responsible for maintaining the structural integrity of the retina and choriocapillaris (4).

With such a variety of metabolic and supportive functions, the RPE plays a vital role in visual function. Therefore, it is not surprising that a deregulation in any of these functions has a critical impact on retinal function and may lead to retinal degeneration, loss of visual function and blindness (4; 9; 10; 11).

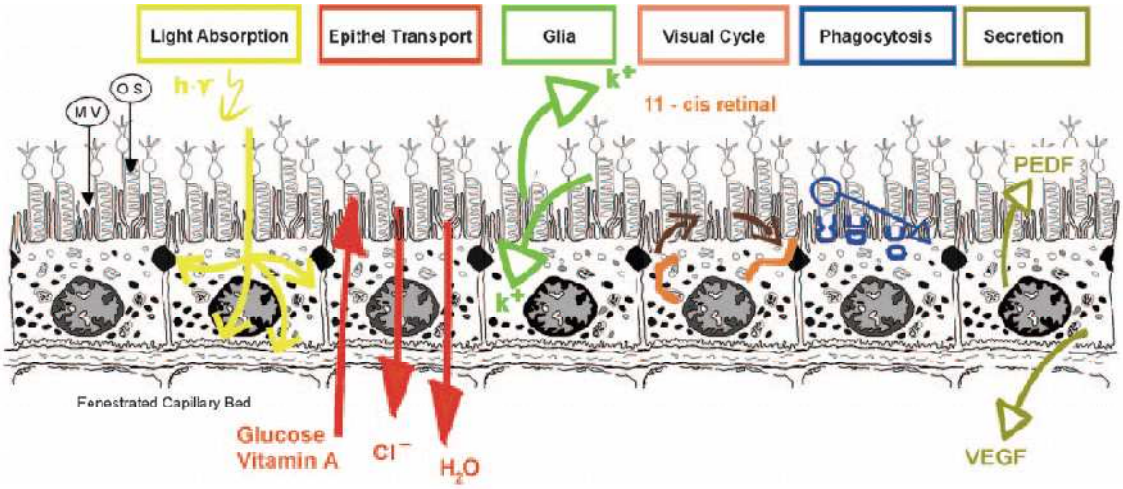

Figure 3: Summary of retinal pigment epithelium (RPE) functions. PEDF, pigment epithelium-derived growth factor; VEGF, vascular epithelium growth factor; Epithel, epithelium. (10)

1.2. Age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness among elderly population (usually over 60) at industrialized countries and its prevalence is likely to rise as a consequence of increasing longevity (6; 12; 13).

AMD is characterized by degenerative changes in the macula, the central region of the retina bearing the highest concentration of photoreceptors (4; 5; 6). Accordingly, this degeneration of the macula leads to loss of central vision (6; 13).

One of the earliest clinical manifestations and pathological features of AMD is the formation of drusen (11; 14; 15). Drusen are accumulations of extracellular amorphous debris located between the RPE and Bruch's membrane (6; 8; 11). Drusen can be found in healthy individuals over 50 years, but the presence of large or numerous drusen confers significant risk for AMD (4; 13; 16).

AMD can be classified into two major subtypes: the atrophic (or dry form) and neovascular (or wet form) (Figure 4).

The atrophic form is characterized by drusen accumulation, geographic atrophy of RPE, and photoreceptor dysfunction and degeneration (Figure 4) (4; 6; 8; 11; 17). The dry form is the most common form of AMD, with more than 80% of all the AMD patients presenting these phenotypes (11; 18).

The neovascular form is more debilitating and often follows the atrophic form. The key feature of wet AMD is choroidal neovascularization (CNV), the growth of new blood vessels from the choroid into the region underlying the RPE or extending past the RPE into the subretinal space and retina (Figure 4) (4; 6; 8; 11; 18). This choroidal neovascularization can lead to leakage of blood into the subretinal space, which, along with RPE atrophy and photoreceptor degeneration, leads to vision loss (4; 11; 17; 18).

As a multifactorial disease, there isn't a known cause for AMD. Instead, several risk factors have been identified. Some of these include smoking, body mass index, diet and light exposure. A number of studies have linked genetics, oxidative stress, RPE senescence, hypoxia and many other factors to AMD (4; 5; 6; 8; 13; 14; 19). Still, the exact etiology and pathogenesis of the disease remain largely unclear.

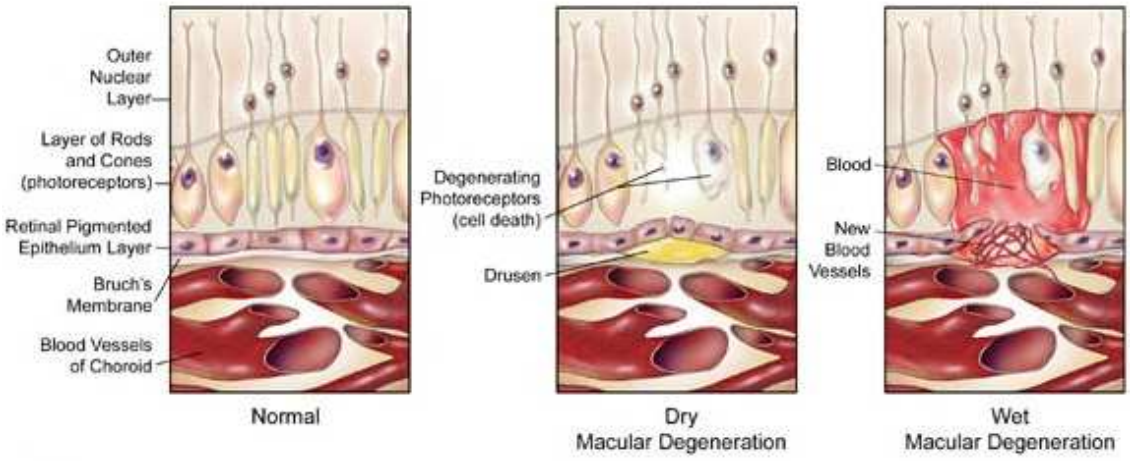

Figure 4: Comparison between the dry and wet forms of Age-related macular degeneration (AMD). Adapted from National Eye Institute (136)

1.3. Oxidative Stress and AMD

Oxidative stress, which refers to cellular injury caused by reactive oxygen species (ROS), has been proposed to play a causative or contributing role in a large number of diseases, such as heart disease, certain types of cancers, neurodegenerative disorders, cataract and AMD (20; 21; 22). In fact, a progressive increase of the oxidative cellular environment, due to the altered redox homeostasis, appears to be one of the hallmarks of the aging process.

ROS include free radicals, molecules that contain one or more unpaired electrons in their orbits, such as superoxide (O^{-2}) and hydroxyl radical ($OH^{\cdot}$) as well as non-radicals, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Figure 5) (20; 23; 24). Many of these oxygen metabolites occur as byproducts of normal physiology or as the result of photochemical reactions (20; 25). An increased oxidative environment inside the cell is known to cause cumulative damage to proteins, lipids, and DNA (26).

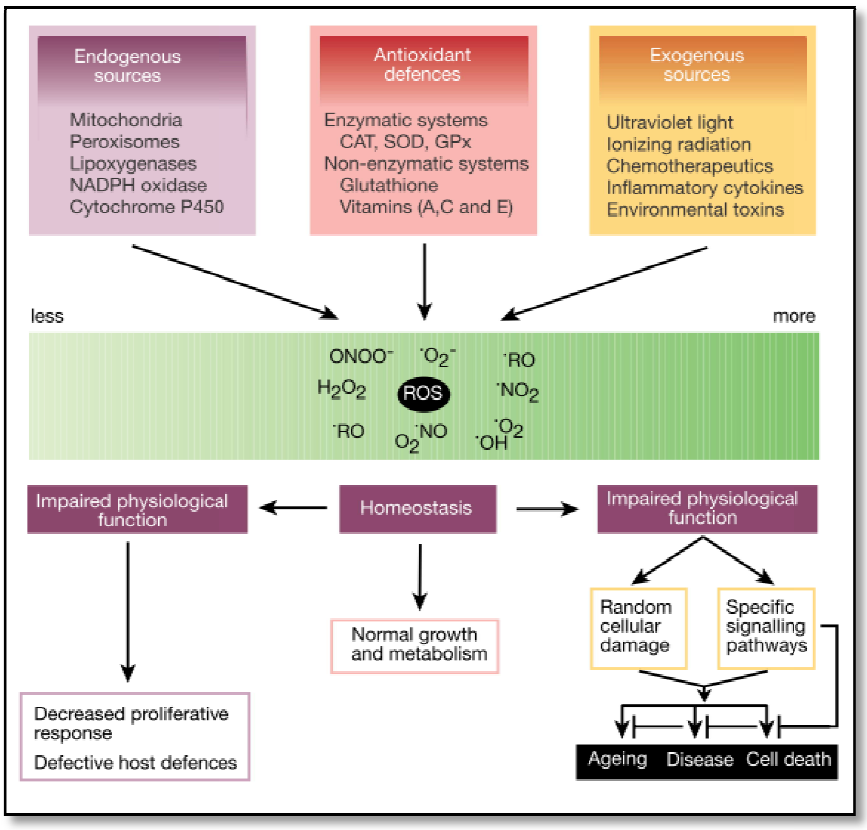

Figure 5: The sources and cellular response to reactive oxygen species (ROS). Oxidant production can be triggered by endogenous or exogenous sources. Also, antioxidant systems, including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), counteract and regulate overall ROS levels to maintain physiological homeostasis. Lowering ROS levels below the homeostatic set point may impair the physiological function of oxidants in cellular proliferation and host defense. In the same way, increased ROS may also be harmful and lead to cell death or to acceleration of ageing and age-related diseases (131).

An increasing body of literature indicates that development of AMD is due to dysfunction of the RPE (14; 27). This dysfunction may be related with the higher susceptibility of the RPE to oxidative stress (21; 23; 28; 29; 30). In fact, a variety of studies suggest that RPE cells can be readily damaged following exposure to an oxidative insult (20; 23).

The retina is an ideal environment for the generation of ROS for several reasons (20; 23; 25). The metabolic rate and oxygen consumption by the retina is much higher than by any other tissue. This high metabolic rate is usually accompanied by generation of ROS. Chronic exposure to light may further increase ROS production (28; 31).

Physiologically, RPE cells phagocytose and digest POS (10). This unique phagocytic function of RPE provides an additional oxidative burden since the shed outer segments are extremely rich in polyunsaturated fatty acids (PUFA), which can be readily oxidized and can initiate a cytotoxic chain-reaction, producing an abundance of ROS. In fact, the process of phagocytosis by the RPE is itself an oxidative stress and results in ROS production (20; 31).

Age-related accumulation of lipofuscin in RPE is another source of oxidative stress. Lipofuscin is a mixture of non-degradable protein-lipid aggregates derived from ingestion of POS (32). A2E is the major fluorophore of lipofuscin and acts as a photosensitizer to generate ROS inside the cells upon exposure to blue light (25; 20; 33; 34; 35).

Consistent with a role of oxidative stress in the development of AMD, an increasing body of literature indicates also that exposure of RPE cells to oxidative stress results in an increase of angiogenic cytokines and growth factors, as well as on other features of AMD (20; 21; 36; 37; 38).

To cope with these toxic oxygen species, the RPE has three lines of defense (39). The first line is absorption and filtering of light (31). The RPE contains various pigments that are specialized in absorbing different wavelengths. General light absorption occurs via melanin in melanosomes (40). Carotenoids lutein and zeaxanthin, the macular pigments of retina, absorb blue light (25). The second line of defense is made by the antioxidants, both enzymatic and non-enzymatic. In the RPE, the major cellular water-soluble non-enzymatic antioxidants found are vitamin C and reduced glutathione (GSH). The main lipid-soluble antioxidants include vitamin E, retinoids and caratenoids (23). The RPE also contains high amounts of enzymatic antioxidants, such as superoxide dismutase (SOD) and catalase (23).The third line of defense is the cell's physiological ability to repair damaged DNA, lipids and proteins (10).

During aging and pathological conditions, the balance between ROS generation and ROS clearance appears to be impaired, due to either a reduction in RPE defense mechanisms and/or an increase in ROS production (20; 23). Consistent with this, an increasing body of literature demonstrates an age-dependent reduction in vitamin E and melanosomes in the RPE (41). Therefore, it is likely that aging RPE cells are more susceptible to oxidative damage (28). In support of this hypothesis, recent studies have shown that mice deficient in Cu,Zn-SOD, an antioxidant enzyme, display typical AMD phenotypes (42; 43).

Currently, the available experimental data provide solid evidence of increased oxidative stress occurring in the retina and RPE upon aging (20; 23; 25; 28; 31). However, it remains unclear whether oxidation is a cause or consequence of retinal degenerations, such as AMD (20; 31).

1.4. Inflammation and AMD

Inflammation is considered a response set by tissues, as a complex network of molecular and cellular interactions directed to facilitate return to physiological homeostasis and tissue repair (44).

Emerging pathological evidence indicates that many age-related diseases, such as AMD, atherosclerosis, arthritis and certain types of cancer are associated with an inflammatory component (21; 45; 46).

The activation of redox-sensitive transcription factors may be involved in triggering the expression of inflammatory cytokines, thus providing a molecular link between oxidative stress and inflammation upon aging (Figure 6) (21; 47; 48).

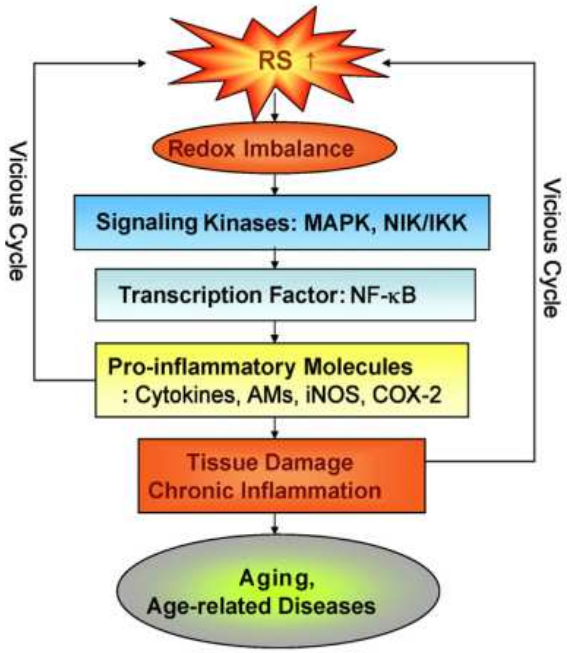

Figure 6: Major molecular pro-inflammatory pathways involved in aging and age-related diseases. RS, reactive species; MAPK, Mitogen-activated protein kinase; NIK, NF-κB kinase; IKK, IKβ kinase; AMs, adhesion molecules; iNOS, inducible NO synthase; COX-2, cyclooxygenase (47).

The complement system is a central part of the innate immunity and its main function is to recognize and eliminate invading microorganisms (21; 49).

The complement system is divided into three main pathways: classical, lectin and alternative. The classical pathway is activated by immune complexes (antibody-antigen complex); the mannose-binding lectin pathway is activated primarily by mannose and *N*-acetyl glucosamine residues particularly abundant on bacterial cell surfaces, and the alternatively pathway is initiated by a variety of substances, including microbial surfaces and polysaccharides (50; 51). Overall, activation of these pathways results in a pro-inflammatory response including generation of membrane attack complexes (MAC), which mediate cell lysis, release of chemokines to attract neutrophils and macrophages to the site of damage and enhancement of capillary permeability (43; 50; 51). A deregulation of the complement, particularly the alternative pathway, results in a defective recognition of these microorganisms and leads to an accumulation of toxic activation products, which can be formed on the surface of host tissues and structures (21).

An increasing body of literature has reported the presence of components of the complement cascade, including C3, C5 and their bioactive fragments C3a, C5a and C5b-9 complex (also known as MAC), in drusen from human eyes, including eyes from AMD patients (20; 49; 52; 50). The observation that immune complexes, complement and/or complement regulatory proteins are present in drusen, both in humans and mice, has led to the hypothesis that AMD results from an atypical inflammatory process that includes inappropriate complement activation (49; 53; 54).

Complement factor H (CFH) is a critical negative regulator of the complement system (45). CFH regulates activation of the alternative pathway by inhibiting several steps of this pathway and promoting degradation of activated complement components (50). CFH binds heparin on cell surfaces to prevent complement-mediated damage to heparin and the cells bearing them. It also binds C-reactive protein (CRP) to inhibit CRP-mediated activation of the alternative pathway in response to damaged tissue (50; 55; 56).

Several recent studies have identified a strong association between polymorphisms in CFH and in complement factor B (CFB), a positive regulator of the alternative pathway of the complement, and an increased risk of AMD (49; 57; 58). Accordingly, genetic polymorphisms in CFH and CFB may result in an abnormal up-regulation of the complement activity and a consequent increase in the inflammatory component of AMD (21; 55; 56).

Consistent with a role for the complement system in the pathogenesis of AMD, a recent report suggests that oxidative stress in RPE can trigger the activation of the complement (59). Moreover, complement activation results in an enhanced expression of interleukin-8 (IL-8), an important pro-inflammatory and angiogenic cytokine (60). The increased expression of IL-8 has also been reported in RPE cells that were fed with oxidized POS, further supporting the link between oxidative stress and inflammation (36; 37; 61).The increased expression and secretion of IL-8 may account, at least in part, for the inflammatory reactions during AMD development (37; 62).

Monocyte chemotactic protein-1 (MCP-1) is a chemokine involved in monocyte infiltration into the sites of inflammation (63). In RPE cells, this chemokine has been suggested to play a role in drusen clearance. A recent study showed that deficiency in MCP-1 or its cognate receptor CCR-2 leads to AMD-like features in mice, including accumulation of lipofuscin and drusen beneath the RPE, photoreceptor atrophy and CNV (50; 64).

The impairment in macrophage recruitment causes accumulation of complement components, such as complement regulatory proteins and IgG in the retina. Since deposition of complement-related proteins and IgG precedes the accumulation of drusen and lipofuscin, it is likely that the AMD-like pathology is due, at least in part, to complement activation and immune complex deposition (59; 64).

However, several studies have demonstrated accumulation of macrophages in histologic specimens from patients with AMD, especially in regions of RPE atrophy, breakdown of Bruch's membrane and CNV (43; 50). Moreover, macrophages in CNV lesions have been shown to express angiogenic factors, such as vascular endothelial growth factor (VEGF) (58). While the presence of macrophages at CNV lesions is recognized, it is still a matter of debate whether macrophages play a pathologic or a restorative/adaptive role in AMD.

Overall, these and other observations strongly suggest that inflammation is an important component in the pathogenesis of AMD.

1.5. The Ubiquitin-Proteasome Pathway

The ubiquitin-proteasome pathway (UPP) is the major non-lysosomal proteolytic pathway within cells (21). In this ATP-dependent pathway, proteins destined for degradation must be marked by a multiubiquitin chain in order to be recognized and degraded by a large protease complex called the proteasome (21; 65; 66; 67). Ubiquitin conjugation, or ubiquitination, is a highly ordered process and involves three different classes of enzymes. An ubiquitin-activating enzyme (E1) first activates and transfers ubiquitin to an ubiquitin-conjugating enzyme (E2), which then acts with one of a large family of ubiquitin protein ligases (E3) to transfer ubiquitin to a lysine residue in the target substrate (21; 66; 68; 69). A chain of at least four ubiquitin moieties is required for substrate recognition by the 26S proteasome complex (Figure 7) (21).

The 26S proteasome complex consists of a 20S catalytic core and two regulatory 19S caps (21; 70). The 20S particle is comprised of four heptameric rings arranged coaxially. The subunits of the 20S core are classified into α and β, with the non-catalytic α-heptameric rings forming each of the two outer rings and the catalytic β-heptamers forming the two inner rings (65; 71). The β-subunits contain three pairs of active sites that perform distinct proteolytic activities. The active sites have been classified as caspase-like, trypsin-like and chymotrypsin-like for cleavage of acid, basic and hydrophobic amino acids, respectively (65; 71). The 19S caps are responsible for the recognition of ubiquitinated substrates and subsequent cleavage of the ubiquitin moieties from those substrates. The protein subunits present in the 19S cap also play an important role in unfolding the protein substrate and feeding it through the proteolytic chamber of the 20S core (21; 72).

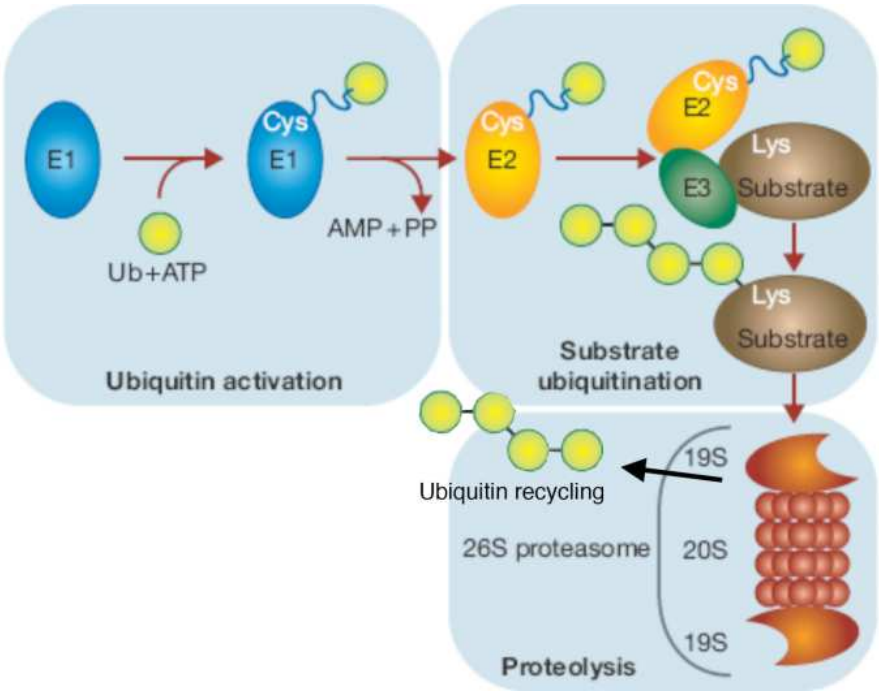

Figure 7: The ubiquitin-proteasome pathway (UPP). A cascade of enzymatic reactions leads to ubiquitination of lysine residues of the substrate. Adapted from Meusseur et al (137).

The UPP is an essential protein quality control system, which selectively degrades mutant, misfolded or damaged proteins (21; 73; 74). Timely removal of abnormal or damaged proteins by the UPP is essential for the cells to recover from various environmental stresses. However, the UPP itself is a target of such stresses (21; 65; 66; 73; 75). All the three classes of ubiquitination enzymes have a cysteine in their active sites, and therefore, their activities are subject to redox regulation. In addition, other types of modifications, such as S-nitrosylation, can inactivate these enzymes (21; 66). ROS and reactive lipid peroxidation products, such as 4-hydroxynonenal (HNE), can also impair the activity of the proteasome (73; 75; 76).

1.5.1. Oxidative inactivation of the UPP: a molecular link between oxidative stress and AMD

Given that the UPP is involved in virtually every aspect of cellular function, it is not surprising that impairment of this pathway is associated with the development of several pathologies. Indeed, an age-related decline in proteasome activity has been implicated in many age-related diseases, such as AMD (66; 68; 71; 73).

The UPP has been involved in a myriad of cellular processes (21; 68; 73), including regulation of signal transduction pathways involved in angiogenesis, regulation of immune response and inflammation.

The hypoxia-inducible factor (HIF) is a key regulator of a wide range of hypoxia-related processes, including angiogenesis (77). Both HIF-1α and HIF-1β subunits exist as a series of isoforms encoded by distinct genetic loci. HIF-1β subunits are constitutive nuclear proteins, whereas HIF-1α subunits are induced by hypoxia. The abundance of HIF-1α subunits is mainly regulated by the rate of degradation. Oxygen-dependent hydroxylation at two proline residues (Pro402 and Pro564) of human HIF-1α mediates its interactions with an ubiquitin ligase (E3), von Hippel-Lindau (VHL), which targets HIF-1α for proteasomal degradation (78; 79), and contributes to the extremely rapid proteolysis of HIF-1α in cells under normoxia conditions (80).

In addition to HIF, other transcription factors, such as nuclear factor κB (NF-κB) are under the control of the UPP. NF-κB proteins are present in the cytoplasm in association with inhibitory proteins called inhibitors of κB (I-κB). Upon activation by a variety of stimuli, the I-κB proteins are phosphorylated, ubiquitinated, and subsequently degraded by the proteasome. Degradation of I-κB allows translocation of NF-κB into the nucleus and binding to its cognate DNA binding sites to regulate the transcription of a large number of genes, including those involved in angiogenesis and inflammation, such as cytokines and stress response proteins (67; 69).

Given that the UPP is involved in regulating a number of signal transduction pathways and transcription factors, including HIF-1α and NF-κB, and its activity can be compromised upon oxidative stress (65; 66; 68; 75), it is therefore plausible that age-related impairment of the UPP in the retina could result in the altered expression of genes involved in angiogenesis and inflammation, such as those under the control of HIF-1α and NF-κB (81). This UPP impairment would, in turn, act as a link between retinal oxidative insult and expression of AMD-like features, such as increased inflammation, accumulation of drusen (caused by a decreased macrophage recruitment) and neovascularization of the retina (Figure 8).

Consistent with this hypothesis, we have shown that the proteasome is a target of oxidative damage in cultured human RPE cells (66) and that oxidative inactivation of the proteasome may provide a link between oxidative stress and increased production of pro-inflammatory cytokines, such as IL-8 (68). Furthermore, we have elucidated a cascade of sequential activation of signaling pathways in response to proteasome inactivation, contributing to an increase in IL-8 production. This cascade is initiated by

proteasome inhibition and involves a downstream activation of MKK3/MKK6, p38 MAPK activation, EGFR phosphorylation, PI3K activation and increased IL-8 expression. Furthermore, we have identified the Itk kinase as a novel regulator of IL-8 expression upon proteasome inhibition (82).

Consistent with a model for AMD-like phenotypes, our studies also show that impairment of the proteasome in RPE increases the production of proangiogenic factors, such as VEGF. This increase is mediated by HIF-1α, which is stabilized upon proteasome inhibition in RPE (72). On the other hand, proteasome inactivation in RPE decreases NF-κB activation and, consequently, attenuates MCP-1 production, an important player in macrophage recruitment to the retina. Interestingly, mice lacking MCP-1 or its cognate receptor, CCR-2, develop features of human AMD (64).

Further support for an involvement of the proteasome in the pathogenesis of AMD comes from recent studies that show a change in proteasome activity in the eyes from AMD patients (26; 71).

Given the function of the UPP in regulating IL-8, VEGF, and MCP-1 in RPE cells and the roles of these chemokines and growth factor in the pathogenesis of AMD, impairment of the UPP in RPE could significantly contribute to the development of AMD (21; 68; 82).

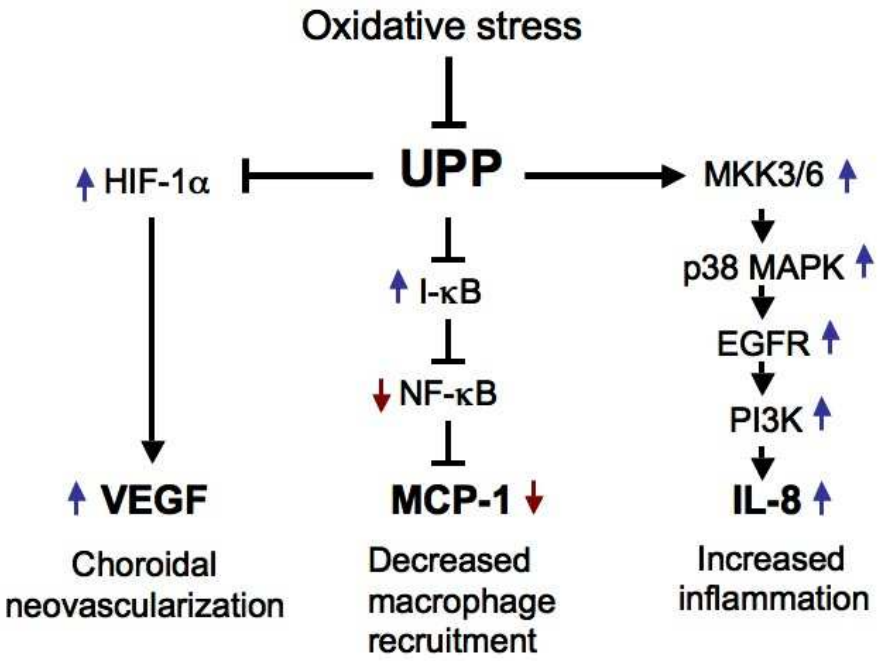

Figure 8: The hypothesized relationship between oxidative impairment of the UPP and AMD. Oxidative stress may inactive the UPP in RPE cells. Impairment of the UPP in RPE cells will decrease the activity of NF-κB (by stabilization of I-κB) and enhance the activity of the HIF system by stabilization of HIF-1α, resulting in VEGF overexpression. On the other hand, proteasome inactivation may also result in the activation of a cascade of sequential activation of signaling pathways, including p38 MAPK, EGFR and PI3K. These changes will result in altered expression of genes involved in AMD-like phenotypes (82).

1.6. Cholesterol and its oxidation products: relevance for AMD

Cholesterol is an essential component of mammalian membranes (83; 84; 85). It alters membrane fluidity, thickness, curvature and permeability (84). In addition, cholesterol is an important constituent of lipid rafts, and because of the large number of signaling proteins that are localized in these structures, it has been postulated that these domains serve as regulatory platforms for some transduction pathways (84; 86; 87; 88). Nevertheless, excess cholesterol is toxic to cells. Therefore, its levels must be tightly regulated (86).

Cholesterol is synthesized in the endoplasmic reticulum (ER) and delivered to other organelles by both vesicular and non-vesicular transport processes (89). Some other sources of cellular cholesterol include endocytic uptake of lipoproteins and hydrolysis of their cholesterol esters in late endosomes and lysosomes (89; 90).

As a hydrophobic molecule, cholesterol must be transported into the plasma by carriers (91). These carriers are classified, according their density, into four main classes: chylomicrons, low-density lipoproteins (LDL), high-density lipoproteins (HDL) and very-high-density lipoproteins (VHDL) (83). LDL is the primary carrier of cholesterol in the blood and delivers cholesterol to peripheral tissues, whereas HDL is involved in the process of reverse cholesterol transport from peripheral tissues to the liver (83).

Cholesterol is composed of three regions: a hydrocarbon tail (also called lateral chain), a ring structure region with four hydrocarbon rings (A, B, C and D), and a hydroxyl group (92). Cholesterol is easily oxidized and this reaction may occur in the ring of the structure or in the lateral chain, resulting therefore in the formation of oxysterols, a large class of 27-carbon atom molecules (92).

Although oxysterols are less abundant than cholesterol, they are absorbed more rapidly in the intestine, have a faster plasma clearance and are quickly collected by tissues, when compared to cholesterol (92).

Oxysterols may be formed enzymatically, such as 25-hydroxycholesterol (25-OH), or as a result of auto-oxidation of cholesterol, such as 7β-hydrocholesterol (7β-OH) and 7-ketocholesterol (7-KC), during the oxidation of the LDL particle (Figure 9) (93; 94). It should be noted that cholesterol auto-oxidation can also take place in various tissues (92). The microsomal cytochrome P450 system is the main enzyme complex involved in the enzymatic formation of oxysterols; however, some other enzymes may be involved in this process. For instance, 25-OH is generated by cholesterol 25-hydroxylase (92).

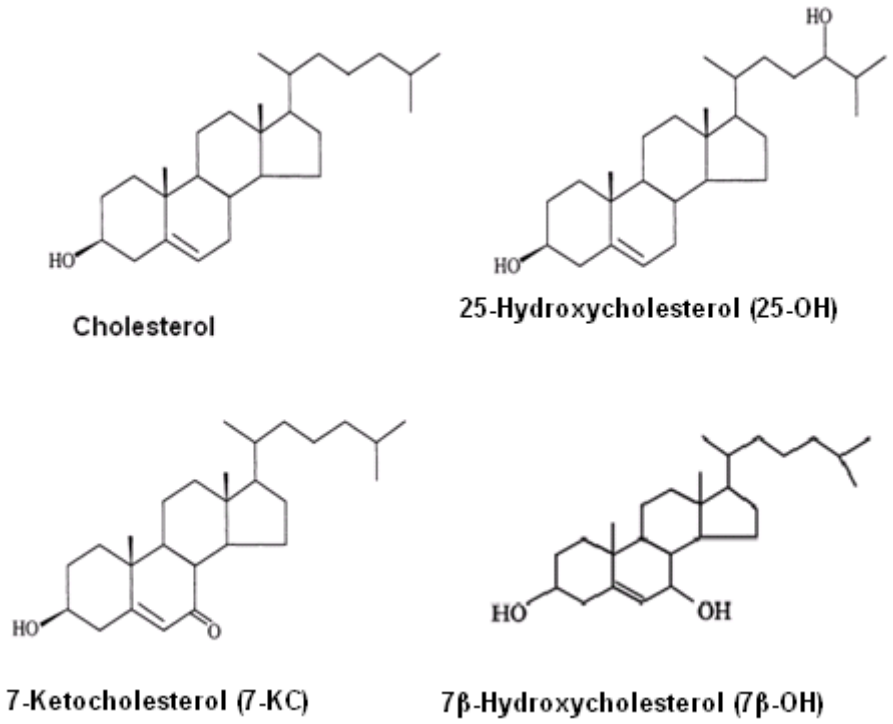

Figure 9: Chemical structures of cholesterol, 25-hydroxycholesterol, 7-ketocholesterol and 7β-hydroxycholesterol. Adapted from Brown et al. (108) and Lordan et al. (109).

In addition to being intermediates in the cholesterol biosynthetic pathway, oxysterols have been shown to possess many important and diverse biochemical and regulatory activities (86; 95; 96; 97; 98; 99; 100; 101). In fact, an increasing body of literature demonstrates that oxysterols may have cytotoxic, oxidative and inflammatory effects in a variety of cell types (96; 102; 103). 7-KC and 7β-OH are considered the most cytotoxic oxysterols, whereas 25-OH has been shown not to be as toxic as these two (92). The different structure of oxysterols and their different affinities for oxysterol-binding proteins may explain the distinct biological activities observed with different oxysterols (104).

Given that the aging process is usually characterized by an increase in the production of oxidized compounds, it is not surprising that an age-dependent accumulation of oxysterols, as cholesterol oxidation products, has been reported in several tissues, including the lens and the retina (105; 106; 107). Furthermore, oxysterols have been associated with the development of many age-related diseases, such as cataract, atherosclerosis and AMD (94; 108; 109). The inflammatory properties

of oxysterols may account for the inflammatory component of these age-related diseases (92; 94; 96).

Although oxysterols have been shown to have inflammatory properties by triggering the production of pro-inflammatory cytokines in several cell types, including the RPE (92; 110; 111; 112; 113), the molecular mechanisms underlying this increased production are still unclear.

1.7. Objectives

Age-related macular degeneration (AMD) is the leading cause of blindness in the western world in the population over 60 years of age (6; 13).

An increasing body of literature indicates that AMD is related to the dysfunction of the RPE (27; 39). Furthermore, recent findings suggest a role for inflammation in the pathogenesis of AMD (21; 45; 51; 56). However, the molecular mechanisms underlying the development of the disease are still far from understood.

Oxysterols, the products of cholesterol oxidation, have been shown to increase the production of pro-inflammatory cytokines, such as IL-8, in several cell types (92; 110; 111). Moreover, previous reports, including work from our laboratory, have demonstrated an age-related accumulation of oxysterols in ocular tissues, such as the lens and the retina (105; 106; 107)

Therefore, the first aim of this study was to test the effect of oxysterols on IL-8 production in cultured RPE. We investigated whether different oxysterols known to have pro-inflammatory properties increased IL-8 expression and secretion in a RPE cell line.

Our recent studies suggest that the oxidative inactivation of the proteasome can constitute a molecular link between oxidative stress and increased IL-8 production in RPE cells (68). Furthermore, oxysterols have been shown to increase the production of ROS in cultured RPE (110; 114). Therefore, the second aim of the study was to assess whether the increased IL-8 production by oxysterols in the RPE involved impairment of the proteasome and whether this was an oxidation-dependent event.

Finally, having established that oxysterols increase IL-8 production in cultured RPE, the third aim of this work was to elucidate the signal transduction pathways involved in this up-regulation.

Altogether, this study aimed at understanding the molecular mechanisms underlying the enhanced IL-8 production induced by oxysterols in cultured RPE.

By understanding the role of oxysterols in the development of AMD, it might be possible to envision new therapeutic approaches to this disease.

2. EXPERIMENTAL PROCEDURES

2.1. Materials

Dulbecco's Modified Eagle's Medium/ Nutrient Mixture F-12 Ham (DMEM-F12), oxysterols (25-OH, 7-KC e 7β-OH), cholesterol, vitamin E, bovine serum albumin (BSA), sodium orthovanadate and the monoclonal antibody against actin were obtained from Sigma-Aldrich (St Louis, MO, USA). The fetal bovine serum (FBS) and the antibiotics for cell culture were purchased from Gibco (Grand Island, NY, USA). MG132, U0126, FR180204, LY294002 were purchased to Calbiochem (La Jolla, CA, USA). Peptidasefluorogenic substrates Succinyl-Leu-Leu-Val-Tyr-amidomethylcoumarin (LLVY-AMC), *N-t*-butyloxycarbonyl-Leu-Ser-Thr-Arg-amidomethylcoumarin (LSTR-AMC) and benzyloxycarbonyl-Leu-Leu-Glu-amidomethylcoumarin (LLE-AMC) were from Biomol (Plymouth Meeting, PA, USA).

SDS-PAGE reagents and the nitrocellulose membrane for Western Blot were from Bio-Rad Laboratories (Hercules, CA, USA). Rabbit polyclonal antibodies to phosphorylated ERK1/2 (extra-cellular signal regulated kinase) and total ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and sheep anti-mouse IgG were from Zymed Laboratories Inc. (San Francisco, CA, USA). The ECL Western Blotting detection kit was obtained from Amersham Biosciences (Buckingamshire, UK). The DuoSet ELISA kit for IL-8 was obtained from R&D Systems (Minneapolis, MN, USA).

The RNA RNeasy mini kit and the SYBR Green PCR Master Mix were from Qiagen (Valencia, CA, USA).

All other chemicals were obtained from Sigma and were of the highest purity available.

2.2. Cell culture and treatments

The retinal pigment epithelial cell line ARPE-19 (115) was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were routinely maintained at 37ºC under 5% CO_2 atmosphere and cultured in DMEM:F12 supplemented with 10% FBS and containing 100 U/mL penicillin G and 100 µg/mL streptomycin.

Before treatments, confluent cells were rinsed once with Phosphate-buffered saline (PBS) and fresh medium was added. For Western Blot assays, the medium was changed the day before the incubations.

The different oxysterols (25-OH, 7-KC, 7β-OH) and cholesterol were prepared in ethanol at 10 mg/mL and diluted to 20 µg/mL (50 µM) in the cell medium immediately before use. Cells incubated only with the vehicle (ethanol) were used as a control. U0126, FR180204 and LY294002 were prepared in dimethyl sulfoxide (DMSO) at 10 mM and diluted to 10 µM in the cell medium just prior to use. Controls included cells incubated only with the vehicle (DMSO). In all experiments, the inhibitors were added to the cell medium 1 hour before the treatments. For proteasome inhibition studies, MG132 was prepared in DMSO at 10 mM and diluted to 20 µM in cell medium immediately before use. Vitamin E (α-tocopherol) was prepared in DMSO at 50 mg/mL and then diluted to 5 µg/mL just prior to use. Vitamin E was added to the cell medium 6 hours before the treatments.

2.3. Western Blot

Whole cell lysates were prepared for Western blot analysis. Following treatment, cells were rinsed once with ice-cold PBS supplemented with 2 mM of sodium orthovanadate (phosphatase inhibitor) and then scrapped and collected in Laemmli buffer**.** Cell lysates were then denatured at 100ºC for 5 min and solubilized by sonication for 3 time periods of 5 seconds. The denatured proteins were separated by electrophoresis in a 10-12% polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE). Proteins in the gel were then transferred to nitrocellulose membranes, during 1 hour at 100V. Next, membranes were blocked in 5% milk during 1 hour at room temperature and then probed with rabbit polyclonal antibodies to phosphorylated ERK1/2 and total ERK1/2 (1:1000 in TST containing 5% of BSA) or mouse monoclonal

antibody to β-actin (1:10000 in TST containing 5% of milk) and incubated overnight at 4ºC. After incubation for 1 hour at room temperature with the corresponding HRP-conjugated secondary antibody, the proteins were visualized using ECL Western Blotting Detection kit, following the manufacturer's instructions. Exposure times were adjusted so that the band intensity fell within the linear range of detection.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Levels of IL-8 secreted into the medium by RPE were determined by ELISA. The medium was diluted 10 times to determine IL-8 levels. All ELISAs were performed according to manufacturer's instructions. Briefly, a monoclonal antibody against IL-8 was diluted to the working concentration in PBS without the carrier protein and a 96-well plate was coated with 100 µL of the diluted antibody per well. The plate was incubated overnight at room temperature. The samples were added to the plate and incubated at room temperature for 2 hours. After the incubation, a biotinylated polyclonal antibody against IL-8 was added and incubated overnight at 4ºC. After incubation with streptavidin-HRP for 20 minutes at room temperature, the TMB substrate Chromogen solution was added and incubated at room for 20 minutes, protecting the plate from direct light. The reaction was stopped by adding H_2SO_4 1M and the optical density of each well was determined using a microplate reader set to 450 nm.

2.5. Real-time RT- PCR

Total RNA was extracted from ARPE-19 cells using the RNeasy mini kit according to the instructions of the manufacturer. The amount of total RNA was determined by absorbance at 260 nm and its purity was assessed by the 260/280 nm ratios. For first strand cDNA synthesis, 2 µg of total RNA from each sample were reverse-transcribed with SuperScriptase III, using Random Primers (300 ng), dNTPs (0.5 mM each), RT buffer (1x) and DTT (0.01 M) in a total volume of 20 µL. The reaction was performed at 25ºC for 10 minutes, followed by 50 minutes at 42ºC, for primer annealing to the RNA template and cDNA synthesis, respectively. The reaction was terminated at 70ºC for 15 minutes, and the samples were cooled to 4ºC and stored at -20ºC until further use.

Real-time RT-PCR analysis was conducted on ABI PRISM 7000 sequence detection system from Applied Biosystems using SYBR Green PCR master mix according to the manufacturer's instructions. The primers used for amplification of the IL-8 gene are as follows: the forward primer was: 5'-AAACCACCGGAAGGAACCAT-3' and the reverse primer was: 5'-CCTTCACACAGAGCTGCAGAAA-3'. Levels of GAPDH were used for normalization of the total mRNA amount. For quantification of GAPDH mRNA, the forward primer was 5'-ATCACCATCTTCCAGGAGCGA-3' and the reverse primer was 5'-CCTTCTCCATGGTGGTGAAGAC-3'. Each primer of a pair was added to the reaction mixture (final volume: 25 µL) at a final concentration of 25 µM, in addition to the SYBR Green PCR master mix (1x) and 1 µl of cDNA sample. The thermal cycling was initiated by denaturation during 10 minutes at 95ºC followed by 45 cycles of a 30 s melting step at 95ºC, a 5 s annealing step at 58ºC and a 25 s elongation step at 72ºC. After amplification for 45 cycles, samples were subjected to a melting curve analysis in order to confirm the absence of unspecific amplification products and primer dimers. The relative changes in the mRNA levels of the genes tested were determined by the ΔΔCt method. The "Cycle threshold" (Ct) values for each target gene given by the ABI PRISM 7000 sequence detection system software were subtracted by the respective Ct value determined for the GAPDH gene from the same sample and condition (ΔCt), in order to normalize for changes in target gene expression. The ΔCt values were subsequently subtracted by the respective values of the control for the target gene giving ΔΔCt. The derivation to the formula 2-(ΔΔCt) sets each control at the unity (or 100%), since ΔΔCt (control) = 0, and the different experimental conditions at a percentage relative to the control.

2.6. Proteasome Activity Assay

ARPE-19 cells were lysed in 25 mM Tris-HCl buffer, pH 7.6. All three peptidase activities of the proteasome were determined using fluorogenic peptides, as described (65). LLVY-AMC was used for the chymotrypsin-like activity, LSTR-AMC was used for the trypsin-like activity, and LLE-AMC was used for the caspase-like activity. The mixture, containing 20 µL of cell supernatant in 25 mM Tris-HCl, pH 7.6, was incubated at 37 °C with the appropriate concentrations of peptide substrate (LLVY-AMC at 25 µM, LLE-AMC and LSTR-AMC at 40 µM) in a buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 3 mM NaN_3 and 0.04% 3-[(3-

Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS). The final volume of the assay was 200 µL. Rates of reactions were measured in a temperature-controlled microplate fluorometric reader. Excitation wavelength was 380 nm and emission wavelength was 460 nm. Proteasome activity was defined as portion of peptidase activity in the cell extracts that was inhibited by 20 µM *N*-Cbz-Leu-Leu-leucinal (MG132), a proteasome inhibitor (116).

2.7. Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 4.00 software (GraphPad Software Inc., U.S.A.). All the results are representative of at least three independent experiments. Data are expressed as mean ± standard deviation (SD). Comparisons between multiple groups were performed with an one-way ANOVA followed by a Tukey's test or Student's t-test (Figure 10C).Significance was accepted at $p<0.05$.

3. RESULTS

3.1. 25-OH increases IL-8 production in the RPE

A number of studies highlight the role of inflammation in the development of AMD (21; 45; 51; 56). Furthermore, oxysterols have been shown to accumulate in ocular tissues, such as the lens and retina, in an age-related manner (105; 106; 107) and to increase the production of pro-inflammatory cytokines, such as IL-8, in several cell types (110; 111; 117).

To investigate the potential link between oxysterols and inflammation, we determined the effect of several oxysterols on the secretion of IL-8, a pro-inflammatory cytokine. The oxysterols tested were selected based on previous studies that have shown their ability to induce inflammatory gene expression in other cell types (93; 102).

As shown in Figure 9A, incubation of ARPE-19 cells with 25-OH resulted in a 2-fold increase in the secretion of IL-8. This effect seems to be specific for 25-OH, as incubation of ARPE-19 cells with both 7-KC and 7β-OH did not increase IL-8 production (Figure 10A). In fact, both oxysterols slightly decreased IL-8 production, when compared with the control (Figure 10A). To rule out the possibility that the increased IL-8 secretion following 25-OH treatment was due to the cholesterol molecule itself, an additional control included the incubation of ARPE-19 cells with cholesterol. As shown in Figure 10B, cholesterol did not have a significant effect on IL-8 secretion in ARPE-19 cells, whereas 25-OH was able to increase IL-8 production. Consistent with the protein levels, incubation of ARPE-19 cells with 25-OH dramatically increased IL-8 mRNA levels (Figure 10C).

Taken together, these data indicate that 25-OH leads to an up-regulation of IL-8 production in RPE.

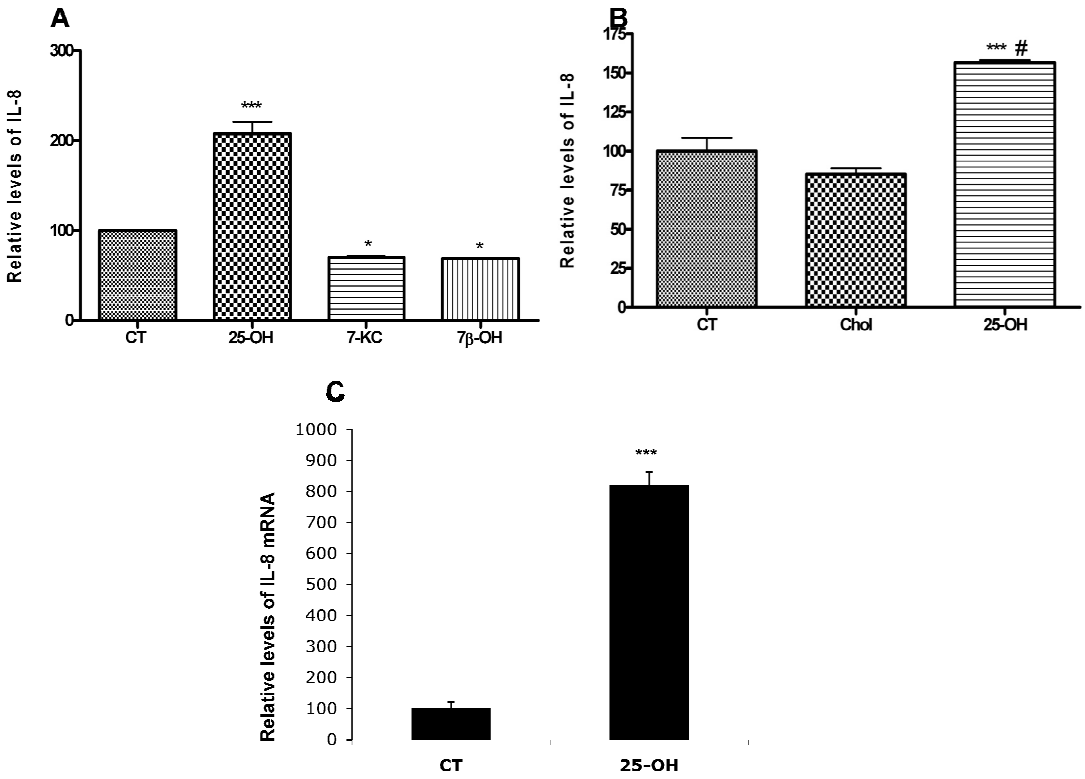

Figure 10: 25-OH increases IL-8 production in RPE cells. ARPE-19 cells were incubated with 7-KC (20 µg/mL), 7β-OH (20 µg/mL), 25-OH (20 µg/mL) and cholesterol (20 µg/mL) for 24 hours. IL-8 protein levels in the medium were determined by ELISA (panels A and B). mRNA levels for IL-8 were determined by real time RT-PCR (panel C). Results are presented as the mean±S.D of three independent experiments. ***p<0.001 and * p<0.05 as compared with control; #p<0.001 as compared with cholesterol.

3.2. 25-OH does not affect proteasome activity in RPE

We have recently shown that oxidative inactivation of the proteasome is a mechanistic link between oxidative stress and up-regulation of IL-8 production in RPE cells (65; 66; 68). Moreover, a recent report indicates that oxysterols induce ROS production in the RPE (110). Therefore, it is conceivable that 25-OH increases IL-8 production in RPE by the oxidative inactivation of the proteasome. To test this hypothesis, we assessed the effect of 25-OH on proteasome activity in ARPE-19 cells.

As shown in Figure 11, 25-OH did not have a significant effect on any of the three proteolytic activities of the proteasome. MG132, a potent proteasome inhibitor, was used as a positive control. The minor inhibitory effect of MG132 towards the trypsin-like activity of the proteasome may be due to a higher selectivity of this inhibitor for the other two activities (118; 119).

Together, these results demonstrate that 25-OH does not affect proteasome activity in the RPE.

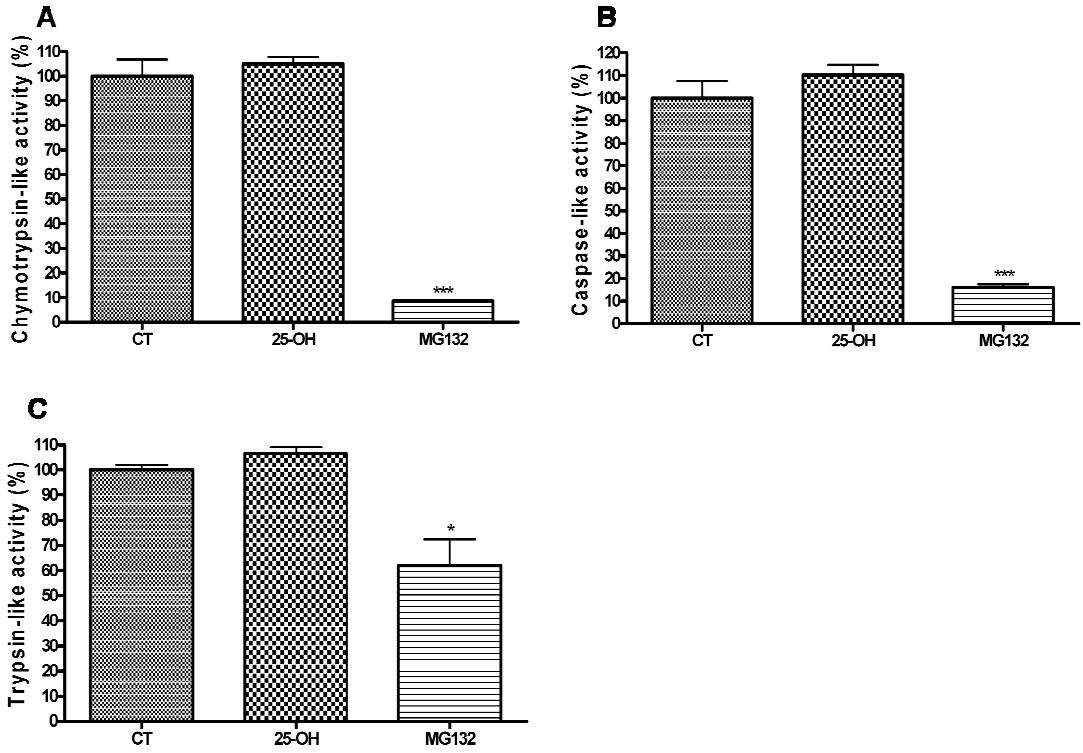

Figure 11: 25-OH does not affect proteasome activity in RPE cells. ARPE-19 cells were incubated with 25-OH (20 µg/mL) or MG132 (20 µM). Proteasome activity was determined using a fluorogenic peptide as substrate. Panel A, B and C show chymotrypsin-like, caspase-like and trypsin-like activities, respectively. Results are presented as the mean±S.D of three independent experiments. ***$p<0.001$ and *$p<0.05$ as compared to control.

3.3. 25-OH-induced IL-8 production in RPE is not associated with increased oxidative stress

A number of studies suggest that oxidative stress can trigger an inflammatory response in several cell types, including the RPE (36; 37; 61; 68). Consistent with these observations, we have recently shown that oxidative stress can increase the production of IL-8, an important pro-inflammatory cytokine, in ARPE-19 cells (68). Given that a previous report (110) and our preliminary data indicate that oxysterols can induce the production of ROS in the RPE, we assessed whether the increased IL-8 production following 25-OH treatment was an oxidative event.

Consistent with our previous results, 25-OH increased IL-8 production in ARPE-19 cells (Figure 12). However, pre-incubation with vitamin E did not prevent 25-OH-induced IL-8 production, suggesting that this effect is not due to increased oxidative stress.

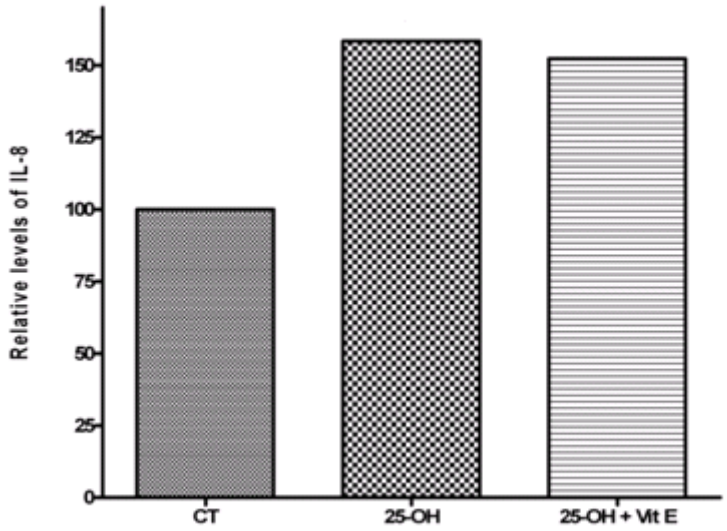

Figure 12: The increased IL-8 production induced by 25-OH is not suppressed by antioxidants. ARPE-19 cells were preincubated with Vitamin E (Vit E) (5 µg/mL) for 6 hours and then exposed to 25-OH (20 µg/mL) for 24 hours. IL-8 protein levels in the medium were determined by ELISA. Results presented are the mean of two independent experiments.

3.4. 25-OH activates ERK in RPE in a PI3K-dependent manner

The data above indicate that 25-OH increases IL-8 production in RPE. Moreover, this increase does not involve oxidative stress nor inhibition of the proteasome. However, the signaling events underlying the enhanced IL-8 production in response to 25-OH are still unclear.

In order to search for the signal transduction pathways that account for the up-regulation of IL-8 upon 25-OH treatment, we examined the effect of 25-OH on the activation of the ERK pathway. This pathway has been shown to regulate IL-8 gene expression (111; 113; 117) and ERK can be activated upon various stresses, including exposure to oxysterols (111; 113; 117; 120).

As shown in Figure 13A, phosphorylated ERK was barely detected in control cells. However, incubation of ARPE-19 cells with 25-OH resulted in the activation of ERK, as assessed by the increased levels of phosphorylated ERK. This effect was most pronounced at 12 hours of incubation. In contrast, the total amount of ERK did not change in any of the experimental conditions. Interestingly, 25-OH seems to primarily activate ERK2. In fact, after a long exposure, we were able to detect a second band, probably corresponding to ERK1 (Figure 13B), suggesting that 25-OH activates both ERK isoforms to different extents.

To rule out the possibility that the ERK activation following 25-OH treatment was due to the cholesterol molecule itself, an additional control included the incubation of ARPE-19 cells with cholesterol. As shown in Figure 13C, cholesterol did not have a significant effect on ERK activation in ARPE-19 cells, whereas 25-OH was able to activate ERK. This is consistent with our previous results showing that cholesterol did not have a significant effect on IL-8 production (Figure 10B) and further suggests a requirement for ERK activation in the increased IL-8 production in RPE cells upon 25-OH treatment.

The data above suggest that 25-OH activates ERK in RPE cells. However, the upstream signaling events leading to this activation are still unclear.

To determine the upstream events that trigger the activation of ERK1/2 in response to 25-OH, we assessed the effect of PI3K inhibition on ERK activation under these conditions. The PI3K pathway has been shown to activate ERK in other cell types (86; 121). Therefore, it is conceivable that the 25-OH-induced ERK activation in RPE cells is PI3K-dependent.

Figure 13D shows that 25-OH activates ERK in RPE, which is consistent with our previous results. LY294002, a PI3K inhibitor, was able to completely prevent the ERK activation induced by 25-OH. The total amount of ERK did not significantly change in any of the experimental conditions.

Taken together, these data indicate that 25-OH activates ERK in a PI3K-dependent manner in cultured RPE cells

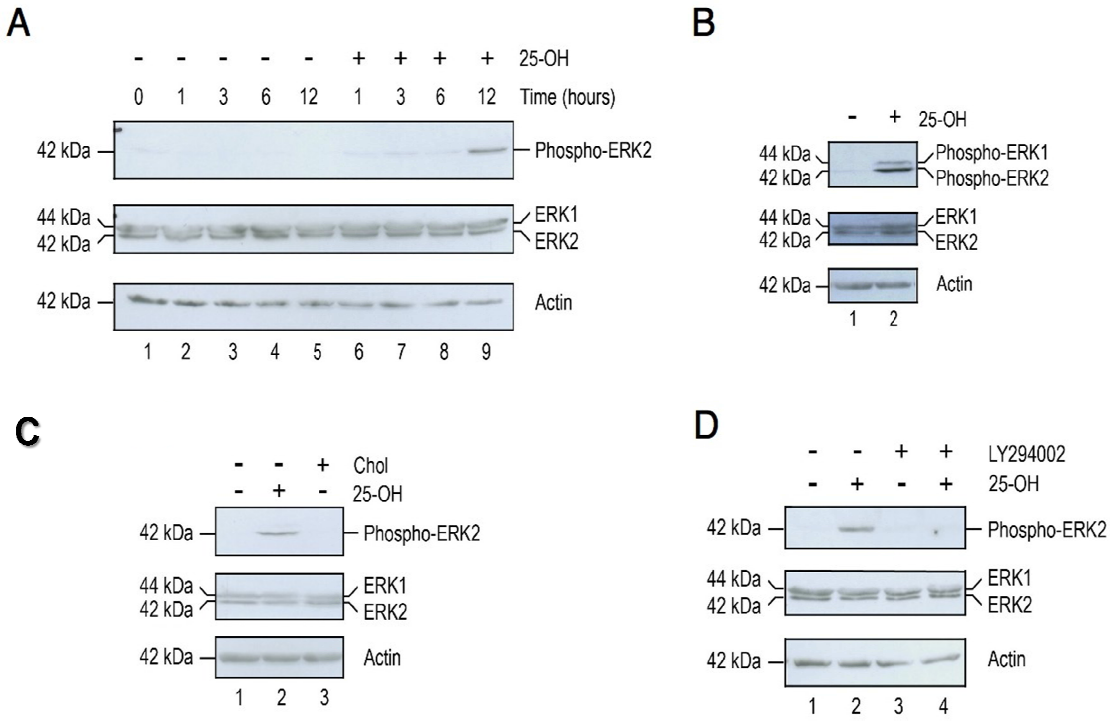

Figure 13: 25-OH activates ERK in a PI3K-dependent manner in RPE. Panel A: ARPE-19 cells were cultured in the presence or absence of 25-OH (20 µg/mL) for 0, 1, 3, 6 and 12 hours. Panels B and C: ARPE-19 cells were incubated in the presence or absence of 25-OH (20 µg/mL) or cholesterol (20 µg/mL) (*panel C*) for 12 hours. Panel D: ARPE-19 cells were incubated in the presence or absence of 25-OH (20 µg/mL) and LY294002 (10 µM), a PI3K inhibitor, for 12 hours. Levels of endogenous phospho-ERK and total ERK were detected by Western Blot using polyclonal antibodies.

3.5. 25-OH increases IL-8 production in RPE in a PI3K- and ERK-dependent manner

The data presented so far suggest that 25-OH up-regulates IL-8 and activates ERK in a PI3K-dependent manner in RPE. To further test the causal relationship between PI3K and ERK activation and up-regulation of IL-8 in response to 25-OH, we evaluated the effect of PI3K and ERK inhibitors on IL-8 production after 25-OH treatment.

As shown in Figure 14A, 25-OH up-regulated IL-8 production in RPE cells. Remarkably, LY294002, a PI3K inhibitor, was able to prevent the IL-8 up-regulation induced by 25-OH. Even in control cells, LY294002 led to a decrease in IL-8 secretion in RPE cells.

Moreover, blocking the ERK pathway by using different inhibitors of this pathway (U0126 and FR180204) also prevented the enhanced IL-8 production triggered by 25-OH (Figure 14B and 14C). A control blot confirmed the inhibition of ERK phosphorylation by U0126 and FR180204 (Figure 14D).

Taken together, these data strongly suggest that the activation of PI3K and ERK plays a major role in the up-regulation of IL-8 production following 25-OH treatment.

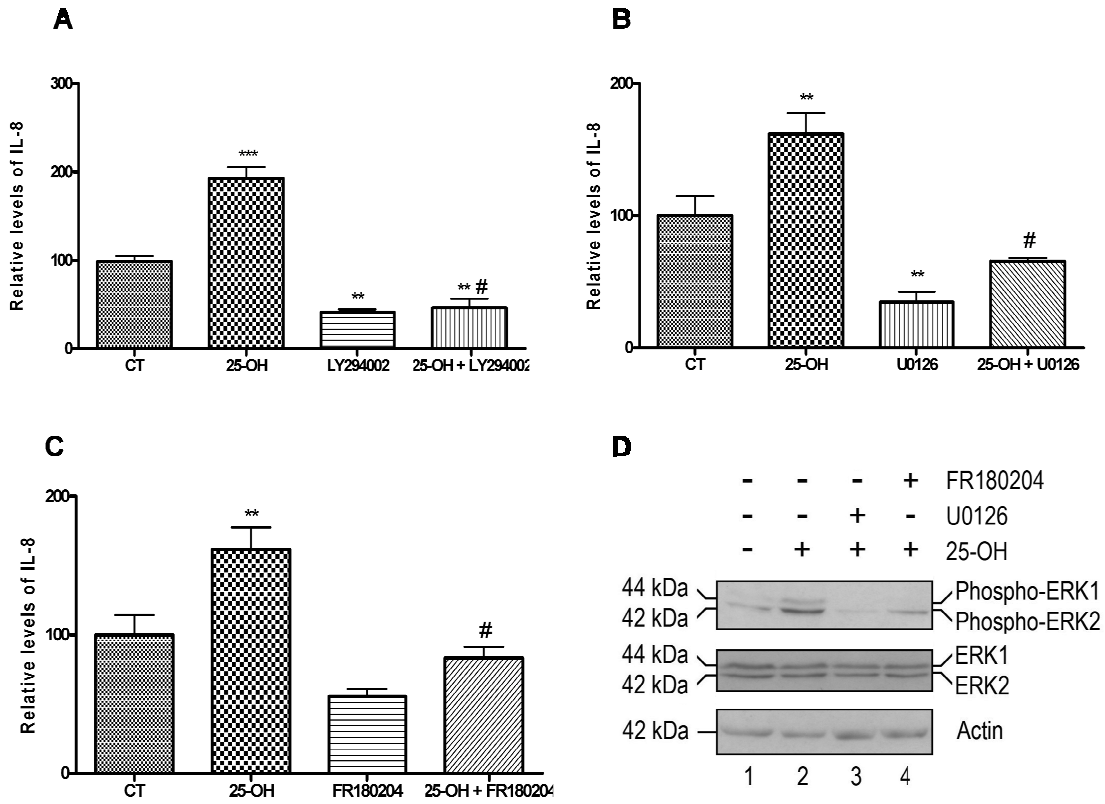

Figure 14: 25-OH-induced IL-8 production is PI3K- and ERK-dependent. ARPE-19 cells were pre-incubated in the presence or absence of LY294002 (*panel A*), U0126 (*panel B*) or FR180204 (*panel C*) 10 µM for 1 hour before the incubation with or without 25-OH (20 µg/mL) for 24 hours. IL-8 protein levels in the medium were determined by ELISA. Results are presented as the mean±S.D of three independent experiments. Panel D: Levels of endogenous phospho-ERK and total ERK were detected by Western Blot using polyclonal antibodies. ***p<0.001 and **p<0.01 as compared with control; #p<0.001 as compared with 25-OH.

4. DISCUSSION

AMD is the main cause of blindness in the elderly population (usually over 60 years old) in the western world (13). Although the cause of AMD is currently unknown, it has been suggested that multiple factors, including genetic, nutritional and environmental factors, are involved in the development of this pathology (4; 5; 6; 8; 14; 13). Most of the known risk factors for AMD, including environmental and nutritional factors, appear to have oxidative stress as a common denominator (5; 13; 20; 23).

Recent findings further suggest a role for inflammation in the pathogenesis of AMD (28; 31; 45; 46).

The activation of redox-sensitive transcription factors may be involved in triggering the expression of pro-inflammatory cytokines, such as IL-8, thus providing a link between oxidative stress and inflammation upon aging (21; 47; 48). Further support for this link between oxidative stress and inflammation in AMD comes from a recent study demonstrating that oxidative damage-induced inflammation initiates AMD (122).

However, the molecular mechanism(s) linking oxidation to inflammation in this pathology is still unclear.

We have recently shown that the oxidative inactivation of the proteasome may be a mechanistic link between oxidative stress and increased production of pro-inflammatory cytokines, such as IL-8 (21; 68; 82).

However, the fact that pro-inflammatory cytokines, such as IL-8, can be regulated by both oxidative and non-oxidative mechanisms adds a new layer of complexity in understanding the molecular mechanisms underlying AMD. In fact, we have also demonstrated that A2E, a major fluorophore of lipofuscin, induces the secretion of IL-8 in RPE, even though it does not cause detectable oxidative damage or proteasome inhibition (68).

Oxysterols, or cholesterol oxides, have been shown to increase IL-8 production in several cell types and, thus, have been implicated in many age-related diseases with a strong inflammatory component (110; 111; 117). Moreover, previous reports, including work from our laboratory, have demonstrated an age-related accumulation of oxysterols in ocular tissues, such as the lens and the retina (105; 107; 106).

However, the molecular mechanisms underlying the production of IL-8 under these conditions remain unclear.

The data presented in this thesis show that exposing ARPE-19 cells to 25-OH results in the increase of IL-8 gene expression and secretion (Figure 10). 25-OH also activates PI3K and ERK (Figure 13). Consistent with this observation, inhibiting either one of these pathways prevented the 25-OH-induced IL-8 production in RPE cells (Figure 14). Together, these data indicate that 25-OH up-regulates IL-8 production in RPE cells by a mechanism dependent on PI3K and ERK activation, as illustrated in Figure 15. In this model, 25-OH activates PI3K by a yet unknown mechanism. Activation of PI3K will result in the phosphorylation and consequent activation of ERK. ERK will then recruit a yet unknown transcription factor, which in turn will lead to an increase in IL-8 gene expression.

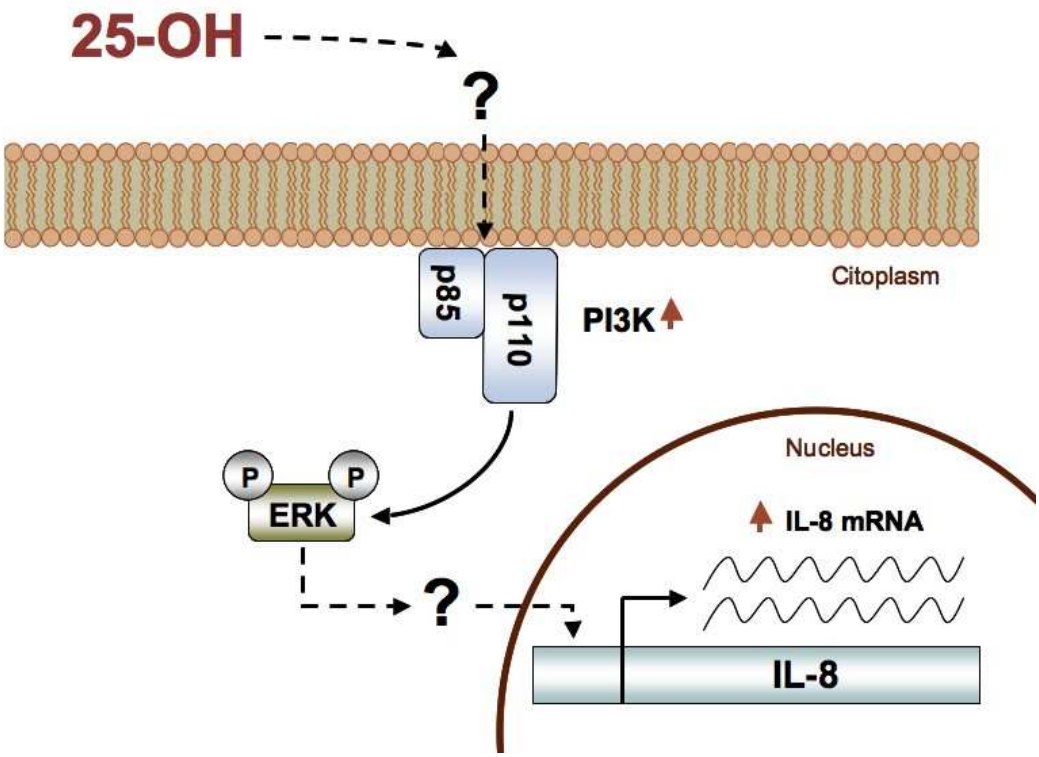

Figure 15: Schematic model of a possible molecular mechanism regulating IL-8 production in response to 25-OH in RPE cells. In this model, 25-OH leads to PI3K activation through a yet unknown mechanism. Once activated, PI3K phosphorylates and consequently activates ERK. Activation of ERK will recruit a yet unknown transcription factor, which in turn will lead to an increase in IL-8 gene expression.

From all the oxysterols tested, 25-OH was the one with the most pronounced effect in terms of IL-8 secretion *(*Figure 10A). These data are consistent with previous

reports that demonstrate that this oxysterol is the most potent inducer of pro-inflammatory gene expression. The fact that the other oxysterols tested (7-KC and 7β-OH) decreased IL-8 levels in RPE cells may be explained by their cytotoxicity. In fact, several reports have consistently shown that these oxysterols are more cytotoxic than 25-OH (110; 111).

This work also demonstrates that the increased IL-8 production in ARPE-19 cells induced by 25-OH is independent of oxidation (Figure 12). This is consistent with a recent report, which demonstrated that addition of vitamin E did not prevent IL-8 production induced by 25-OH in ARPE-19 cells (110).

Furthermore, the present study also indicates that 25-OH does not have an effect on proteasome activity in RPE cells (Figure 11). Together, these results indicate that 25-OH increases IL-8 production in the RPE by a mechanism that does not involve oxidative stress nor inhibition of the proteasome. This is consistent with the existence of non-oxidative mechanisms regulating IL-8 production under stress conditions (68).

The increased IL-8 production in ARPE-19 cells upon 25-OH exposure results from ERK activation. This effect seems to be specific for ERK, as 25-OH did not have any effect on the activation of other MAPKs tested, such as p38 MAPK or JNK. Consistent with this, a recent study has demonstrated a similar effect on macrophages (113).

The mechanism whereby 25-OH activates PI3K and, consequently, ERK activation is still not clear.

A possible candidate to mediate the effects of 25-OH in RPE cells is OSBP (Oxysterol Binding Protein). OSBP are a family of sterol-binding proteins implicated in various cellular processes, such as vesicle transport, lipid metabolism, and signal transduction (123) Interestingly, 25-OH has been shown to be the oxysterol that binds OSBP with the highest affinity (123; 124). Moreover, a recent study has uncovered an important role for OSBP in ERK activation (125). It is therefore possible that OSBP may be mediating the effects of 25-OH in the activation of this signaling pathway.

Another plausible mechanism to explain the effects of 25-OH may be its capacity to interfere with cholesterol homeostasis. In fact, several studies suggest that 25-OH may induce alterations in the lipid rafts in the membranes of cultured cells (98; 110). In addition, cholesterol depletion from the membranes results in the PI3K-mediated ERK activation (126). It is therefore possible that 25-OH exerts its effects by depleting membrane cholesterol.

The transcription factors involved in the enhanced IL-8 production under these conditions remain unknown.

IL-8 gene expression is regulated by several transcription factors, such as NF-κB and AP-1 (61; 127). Preliminary data from our laboratory suggests that NF-κB is not involved in the increased IL-8 production induced by 25-OH, as inhibition of this pathway does not have a significant effect on IL-8 levels under these conditions. This is consistent with several reports that described a minor role for NF-κB in the regulation of IL-8 production upon oxysterol treatment (74).

A plausible candidate mediating IL-8 expression following 25-OH exposure is the transcription factor AP-1. AP-1 has been involved in the regulation of IL-8 expression in several cell types (61; 74; 127). Moreover, a recent study suggests that 25-OH increases IL-8 production in macrophages through AP-1 activation (113).

Another possible candidate responsible for the increased IL-8 production under these conditions is the Liver X receptor (LXR). LXR belong to the nuclear hormone receptor family and some LXR isoforms are activated by oxysterols (110). Furthermore, LXR have been shown to up-regulate IL-8 production in atherosclerosis (110), so it is plausible that they may play a role in the regulation of IL-8 expression in RPE cells following 25-OH exposure (110; 128).

Taken together, the results presented in this thesis elucidate a novel signaling network that leads to overproduction of IL-8 in response to 25-OH. However, further work is required to elucidate the molecular mechanisms underlying the increased IL-8 production under these conditions. This information will not only shed light onto understanding how 25-OH triggers IL-8 production, but also will provide clues how to control IL-8 production in order to reduce inflammation and inflammation-related diseases.

5. REFERENCES

1. **Edward, DP; Kaufman, LM.** Anatomy, development, and physiology of the visual system. *Pediatr Clin N Am.* 2003, Vol. 50, pp. 1-23.

2. **Parier, V; Soubrane, G.** Age-related macular degeneration. *La Revue de médecine interne.* 2008, Vol. 29, pp. 215–223.

3. **Wolff, E.** Wolff's Anatomy of the Eye and Orbit. *Br J Ophthalmol.* 4, 1977, Vol. 61, p. 302.

4. **Jager, RD; Mieler, WF; Miller, JW.** Age-Related Macular Degeneration. *N Engl J Med.* 2008, Vol. 358, pp. 2606-2617.

5. **Chopdar, A; Chakravarthy, U; Verma, D.** Age related macular degeneration. *BMJ.* 2003, Vol. 326, pp. 485-488.

6. **Fine, SL; Berger, JW; Maguire, MG; Ho, AC.** Age-related macular degeneration. *Drug Therapy.* 7, 2000, Vol. 342.

7. **Arden, GB.** Age-related macular degeneration. *Journal of the British Menopause Society.* 2, 2006, Vol. 12, pp. 64-70.

8. **Rattner, A; Nathans, J.** Macular degeneration: recent advances and therapeutic opportunities. *Nature.* 2006, Vol. 7, pp. 860-872.

9. **Burke, JM; Hjelmeland, LM.** Tissue Mosaicism in the RPE. *Molecular Interventions.* 4, 2005, Vol. 5.

10. **Strauss, O.** The Retinal Pigment Epithelium in Visual Function. *Physiol Rev.* 2005, Vol. 85, pp. 845-881.

11. **Nowak, JZ.** Age-related macular degeneration (AMD): pathogenesis and therapy. *Pharmacological Reports.* 2006, Vol. 58, pp. 353-363.

12. **Neelam, K; Nolan, J; Chakravarthy, U; Beatty, S.** Psychophysical Function in Age-related Maculopathy. *Surv Ophthalmol.* 2, 2009, Vol. 54, pp. 167--210.

13. **Leeuwen, RV;Klaver, CCW ; Vingerling, JR ; Hofman, A.** Epidemiology of age-related maculopathy: a review. *European Journal of Epidemiology.* 2003, Vol. 18, pp. 845–854.

14. **Boulton, M; Różanowska, M; Wess, T.** Ageing of the retinal pigment epithelium: implications for transplantation. *Graefe's Arch Clin Exp Ophthalmol.* 2004, Vol. 242, pp. 76–84.

15. **Nolan, JM; Stack, J; O' Donovan, O; Loane, E; Beatty, S.** Risk factors for age-related maculopathy are associated with a relative lack of macular pigment. *Experimental Eye Research.* 2007, Vol. 84, pp. 61-74.

16. **Midena, E; Angeli, CD; Blarzino, MC; Valenti, M; Segato, T.** Macular Function Impairment in Eyes With Early Age-Related Macular Degeneration. *Investigative Ophthalmology & Visual Science.* 2, 1997, Vol. 38.

17. **Haddad, S; Chen, CA; Santangelo, SL; Seddon, JM.** The Genetics of Age-Related Macular Degeneration: A Review of Progress to Date. *Survey of ophtalmology.* 4, 2006, Vol. 51.

18. **Moshfeghi, DM.** Age-related macular degeneration:Evaluation and treatment. *Cleveland Clinic Journal of Medicine.* 12, 2003, Vol. 70, pp. 1017-1037.

19. **Bird, AC.** Towards an understanding of age-related macular disease. *Eye.* 2003, Vol. 17, pp. 457–466.

20. **Qin, S.** Oxidative Damage of Retinal Pigment Epithelial Cells and Age-Related Macular Degeneration. *Drug development research.* 2007, Vol. 68, pp. 213–225.

21. **Fernandes, AF; Pereira, P.** Oxidative Stress, Inflammation and Age-Related Macular Degeneration: a Bizarre Love Triangle. *Exp. Ophthalmol.* 1, 2007, Vol. 33, pp. 1-8.

22. **Olinski, R; Siomek, A; Rozalski, R; Gackowski, D; Foksinski, M; Guz, J; Dziaman, T; Szpila, A; Tudek, B.** Oxidative damage to DNA and antioxidant status in aging and age-related diseases. *Acta Biochimica Polonica.* 1, 2007, Vol. 54, pp. 11–26.

23. **Cai, J; Nelson, KC; Wu, M; Sternberg Jr, P; Jones, DP.** Oxidative Damage and Protection of the RPE. *Progress in Retinal and Eye Research.* 2, 2000, Vol. 19, pp. 205-221.

24. **Droege, W.** Free Radicals in the Physiological Control of Cell Function. *Physiol Rev.* 2002, Vol. 82, pp. 47-95.

25. **Winkler, BS; Boulton, ME; Gottsch, JD; Sternberg, P.** Oxidative damage and age-related macular degeneration. *Mol Vis.* Vol. 5, pp. 1-32.

26. **Decanini, A; Nordgaard, CL; Feng, X; Ferrington, DA; Olsen, TW.** Changes in Select Redox Proteins of the Retinal Pigment Epithelium in Age-related Macular Degeneration. *Am J Ophthalmol.* 2007, Vol. 143, pp. 607–615.

27. **Zarbin, MA.** Current Concepts in the Pathogenesis of Age-Related Macular Degeneration. *Arch Ophthalmol.* 2004, Vol. 122, pp. 598-614.

28. **Liang, F; Godley, BF.** Oxidative stress-induced mitochondrial DNA damage in human retinal pigment epithelial cells: a possible mechanism for RPE aging and age-related macular degeneration. *Experimental Eye Research.* 2003, Vol. 76, pp. 397–403.

29. **Klein, R; Peto, T; Bird, A; Vannewkirk, M.** The Epidemiology of Age-Related Macular Degeneration. *Am J Ophthalmol.* 2004, Vol. 137, pp. 486-495.

30. **Feigl, B.** Age-related maculopathy – Linking aetiology and pathophysiological changes to the ischaemia hypothesis. *Progress in Retinal and Eye Research.* 2009, Vol. 28, pp. 63-86.

31. **Beatty, S; Koh, H; Henson, D; Boulton, M.** The Role of Oxidative Stress in the Pathogenesis of Age-Related Macular Degeneration. *Surv Ophthalmol 45.* 2000, Vol. 45, pp. 115–134.

32. **Sparrow, JR; Boulton, M.** RPE lipofuscin and its role in retinal pathobiology. *Experimental Eye Research.* 2005, Vol. 80, pp. 595–606.

33. **Sparrow, JR; Fishkin, N;Zhou, J; Cai , B; Jang, YP; Krane, S; Itagaki, Y; Nakanishi, K.** A2E, a byproduct of the visual cycle. *Vision Research.* 2003, Vol. 43, pp. 2983–2990.

34. **Sparrow, JR; Vollmer-Snarr, HR; Zhou, J; Jang, YP; Jockusch, S.** A2E-epoxides Damage DNA in Retinal Pigment Epithelial Cells. *The Journal of Biological Chemistry.* 20, 2003, Vol. 278, pp. 18207–18213.

35. **Gray, DA; Woulfe, J.** Lipofuscin and Aging: A Matter of Toxic Waste. *Sci. Aging Knowl. Environ.* 2005, pp. 1-5.

36. **Verhasselt, V; Goldman, M; Willems, F.** Oxidative stress up-regulates IL-8 and TNF-a synthesis by human dendritic cells. *Eur. J. Immunol.* 1998, Vol. 28, pp. 3886–3890.

37. **Higgins,GT; Wang, JH; Dockery, P; Cleary, PE; Redmond, HP.** Induction of angiogenic cytokine expression in cultured RPE by ingestion of oxidized photoreceptor outer segments. *Invest Ophthalmol Vis Sci.* 2003, Vol. 44, pp. 1775–1782.

38. **Kannan, R; Zhang, N; Sreekumar, PG; Spee, CK; Rodriguez, A; Barron, E; Hinton, DR.** Stimulation of apical and basolateral VEGF-A and VEGF-C secretion by oxidative stress in polarized retinal pigment epithelial cells. *Mol Vis.* 2006, Vol. 12, pp. 1649-1659.

39. **Boulton, M; Dayhaw-Barker, P.** The role of the retinal pigment epithelium: topographical variation and ageing changes. *Eye.* 2001, Vol. 15, pp. 384-389.

40. **Sundelin, SP; Nilsson, SE; Brunk, UT.** Liposfuscin-formation in cultured retinal pigment epithelial cells is related to their melanin content. *Free Radical Biology & Medicine.* 1, 2001, Vol. 30, pp. 74–81.

41. **Snodderly, DM.** Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *Am J Clin Nutr.* 1995, Vol. 62, pp. 1448-1461.

42. **Imamura, Y; Noda, S; Hashizume, K; Shinoda, K; Yamaguchi, M; Uchiyama, S; Shimizu, T; Mizushima, Y; Shirasawa, T; Tsubota, K.** Drusen, choroidal neovascularization, and retinal pigment epithelium dysfunction in SOD1-deficient mice: A model of age-related macular degeneration. *PNAS.* 30, 2006, Vol. 103, pp. 11282–11287 .

43. **Dong, X; Patel, M; Chan, C.** Molecular pathology of age-related macular degeneration. *Progress in Retinal and Eye Research.* 2009, Vol. 28, pp. 1–18.

44. **Vasto, S; Candore, G; Balistreri, CR; Caruso, M; Colonna-Romano, G; Grimaldi, MP; Listi, F; Nuzzo, D; Lio, D; Caruso, C.** Inflammatory networks in ageing, age-related diseases and longevity. *Mechanisms of Ageing and Development.* 2007, Vol. 128, pp. 83–91.

45. **McGeer, EG; Klegeris, A; McGeer, PL.** Inflammation, the complement system and the diseases of aging. *Neurobiology of Aging.* 2005, Vol. 26, pp. 94–97.

46. **Anderson, DH; Mullins, RF; Hageman, GS.** A Role for Local Inflammation in the Formation of Drusen in the Aging Eye. *Am J Ophthalmol.* 2002, Vol. 134, pp. 411-431.

47. **Chung, HY; Cesari, M; Anton, S; Marzetti, E; Giovannini, S; Seo, AY; Carter, C; Yu, BP; Leeuwenburgh, C.** Molecular inflammation: Underpinnings of aging and age-related diseases. *Ageing Research Reviews.* 2009, Vol. 8, pp. 18–30.

48. **Sarkar, D; Fisher, PB.** Molecular mechanisms of aging-associated inflammation. *Cancer Letters.* 2006, Vol. 236, pp. 13–23.

49. **Klein, RJ; Zeiss, C;Chew, EY; Tsai, J; Sackler, RS ; Haynes, C.; Henning, AK; SanGiovanni, JP; Mane, SM; Mayne, ST; Bracken, MB; Ferris, FL;Jott, J; Barnstable, C; Hoh, J.** Complement Factor H Polymorphism in Age-Related Macular Degeneration. *Science.* 2005, Vol. 308, pp. 385–389.

50. **Patel, M; Chan, C.** Immunopathological aspects of age-related macular degeneration. *Semin Immunopathol.* 2, 2008, Vol. 30, pp. 97–110.

51. **Kijlstra, A; La Heij, EC; Hendrikse, F.** Immunological Factors in the Pathogenesis and Treatment of Age-Related Macular Degeneration. *Ocular Immunology & Inflammation.* 1, Vol. 13, pp. 3 — 11.

52. **Mullins, RF; Russel, SR; Anderson, DH; Hageman, GS.** Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis,elastosis, amyloidosis, and dense deposit disease. *Faseb J.* Vol. 14, pp. 835–846.

53. **Hageman, GS; Luthert, PJ; Chong, VN; Johnson, LV; Anderson, DH; Mullins, RF.** An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. *Prog Retin Eye Res.* 6, 2001, Vol. 20, pp. 705-732.

54. **Johnson, LV; Ozaki, S; Staples, M; Erickson, PA; Anderson, DH.** A Potential Role for Immune Complex Pathogenesis in Drusen Formation. *Exp. Eye Res.* 2000, Vol. 70, pp. 441-449.

55. **Donoso, LA; Kim, D; Frost, A; Callahan, A; Hageman, G.** The Role of Inflammation in the Pathogenesis of Age-related Macular Degeneration. *Survey of ophtalmology.* 2, 2006, Vol. 51, pp. 137-152.

56. **Rodrigues, EB.** Inflammation in Dry Age-Related Macular Degeneration. *Ophthalmologica.* 2007, Vol. 221, pp. 143–152 .

57. **Haines, JL.** Complement Factor H Variant Increases the Risk of Age-Related Macular Degeneration. *Science.* 2005, Vol. 308, pp. 419-421.

58. **Shen, D; Tuo, J; Patel, M; Herzlich, AA; Ding, X; Chew, EY; Chan, C.** Chlamydia pneumoniae infection, complement factor H variants, and age-related macular degeneration. *Br J Ophthalmol. 2009 Mar;.* 3, 2009, Vol. 93, pp. 405-408.

59. **Zhou, J; Jang, YP; Kim, SR; Sparrow, JR.** Complement activation by photooxidation products of A2E, a lipofuscin constituent of the retinal pigment epithelium. *Proc Natl Acad Sci USA.* 2006, Vol. 103, pp. 16182-16187.

60. **Brat, DJ; Bellail, AC; Van Meir, EG.** The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro-Oncology.* 2005, Vol. 7, pp. 122–133.

61. **DeForge, LE; LauPreston, AM; Takeuchi, E; Kenney, J; Boxer, LA; Remick, DG.** Regulation of Interleukin 8 Gene Expression by Oxidant Stress. *The journal of biological chemistry.* 34, 1993, Vol. 268, pp. 25568-25576.

62. **Kalayoglu, MV; Bula, D; Arroyo, J; Gragoudas, ES; D'Amico, D; Miller, JW.** Identification of Chlamydia pneumoniae within human choroidal neovascular

membranes secondary to age-related macular degeneration. *Graefes Arch Clin Exp Ophthalmol.* 11, 2005, Vol. 243, pp. 1080-90.

63. **Forrester, JV.** Macrophages eyed in macular degeneration. *Nature medicine.* 11, 2003, Vol. 9, pp. 1350-1351.

64. **Ambati, J; Ambati, BK; Yoo, SH; Ianchulev, S ; Adamis, AP.** Age-Related Macular Degeneration: Etiology, Pathogenesis, and Therapeutic Strategies. *Survey of ophthalmology.* 3, 2003, Vol. 48, pp. 257–293.

65. **Bulteau, AL; Lundberg, KC; Humphries, KM; Sadek, HA; Szweda, PA; Friguet, B; Szweda, LI.** Oxidative modification and inactivation of the proteasome during coronary occlusion/reperfusion. *J Biol Chem.* 32, 2001, Vol. 276, pp. 30057-30063.

66. **Zhang, X; Zhou, J; Fernandes, AF; Sparrow, JR; Pereira, P; Taylor, A; Shang, F.** The proteasome: a target of oxidative damage in cultured human retina pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 8, 2008, Vol. 49, pp. 3622-3630.

67. **Alkalay, I; Yaron, A; Hatzubai, A; Oriani, A; Ciechanover, A; Ben-Neriah, Y.** Stimulation-dependent IkBa phosphorylation marks the NF-kB inhibitor for degradation via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci.* Vol. 92, pp. 10599-10603.

68. **Fernandes, AF; Zhou, J; Zhang, X; Sparrow, JR; Taylor, A ; Pereira, P; Shang, F.** Oxidative inactivation of the proteasome in RPE: A potencial link between oxidative stress and upregulation of IL-8. *J Biol Chem.* 30, 2008, Vol. 283, pp. 20745-20753.

69. **Tanaka, K; Kawakami, T; Tateishi, K; Yashiroda, H ; Chiba, T;.** Control of IjBα proteolysis by the ubiquitin-proteasome pathway. *Biochimie.* 2001, Vol. 83, p. 351−356.

70. **Ciechanover, A.** The ubiquitin proteolytic system and pathogenesis of human diseases: a novel platform for mechanism-based drug targeting. *Biochemical Society Transactions.* 2003, Vol. 31, pp. 474-481.

71. **Ethen, CM; Hussong, SA; Reilly, C; Feng, X; Olsen, TW; Ferrington, DA.** Transformation of the proteasome with age-related macular degeneration. *Febs Letters.* 2007, Vol. 581, pp. 885–890.

72. **Fernandes, AF; Guo, W; Zhang, X; Gallagher, M; Ivan, M; Taylor, A; Pereira, P; Shang, F.** Proteasome-dependent regulation of signal transduction in retinal pigment epithelial cells. *Experimental Eye Research.* 2006, Vol. 83, pp. 1472-1481.

73. **Caballero, M; Liton, PB; Epstein, DL; Gonzalez, P.** Proteasome inhibition by chronic oxidative stress in human trabecular meshwork cells. *Biochemical and Biophysical Research Communications.* 2003, Vol. 308, pp. 346–352.

74. **Joshi-Barve, S; Barve, SS; Butt, W; Klein, J; McClain, CJ.** Inhibition of Proteasome Function Leads to NF-kB Independent IL-8 Expression in Human Hepatocytes. *Hepatology.* 2003, Vol. 38, pp. 1178-1187.

75. **Ishii,T; Sakurai,T; Usami, H; Uchida, K.** Oxidative Modification of Proteasome: Identification of an Oxidation-Sensitive Subunit in 26 S Proteasome. *Biochemistry.* 42, 2005, Vol. 44, pp. 13893-13901.

76. **Conconi, M; Petropoulos, I; Emod, I; Turlin, E; Biville, F; Friguet, B.** Protection from oxidative inactivation of the 20S proteasome by heat-shock protein . *Biochem J.* Pt 2, 1998, Vol. 333, pp. 407-415.

77. **Wenger, RH.** Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. *FASEB J.* 10, 2002, Vol. 16, pp. 1151-1162.

78. **Ivan, M; Kondo, K; Yang, H; Kim, W; Valiando, J ; Ohh, M; Salic, A ; Asara, JM ; Lane, WS; Kaelin, WG.** HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science.* 5516, 2001, Vol. 292, pp. 464-468.

79. **Jaakkola, P; Mole, DR; Tian, YM; Wilson, MI; Gielbert, J; Gaskell, SJ; Kriegsheim, A; Hebestreit, HF.** Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science.* 5516, 2001, Vol. 292, pp. 468-472.

80. **Masson, N; Willam, C; Maxwell, PH; Pugh, CW; Ratcliffe, PJ.** Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *EMBO J.* 18, 2001, Vol. 20, pp. 5197-5206.

81. **Wu, M; Bian, Q; Liu, Y; Fernandes, AF; Taylor, A; Pereira, P; Shang, F.** Sustained oxidative stress inhibits NF-κB activation partially via inactivating. *Free Radical Biology & Medicine.* 2009, Vol. 46, pp. 62-69.

82. **Fernandes, AF, et al.** Proteasome inactivation promotes p38 MAPK-dependent- PI3K and increase IL-8 production in retinal pigment epithelial cells. *Mol Biol Cell.* 2009.

83. **Kaul, D.** Molecular link between cholesterol, cytokines and atherosclerosis. *Molecular and Cellular Biochemistry.* 2001, Vol. 219, pp. 65–71.

84. **Westover, EJ; Covey, DF; Brockman, HL; Brown, RE; Pike, LJ.** Cholesterol Depletion Results in Site-specific Increases in Epidermal Growth Factor Receptor Phosphorylation due to Membrane Level Effects. *The journal of Biological Chemistry.* 51, 2003, Vol. 278, pp. 51125–51133.

85. **Róg, T; Pasenkiewicz-Gierula, M; Vattulainen, I; Karttunen, M.** Ordering effects of cholesterol and its analogues. *Biochim Biophys Acta.* 1, 2009, Vol. 1788, pp. 97-121.

86. **Chen, X; Resh, MD.** Cholesterol Depletion from the Plasma Membrane Triggers Ligand-independent Activation of the Epidermal Growth Factor Receptor. *J Biol Chem.* 51, 2002, Vol. 277, pp. 49631-49637.

87. **Pike, LJ; Miller, JM.** Cholesterol Depletion Delocalizes Phosphatidylinositol Bisphosphate and Inhibits Hormone-stimulated Phosphatidylinositol Turnover. *J Biol Chem.* 35, 1998, Vol. 273, pp. 22298-22304.

88. **Edidin, M.** The state of lipid rafts: from model membranes to cells. *Annu Rev Biophys Biomol Struct.* 2003, Vol. 32, pp. 257-83.

89. **Maxfield, FR; Tabas, I.** Role of cholesterol and lipid organization in disease. *Nature.* 2005, Vol. 438, pp. 612-621.

90. **Ho, YK; Brown, S; Bilheimer, DW; Goldstein, JL.** Regulation of cholesterol synthesis by low density lipoproteins in isolated human lymphocytes. *J Clin Invest.* 6, 1976, Vol. 58, pp. 1465-74.

91. **Maxfield, Frederick R.; Tabas, Ira.** Role of cholesterol and lipid organization in disease. *Nature.* 2005, Vol. 438, pp. 612-621.

92. **Vejux, A; Malvitte, L; Lizard, G.** Side effects of oxysterols: cytotoxicity, oxidation, inflammation, and phospholipidosis. *Brazilian journal of medical and biologial research.* 2008, Vol. 41, pp. 545-556.

93. **Erridge, C; Webb, DJ; Spickett, CM.** 25-Hydroxycholesterol, 7-hydroxycholesterol and 7-ketocholesterol upregulate interleukin-8 expression independently of Toll-like receptor 1, 2, 4 or 6 signalling in human macrophages. *Free Radical Research.* 3, 2007, Vol. 41, pp. 260-266.

94. **Björkhem, I; Diczfalusy, U.** Oxysterols: Friends, Foes, or Just Fellow Passengers? *Arterioscler Thromb Vasc Biol.* 2002, Vol. 22, pp. 734-742.

95. **Leonarduzzi, G; Sottero, B; Poli, G.** Oxidized products of cholesterol: dietary and metabolic origin, and proatherosclerotic effects. *Journal of Nutritional Biochemistry.* 2002, Vol. 13, pp. 700 –710.

96. **Lemaire-Ewing, S; Prunet, S; Montage, T; Vejux, A; Berthier, A; Bess'ede, G; Corcos, L; Gambert, P; N'eel, D; Lizard, G.** Comparison of the cytotoxic, pro-oxidant and pro-inflammatory characteristics of different oxysterols. *Cell Biology and Toxicology.* 2005, Vol. 21, pp. 97–114.

97. **Schroepfer, GJ.** Oxysterols: Modulators of Cholesterol Metabolism and Other Processes. *Physiological reviews.* 1, 2000, Vol. 80.

98. **Björkhem, I.** Do oxysterols control cholesterol homeostasis? *J. Clin. Invest.* 2002, Vol. 110, pp. 725–730 .

99. **Christ, M; Luu, B; Mejia, JE; Moosbrugger, I; Bischof, P.** Apoptosis induced by oxysterols in murine lymphoma cells and in normal thymocytes. *Immunology.* 1993, Vol. 78, pp. 455-460.

100. **Yin, J; Chaufour, X; McLachlan, C; McGuire, M; White, G; King, N; Hambly, B.** Apoptosis of vascular smooth muscle cells induced by cholesterol and its oxides in vitro and in vivo. *Atherosclerosis.* 2, 2000, Vol. 148, pp. 365-74.

101. **Girão, H; Pereira, P; Ramalho, J; Quinlan, R; Prescott, A.** Cholesterol oxides mediated changes in cytoskeletal organisation involves Rho GTPases. *Experimental Cell Research.* 2003, Vol. 291, pp. 502–513.

102. **Girão, H; Shang, F; Pereira, P.** 7-Ketocholesterol stimulates differentiation of lens epithelial cells. *Molecular Vision.* 2003, Vol. 9, pp. 497-501.

103. **Wielkoszyński, T; Gawron, K; Strzelczyk, J; Bodzek, P; Zalewska-Ziob, M; Trapp, G; Srebniak, M; Wiczkowski, A.** Cellular toxicity of oxycholesterols. *Bioessays.* 4, 2006, Vol. 28, pp. 387-398.

104. **Olkkonen, VM ; Hynynen, R.** Interactions of oxysterols with membranes and proteins. *Mol Aspects Med.* 3, 2009, Vol. 30, pp. 123-33.

105. **Girão, H; Mota, MC; Ramalho, J; Pereira, P.** Cholesterol Oxides Accumulate in Human Cataracts. *Exp Eye Res.* 1998, Vol. 66, pp. 645-652.

106. **Curcio, CA; Presley, JB; Malek, G; Medeiros, NE; Avery, DV; Kruth, HS.** Esterified and unesterified cholesterol in drusen and basal deposits of eyes with age-related maculopathy. *Experimental Eye Research.* 2005, Vol. 81, pp. 731–741.

107. **Curcio, CA; Millican, CL; Bailey, T ; Kruth, HS.** Accumulation of Cholesterol with Age in Human Bruch's Membrane. *Invest Ophthalmol Vis Sci.* 2001, Vol. 42, pp. 265-274.

108. **Brown, AJ; Jessup, W.** Oxysterols and atherosclerosis. *Atherosclerosis.* 1999, Vol. 142, pp. 1-28.

109. **Lordan, S; Mackrillb, JJ; O'Brien, NM.** Oxysterols and mechanisms of apoptotic signaling: implications in the pathology of degenerative diseases. *Journal of Nutritional Biochemistry.* 2009, Vol. 20, pp. 321–336.

110. **Joffre, C; Leclère, L; Buteau, B; Martine, L; Cabaret, S; Malvitte, L; Acar, N; Lizard, G; Bron, A; Creuzot-Garcher, C; Bretillon, L.** Oxysterols Induced Inflammation and Oxidation in Primary Porcine Retinal Pigment Epithelial Cells. *Current Eye Research.* 3, Vol. 32, pp. 271-280.

111. **Rydberg, EK; Salomonsson, L; Hultén, LM; Norén, K; Bondjers, G; Wiklund, O; Björnheden, T; Ohlsson, BG.** Hypoxia increases 25-hydroxycholesterol-induced interleukin-8 protein secretion in human macrophages. *Atherosclerosis.* 2, 2003, Vol. 170, pp. 245-252.

112. **Wang, N; Tabas, I; Winchester, R; Ravalli, S; Rabbani, LE; Tall, A.** Interleukin 8 Is Induced by Cholesterol Loading of Macrophages and Expressed by Macrophage Foam Cells in Human Atheroma. *The journal of biological chemistry.* 15, 1996, Vol. 271, pp. 8837–8842.

113. **Lemaire-Ewing, S; Berthier, A; Royer, MC; Logette, E; Corcos, L; Bouchot, A; Monier, S; Prunet, C; Raveneau, M; Rébé, C; Desrumaux, C; Lizard, G; Néel, D.** 7β-Hydroxycholesterol and 25-hydroxycholesterol-induced interleukin-8 secretion involves a calcium-dependent activation of c-fos via the ERK1/2 signaling pathway in THP-1 cells. *Cell Biol Toxicol.* 2, 2009, Vol. 25, pp. 127-139.

114. **Javitt, NB.** Oxysterols: Novel biologic roles for the 21st century. *Steroids.* 2008, Vol. 73, pp. 149–157.

115. **Dunn, KC; Aotaki-Keen, E; Pukey, FR; Hjelmeland, LM.** ARPE-19, A Human Retinal Pigment Epithelial Cell Line with Differentiated Properties. *Exp. Eye Res.* 1996, Vol. 62, pp. 155-169.

116. **Lee, DH; Goldberg, AL.** Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in Saccharomyces cerevisiae. *J Biol Chem.* 1996, Vol. 271, pp. 27280-27284.

117. **Vejux, A; Lizard, G.** Cytotoxic effects of oxysterols associated with human diseases: Induction of cell death (apoptosis and/or oncosis), oxidative and inflammatory activities, and phospholipidosis. *Molecular Aspects of Medicine.* 3, 2009, Vol. 30, pp. 153-170.

118. **Stressgen Heat Shock, Products.** Assay Designs. [Online] http://www.assaydesigns.com/ccpa1048-3229-3229-mg-132--proteasome-inhibitor.htm.

119. **Cho, N; Staszczak; Rogalski, J.** Effect of Inhibition of Proteasome–Mediated Proteolysis on Ligninolytic Activities of White–Rot Fungi. *J. Fac. Agr.* 2, 2008, Vol. 53, pp. 399–404.

120. **Raman, M; Chen, W; Cobb, MH.** Differential regulation and properties of MAPKs. *Oncogene.* 2007, Vol. 26, pp. 3100–3112.

121. **Chen, X ; Resh, MD.** Activation of mitogen-activated protein kinase by membrane-targeted Raf chimeras is independent of raft localization. *J Biol Chem.* 37, 2001, Vol. 276, pp. 34617-34623.

122. **Hollyfield, JG; Bonilha, VL; Rayborn, ME; Yang, X; Shadrach, KG; Lu, L; Ufret, RL; Salomon, RG; Perez, VL.** Oxidative damage-induced inflammation initiates age-related macular degeneration. *Nat Med.* 2008, pp. 194-198.

123. **Lehto, M; Olkkonen, VM.** The OSBP-related proteins: a novel protein family involved in vesicle transport, cellular lipid metabolism, and cell signalling. *Biochimica et Biophysica Acta.* 2003, Vol. 1631, pp. 1 – 11.

124. **Massey, JB.** Membrane and protein interactions of oxysterols. *Curr Opin Lipidol.* 2006, Vol. 17, pp. 296–301.

125. **Wang, P ; Weng, J; Anderson, RG.** OSBP Is a Cholesterol-Regulated Scaffolding Protein in Control of ERK1/2 Activation. *Science.* Vol. 307.

126. **Kandutsch, AA; Chen, HW; Shown, EP.** Binding of 25-hydroxycholesterol and cholesterol to different cytoplasmic proteins. *Proc Natl Acad Sci U S A.* 6, 1977, Vol. 74, pp. 2500-2503.

127. **Roebuck, KA.** Regulation of Interleukin-8 Gene Expression. *Journal of interferon and cytokine research.* 1999, Vol. 19, pp. 429-438.

128. **Malvitte, L; Montange, T; Joffre, C; Vejux, A; Maïza, C; Bron, A; Creuzot-Garcher, C; Lizard, G.** Analogies between atherosclerosis and age-related maculopathy: expected roles of oxysterols. *J. Fr. Ophtalmol.* 5, 2006, Vol. 29, pp. 570-578.

129. **Yu, AL; Lorenz, RL; Haritoglou, C ; Kampik, A; Welge-Lussen, U.** Biological effects of native and oxidized low-density lipoproteins in cultured human retinal pigment epithelial cells. *Experimental Eye Research.* 2009, Vol. 88, pp. 495–503.

130. **Hoffmann, E; Dittrich-Breiholz, O; Holtmann, H; Kracht, M.** Multiple control of interleukin-8 gene expression. *J. Leukoc. Biol.* 2002, Vol. 72, pp. 847–855.

131. **Finkel, T; Holbrook, NJ.** Oxidants, oxidative stress and the biology of ageing. *Nature.* 2000, Vol. 408.

132. **Wu, H; Wen, H; Lin, W.** Proteasome Inhibitors Stimulate Interleukin-8 Expression via Ras and Apoptosis Signal-Regulating Kinase-dependent Extracellular Signal-Related Kinase and c-Jun N-Terminal Kinase Activation. *Am. J. Respir. Cell Mol. Biol.* 2002, Vol. 27, pp. 234–243.

133. **Wu, H; Chib, K; Lin, W.** Proteasome inhibitors stimulate activator protein-1 pathway via reactive oxygen species production. *Febs Letters.* 2002, Vol. 526, pp. 101-105.

134. **Joseph, A; Li, Y; Koo, H; Davis, JM; Pollack, S; Kazzaz, JA.** Superoxide dismutase attenuates hyperoxia-induced interleukin-8 induction via AP-1. *Free Radical Biology & Medicine.* 2008, Vol. 45, pp. 1143–1149.

135. **Emerald coast eye, institute.** Emerald coast eye institute. [Online] http://emeraldeye.com/images/illustrations/eye.jpg.

136. **National Eye Institute.** National Institutes of Health. [Online] http://www.nei.nih.gov.

137. **Meusser, B; Hirsch, C; Jarosch, E; Sommer, T.** ERAD: the long road to destruction. *Nat Cell Biol.* 2005, Vol. 7, pp. 766-772.