Oxidative stress-related damage of retinal pigment epithelial cells

- possible protective properties of
autophagocytosed iron-binding proteins

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Linköping 2014

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The cover picture illustrates confluent ARPE-19 cells in culture.
Per Lagman designed the cover and the figures on page 10 and 69.

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Printed in Sweden by LiU-Tryck, Linköping, Sweden, 2014

ISBN 978-91-7519-209-3

ISSN 0345-0082

To my family

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ABSTRACT

Oxidative stress is a major pathogenic factor in the development of age-related macular degeneration (AMD), which is the most common cause of severe central visual impairment in the elderly population in the western world.

It is believed that the degenerative process starts in the retinal pigment epithelium (RPE). The post-mitotic RPE is a single layer of pigmented cells located behind the photoreceptors – rods and cones – of the retina. Daily, these cells phagocytose and recycle the expended tips of the photoreceptor outer segments. This heavy phagocytic burden leads to substantial oxidative stress in the cells, which is further enhanced by intense illumination and a high oxygen tension. A hallmark of early AMD is a progressive build-up of the non-degradable age pigment lipofuscin (LF) in lysosomes of the RPE. LF accumulation hampers phagocytosis and autophagy in the RPE, resulting in increased amounts of cellular debris in and around the cells. This decreases the function and viability of both RPE cells and photoreceptors.

Iron is known to accumulate in the retina with increasing age, particularly in AMD-affected eyes, and amplifies oxidative stress by acting as a potent catalyst in the generation of hydroxyl radicals. These highly reactive radicals contribute to LF formation and may, if abundantly present, also directly damage lysosomal membranes. The subsequent leakage of degrading enzymes to the cytosol initiates cell death via apoptosis or necrosis.

In this thesis, we have investigated the oxidative stress response of human RPE (ARPE-19) cells compared to murine J774 cells, another type of lysosome-rich cells with a high phagocytic capacity. The ARPE-19 cells were found to be extremely resistant to oxidative stress and tolerated exposure to single doses of H_2O_2 in concentrations up to 150 times higher than the J774 cells before lysosomal rupture and ensuing cell death occurred. This resistance was increased even further when the cells were protected with a potent iron chelator that prevents redox-active iron to participate in hydroxyl radical generation. Both cell lines were shown to be equally effective in degrading H_2O_2 and seem to contain comparable amounts of total as well as intralysosomal iron.

Therefore, we reasoned that the insensitivity of ARPE-19 cells to H_2O_2 exposure might be related to a mechanism which keeps their intralysosomal iron bound in a non redox-active form. This theory was supported by our finding of very high basal expression levels of metallothionein (MT), heat shock-protein 70 (HSP70) and ferritin (FT) in ARPE-19 cells compared to J774 cells. All of these

proteins have previously been shown to possess potent iron-binding properties. The ARPE-19 cells were also shown to have a higher basal rate of autophagy. SiRNA-mediated attenuation of MT, HSP70 and FT levels in the ARPE-19 cells resulted, to some degree, in an increased sensitivity to H₂O₂ treatment. Furthermore, a human cell stress array showed several other stress-related proteins to be up-regulated in ARPE-19 cells.

Additionally, we have evaluated the commonly used, but frequently misinterpreted, H₂DCF test for oxidative stress. It was demonstrated that oxidation of H₂DCF into fluorescent DCF mainly reflects relocation to the cytosol of lysosomal iron and mitochondrial cytochrome *c*, rather than being the result of some poorly defined “general” oxidative stress.

In conclusion, our results indicate that the extreme resistance to oxidative stress exhibited by the ARPE-19 cells might be related to a high continuous autophagic influx of iron-binding proteins into the lysosomal compartment. Before being degraded, such proteins will temporarily keep intralysosomal iron bound in a non redox-active form, thereby inhibiting hydroxyl radical formation. This may partly explain why RPE cells, in spite of their exposed location and heavy burden of phagocytosis, usually manage to survive and evade significant LF accumulation until late in life.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

- I.** Kurz T, Karlsson M, Brunk UT, Nilsson SE, Frennesson C. ARPE-19 retinal pigment epithelial cells are highly resistant to oxidative stress and exercise strict control over their lysosomal redox-active iron. *Autophagy*, 2009; 5(4):494-501.
- II.** Karlsson M, Kurz T, Brunk UT, Nilsson SE, Frennesson C. What does the commonly used DCF test for oxidative stress really show? *Biochem J*, 2010; 428(2):183-90.
- III.** Karlsson M, Frennesson C, Gustafsson T, Brunk UT, Nilsson SE, Kurz T. Autophagy of iron-binding proteins may contribute to the oxidative stress resistance of ARPE-19 cells. *Exp Eye Res*, 2013; 116:359-65.
- IV.** Karlsson M and Kurz T. Attenuation of iron-binding proteins in ARPE-19 cells reduces their resistance to oxidative stress. Manuscript.

ABBREVIATIONS

AMD	Age-related macular degeneration
AO	Acridine orange
ARM	Age-related maculopathy
BRB	Blood-retinal barrier
CA-9	Carbonic anhydrase IX
CMA	Chaperone-mediated autophagy
CS	Control siRNA
DCF	2', 7'-dichlorofluorescein
FAC	Ferric ammonium citrate
FRS	Free radical scavengers
FT	Ferritin
FTH	Ferritin heavy chain
FTL	Ferritin light chain
H ₂ DCF	Dihydro-dichlorofluorescein
H ₂ DCF-DA	Dihydro-dichlorofluorescein diacetate
HBSS	Hank's balanced salt solution
hfrPE	Human fetal retinal pigment epithelium
HPV	Human papilloma virus
HSP70	Heat shock-protein 70
LC3	Microtubule-associated protein 1 light chain 3
LF	Lipofuscin
LMP	Lysosomal membrane permeabilization
MMP	Mitochondrial membrane permeabilization
MSDH	<i>O</i> -methylserine dodecylamide hydrochloride

MT	Metallothionein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2 <i>H</i> -tetrazolium bromide
OCT	Optical coherence tomography
OS	Oxidative stress
PBS	Phosphate buffered saline
PEDF	Pigment epithelial-derived factor
POS	Photo-receptor outer segments
PUFA	Polyunsaturated fatty acid
RISC	RNAi-induced silencing complex
RNAi	RNA interference
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
SIH	Salicylaldehyde isonicotinoyl hydrazone
SiRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
TMRE	Tetramethylrhodamine ethyl ester
TRX-1	Thioredoxin-1
VEGF	Vascular endothelial growth factor

INTRODUCTION

Age-related macular degeneration (AMD) is a leading cause of legal blindness in the elderly population of developed countries [1-3]. However, not known in detail, the pathogenesis of this common disease is multi-factorial with a combination of genetic, environmental, inflammatory and oxidative components partaking in the process. The development of AMD is believed to start in the retinal pigment epithelium (RPE), which in advanced stages of the disease eventually will succumb, leading to consequential degeneration and atrophy of the light-sensitive photoreceptors in the neuroretina, particularly in its most central part – the macula – causing irreversible central vision loss. This thesis focuses on investigating the protective role of iron-binding proteins in the prevention of oxidative stress-related damage to cultured RPE and what implications this might have on AMD development.

The normal macula

The macula, or macula lutea (from latin, meaning “yellow spot”) is located at the center of the retina, slightly temporal to the optic nerve head. In the middle of the macular area lies the fovea, containing the densest concentration of light-sensitive photoreceptors within the retina. Measuring 1.5-2 mm in diameter, the fovea is responsible for the high-resolution central visual acuity needed for fine detail work, reading and face recognition. Although the macular region comprises a mere 4% of the total retinal area, it accounts for the majority of useful photopic vision. Posterior to the neuroretina lies the RPE, discussed in further detail below, which is separated from the highly vascularized choroid by the five-layered Bruch’s membrane (Figure 1).

Figure 1. Layers of the retina. Several cell types are present in the retina and its surrounding tissues. The present study focuses on the retinal pigment epithelium (RPE), which is located posterior to the light-sensitive photoreceptors. Bruch's membrane separates the RPE from the capillaries of the choroid. Note how the RPE cells envelope the outer segment tips of the photoreceptors.

Age-related macular degeneration

Prevalence and risk factors

AMD presently affects more than 50 million people worldwide. In Scandinavia, approximately 187,000 individuals suffer from severe AMD-related visual impairment. The prevalence is expected to increase dramatically over the next decades as a consequence of a rapidly growing ageing population [4]. By the year 2040, it is estimated that the global number of people with AMD will exceed 280 million [5]. As the name implies, the main risk factor for AMD development is ageing. While signs of AMD are rare in individuals younger than 50 years of age, several studies have indicated that up to one third of the population over the age of 75 show clinical hallmarks of various stages of the disease. Late stage AMD with severely affected visual acuity is present in up to 10% of subjects over 80 years [2, 3, 6, 7]. Apart from ageing, several other risk factors such as smoking, a positive family history of AMD, hypertension, obesity, previous cataract surgery and hyperopia have also been consistently identified [8-10]. There are several genetic variations associated with AMD development, where single nucleotide polymorphisms in the ARMS2/HTRA1 gene and the gene coding for factor H of the alternative complement pathway currently are considered to be the strongest genetic risk factors [10-13].

Classification

In a commonly used classification system, the term age-related maculopathy (ARM) is used for the disease, further subdivided into early and late ARM [14]. Early ARM is always dry and accounts for the majority of cases. Patients with early ARM are typically asymptomatic with mild or no visual impairment and disease progression is usually slow. Yearly, approximately 4% of individuals with early ARM progress to the more advanced stage [15], i.e. late ARM, which - according to the classification - is equal to age-related macular degeneration (AMD). Early ARM presents clinically with pigmentary changes and/or drusen formation in the macular area (Figure 2B). Drusen are seen ophthalmoscopically as small yellowish dots and are made up of extracellular deposits of undegraded proteins, lipids and the age pigment lipofuscin [16-18]. They are located between the basal lamina of the RPE and the inner collagenous layer of Bruch's membrane. Morphologically, drusen are classified as hard or soft. Hard drusen typically have sharp edges and a diameter $< 63 \mu\text{m}$ and are considered harmful only if they are abundantly present. Soft drusen, on the other hand, are more indistinctly delineated with a size exceeding $63 \mu\text{m}$. They tend to become confluent over time and are more related to progression into manifest AMD than hard

ones [19]. AMD is further subdivided into the dry form geographic atrophy and the wet form neovascular AMD.

Geographic atrophy constitutes the smaller group of patients with severe AMD-related vision loss, the neovascular group being twice as common [6]. The gradual deterioration of the RPE with ensuing death of the overlying photoreceptors and underlying choriocapillary layer results in the formation of local areas with atrophic retina, predominantly in the perifoveal region in the initial stages. Over the years, these patches become larger and more numerous. Eventually, they merge and spread out over the entire central macular region, exhibiting the typical pattern of geographic atrophy (Figure 2C) which is the end-stage of dry AMD. Since the most central part of the macula – the fovea – is often spared until late in the degenerative process, visual acuity may be remarkably preserved for a long time even though the clinical appearance of the lesions may be advanced. Currently, no approved curative treatment for atrophic AMD is available, but nutritional supplements such as anti-oxidants (vitamin C and E, lutein and zeaxanthin), zinc and omega-3 fatty acids have been suggested to exert, to some degree, a protective effect against AMD progression [20-23]. New therapies, e. g. stem cell-based treatments to replace degenerated RPE cells, and pharmacological treatments, such as fenretinide, are currently being investigated and seem promising for future treatment of geographic atrophy, although many obstacles yet remain to be solved [24, 25].

Neovascular (exudative, wet) AMD is characterized by the development of choroidal neovascularization. Vascular endothelial growth factor (VEGF), derived from choroidal fibroblasts, macrophages and RPE cells, stimulates the proliferation of new blood vessels that eventually extend through Bruch's membrane into the sub-retinal space [26]. These vessels are fragile and easily leak or break, causing the typical clinical signs with macular edema, intraretinal hemorrhages and lipid exudates, as well as RPE detachment (Figure 2D). The progression is rapid, sometimes with an acute onset, and common symptoms include distortion of lines (metamorphopsia) and a central scotoma. If left untreated, a fibrotic disciform scar will eventually form in the macula resulting in devastating, irreversible damage to central vision. Although it accounts for only 15% of all ARM/AMD cases, neovascular AMD has up until recently been responsible for up to 90% of severe AMD-related visual loss [27]. However, new therapeutic possibilities with intravitreally injected anti-angiogenic drugs targeting VEGF have revolutionized the treatment outcome, thereby reducing this proportion substantially [28-31].

Figure 2. Examples of a normal macula, early ARM and the two forms of AMD. Images of the eye fundus and corresponding OCT (optical coherence tomography) cross-sections of the macula are shown. (A) Normal macula. (B) Early ARM with soft drusen in the macular region. Note the corresponding irregularities at the RPE level of the OCT image. (C) Severe geographic atrophy exhibiting central loss of the RPE and degeneration of the photoreceptor layer. (D) Neovascular AMD displaying typical signs, such as haemorrhages, pigment epithelial detachment and subretinal fluid.

The retinal pigment epithelium

The RPE originates embryologically from the neuroectoderm and makes up a single layer of richly melanin-pigmented cells located between the light-sensitive photoreceptors of the neuroretina and the capillary bed of the choroid. The RPE cells are densely adherent to one another in a hexagonal pattern with their basal portion attached to a basal lamina that constitutes the innermost layer of Bruch's membrane. Tight junctions joining the cells laterally form the outer blood-retinal barrier (BRB), hindering free diffusion of ions and large molecules between the blood vessels of the choroid and the neuroretina [32]. The apical part of each RPE cell exhibits villous processes that envelope the tips of up to 30 photoreceptor outer segments (POS). The number of photoreceptors cared for per RPE cell is highest in the foveal region and decreases somewhat towards the peripheral retina [33]. The dark pigmentation of the RPE originates from melanin-filled granules (melanosomes) located predominantly in the apical portion of the cells [34].

The RPE is one of the metabolically most active tissues in the body and is responsible for maintaining the survival and functionality of the photoreceptors [35]. Apart from managing the controlled transport of nutrients, water and metabolites through the BRB, RPE cells also play a central role in the metabolism and recycling of retinal, which is derived from retinol (vitamin A) [36]. Retinal is a key component of the visual pigment rhodopsin which, upon light exposure, initiates the phototransductive process in the photoreceptors [37]. Furthermore, the RPE performs immunoregulatory tasks as well as synthesis and secretion of growth factors, such as pigment epithelial-derived factor (PEDF) and VEGF, which at normal levels participate in maintaining the structure and function of the neuroretina and choriocapillary endothelium [36].

Another important function of the RPE cells in the maintenance of photoreceptor health is their remarkable capacity for phagocytosis of worn-out POS material. Daily, up to 15% of the membranous outer segment disks are shed from the distal end of the outer segments, while new stacks are constantly added from their basal side [38, 39]. This process follows a light-dependent circadian rhythm, where the rods discard their disks in the morning and the cones at nightfall [40, 41]. Outer segment tips that have been expended are taken up into the RPE by a process known as heterophagy (phagocytosis). Phagosomes engulf the POS and transport them to the basal side of the cell, where they fuse with pre-existing lysosomes. Degrading enzymes in the resulting phagolysosome then begin a digestive process where retinoids, polyunsaturated fatty acids (PUFAs) and amino acids are being recycled and returned to the photoreceptors [42].

It is estimated that each RPE cell during a life-time will phagocytose around three hundred million outer segment disks [17]. The uptake and degradation of so much oxidized, lipid-rich material makes up a massive metabolic challenge for the endolysosomal system of the RPE cells, particularly considering that they, unlike most other cells with a high phagocytic capacity, are post-mitotic and essentially never get replaced. The number of RPE cells gradually declines with increasing age, forcing the remaining ones to stretch out, thereby even further increasing the number of photoreceptors each cell has to care for [17, 43].

General concepts of AMD pathogenesis

The etiology of AMD development is complex, multi-factorial and not yet completely understood. Chronic oxidative stress, inflammatory activity and genetic predispositions are all strongly associated with AMD pathogenesis. Since the present work focuses primarily on the oxidative stress-related components of the disease, particularly the iron-mediated ones, these will be discussed in more detail later on. Initially, a general overview of the different important biological pathways leading to AMD is given. There is consensus that AMD development starts with a progressive dysfunction and subsequent degeneration of the RPE.

Oxidative stress-related injury

The environment in which the RPE resides is rather unfavorable, in particular for a cell type that is supposed to last for a whole lifespan. In addition to their massive burden of phagocytosing POS, RPE cells also have to cope with an abundant light influx and exposure to one of the highest oxygen concentrations in the body [44]. These are all sources for substantial chronic oxidative stress [45]. With increasing age, the lysosomes of the RPE fail to completely degrade all of the ingested oxidized photoreceptor material, leading to iron-mediated formation and accumulation of the non-degradable age pigment lipofuscin (LF) inside the lysosomal compartment. Once formed, LF can neither be degraded by lysosomal enzymes, nor be transported out of the cell via exocytosis. Since the post-mitotic RPE cells are unable to dilute their contents by division, a gradual LF build-up takes place intralysosomally. In older individuals, LF may occupy up to 25% of the RPE cell volume, significantly limiting the room for the normal cellular machinery [46, 47]. Oxidative processes are involved in LF formation. However, LF by itself also further sensitizes RPE cells to various kinds of oxidative stress. This will be more profoundly discussed in later sections, as will the important role of redox-active iron in the oxidative process due to its capacity for catalyzing the formation of highly toxic hydroxyl radicals.

Structural changes in Bruch's membrane

When LF-containing RPE cells decline in number and function, LF and other undegraded waste material build up underneath the cells forming basal laminar and linear deposits, which eventually may evolve into drusen [47]. An age-related thickening of Bruch's membrane also takes place due to accumulation of lipids and collagen, which impairs the transport of fluids and nutrients to the retina from the choroid, thereby inflicting even more stress to the already strained RPE cells [48]. There is a correlation between the amount of debris accumulated in Bruch's membrane and LF load of the RPE [49].

Inflammation

Chronic inflammation has been linked to many degenerative disorders including atherosclerosis, AMD, Parkinson's and Alzheimer's diseases [50]. Several findings indicate the presence of inflammatory activation in AMD. For example, histological studies have found macrophages and dendritic cells to be present in choroid, retina and Bruch's membrane, and drusen are known to contain many pro-inflammatory cells and markers, such as acute phase proteins and complement components [51, 52]. As noted above, the main genetic polymorphisms associated with AMD are found in genes regulating inflammation, particularly in the gene coding for complement factor H, which acts as an inhibitor of the alternative complement cascade [11]. Oxidatively stressed RPE cells initiate activation of the alternative pathway of the complement system, which ultimately forms cytolytic membrane attack complexes that promote cell death. Furthermore, complement factors C3A and C5A are involved in the development of neovascularization in exudative AMD since they induce RPE secretion of VEGF and act as potent chemotactic attractors for macrophages to the choroid [53]. Apart from stimulating even more VEGF production, macrophages also secrete enzymes that break up a passage in Bruch's membrane through which the proliferating vessels may enter the subretinal space [54, 55]. Immunohistochemical studies have shown VEGF and inflammatory cells, including macrophages, to be abundantly present in subfoveal fibrovascular membranes of patients with exudative AMD [56, 57].

Additionally, the role of the ribonuclease DICER1 as governor of RPE health and function via several mechanisms, including inflammation, has gained much interest in recent years. For example, a dramatic reduction of DICER1 levels has been found in RPE cells of patients with geographic atrophy. This was accompanied by an intracellular over-abundance of noxious *Alu* RNA, normally cleaved enzymatically by DICER1 [58]. In the same study, knockdown of DICER1 expression was shown to induce retinal degeneration in a mouse model. *Alu* RNA

toxicity is mediated via the NLRP3 inflammasome, which, when activated, induces an inflammatory cascade which eventually may lead to cell death [59]. Interestingly, the NLRP3 inflammasome may also be triggered by several other factors, e. g. drusen material, oxidative stress and lysosomal rupture [42].

Lysosomes

Lysosomes are ubiquitous, membrane-bound organelles present in the cytosol of virtually all eukaryotic cell types (except erythrocytes). Described first in the 1950's, a discovery that led to the Nobel Prize for Christian de Duve, they comprise the major intracellular digestive system [60]. The lysosomal vesicles, limited by a single phospholipid bilayer, are very heterogeneous and differ substantially in shape and size both within and in-between cells [61]. ATP-dependent proton pumps situated in the lysosomal membrane maintain an acidic environment intralysosomally with pH 4-5, as opposed to the slightly basic pH of the cytosol [62, 63]. This acidic interior provides the necessary conditions for optimal function of the more than 50 hydrolytic enzymes contained intralysosomally (including proteases, lipases, peptidases, phosphatases, nucleases, glycosidases and sulfatases). These enzymes are responsible for the degradation and recycling of all the material entering the cell via phagocytosis, as well as for the autophagic turnover of worn-out intracellular organelles and long-lived proteins [64]. Following digestion within the lysosome, the amino acids and other degradation products diffuse or are actively transported into the cytosol for reutilization [65].

The lysosomal enzymes are produced in the endoplasmatic reticulum and mature within the Golgi apparatus, from which they then are transported in secretory vesicles. These vesicles release their content into late endosomes, forming a mature lysosome, which then may fuse with phagosomes (containing e.g. phagocytosed POS), autophagosomes or other endosomes [66]. There is a continuous delivery to the lysosomes of more substrates bound for degradation from either the inside or the outside of the cell, as well as of newly synthesized degrading enzymes from the trans-Golgi network. By constantly fusing and dividing, the mature lysosomes allow their content to be distributed throughout the whole lysosomal compartment [67, 68].

Autophagy

Unlike heterophagy, where material is entering the cell from the outside, autophagy is a strictly intracellular event. A well-functioning clearance and recycling of worn-out organelles and macromolecules is crucial for the function and vitality of all cells. However, in long-lived post-mitotic cells, such as neurons, cardiac myocytes and RPE cells, this is of particular importance in order to avoid progressive accumulation of defective mitochondria, lipofuscin and aggregate-prone malfunctioning proteins. Short-lived proteins are degraded mainly by proteasomes after being tagged for destruction by ubiquitin, whereas all organelles and larger, long-lived proteins are digested within the lysosomal compartment in a process called autophagocytosis, or autophagy (from greek, meaning “self-eating”).

Autophagy is commonly sub-divided into three major groups: macro-, micro- and chaperone-mediated autophagy [69]. The objective of all three variants is the same, namely to get the substrate bound for degradation into the lysosome, but they differ in mechanism, regulation and selectivity. In macroautophagy, which is the best characterized and probably most important form, cytosolic organelles and proteins are enveloped by a double membrane-bounded vacuole, creating an autophagosome. This will then fuse with a late endosome or lysosome, in which the degradation process takes place [70]. Macroautophagy was long considered to be a random process, engulfing and recycling parts of the cytoplasmic content in a non-selective manner. There is, however, growing evidence indicating that a more specific regulation may sometimes also be involved, where organelles are “tagged” for destruction by marker proteins in order to be recognized and autophagocytosed [71, 72]. Microautophagy, on the other hand, occurs when the lysosome itself sequesters small portions of cytoplasm through invagination of the lysosomal membrane. Once pinched off inside the lumen of the lysosome, the vacuole is degraded [73]. The third variant, chaperone-mediated autophagy (CMA) is much more specific than the other two forms. In CMA, cytosolic chaperones (e.g. HSP73) selectively bind the target protein in the cytoplasm and direct it towards the lysosomal membrane. There the substrate/chaperone-complex docks with the membrane-bound receptor protein LAMP-2A for further transport into the lysosomal lumen [74]. The principles of endocytosis and autophagy are outlined in Figure 3.

Figure 3. Schematic representation of endocytosis and the different autophagic pathways. Extracellular material enters the cell via endocytosis by invagination of the plasma membrane and formation of an early endosome, which then evolves into a late endosome. Lysosomal enzymes are transported from the trans-Golgi network and released in late endosomes that mature to lysosomes. In macroautophagy, cytosolic proteins and organelles such as mitochondria are surrounded by a phagophore, thereby creating an autophagosome. This may fuse with a lysosome or a late endosome. During microautophagy, small portions of the cytosol are invaginated by the lysosomal membrane. Molecular chaperones, e.g. Hsp73, bind to proteins tagged for degradation and transport them to the lysosome (chaperone-mediated autophagy).

Many stressors such as inflammation, oxidative stress or exposure to toxic compounds may induce increased reparative autophagy as a means to replace altered and malfunctioning structures. Autophagy is also stimulated by starvation where it helps the cell to survive by degrading less important cytosolic compounds in order to provide new building blocks for the more vital functions [71]. The metabolically active RPE cells have been shown to exhibit a high basal rate of autophagy [42, 75, 76] that seems to become less effective with ageing [77]. There is increasing evidence that disturbed autophagy is involved in AMD pathogenesis and RPE damage [42, 78, 79]. Suppression of lysosomal function, as seen during increasing LF accumulation, leads to impaired autophagic clearance

of damaged intracellular content. In a futile attempt to degrade the non-degradable LF, much of the newly synthesized lysosomal enzymes intended for autophagic use, is directed to LF-containing lysosomes instead of to autophagolysosomes [80, 81]. The resulting build-up of dysfunctional mitochondria and aggregated proteins may further aggravate cellular oxidative stress with ensuing lysosomal rupture and inflammatory response [42]. Moreover, a decreased rate of autophagy may also be involved in drusen formation [79].

Iron

Iron is the most abundant trace element in the human body with a total amount of approximately 2.5-4.5 g in an average adult [82]. It is essential for our survival due to its participation in vital physiological functions, such as oxygen transport and mitochondrial respiration. In contrast to most other trace elements, the majority of iron in mammals is found in the blood stream where it comprises the central component of the oxygen-carrying hemoglobin. Other examples of iron-containing metalloproteins are myoglobin in muscle tissue, cytochrome *c* in mitochondria and enzymes needed for cell proliferation. Iron homeostasis is a very tightly controlled procedure. Apart from bleeding and shedding of dead cells, the body lacks a regulatory mechanism for iron excretion. Since iron is always bound and transported in larger proteins like transferrin or hemoglobin within the bloodstream, only minute amounts escape to the urine in glomerular filtration. Therefore, since almost all iron is recycled within the body, only a small fraction, about 1-2 mg, of daily dietary iron intake needs to be absorbed from the intestines [82].

“Free”, redox-active iron is highly toxic because of its capacity to catalyze the formation of aggressive hydroxyl radicals. Hence, it is of great importance for all organisms to keep their iron bound within proteins in a non redox-active state. In serum, iron under transport to cells is carried by transferrin. Upon arriving at the plasma membrane, the iron-transferrin complex is delivered to the cell via receptor-mediated endocytosis. In the acidic environment of the late endosome, iron is released and then transported across its membrane to the labile iron pool of the cytosol. Since this iron is in its redox-active form and potentially harmful, it is either rapidly incorporated into iron-containing molecules under construction, or taken up by ferritin complexes for storage [81]. Keeping the labile iron pool to an absolute minimum is a way for the cell to avoid Fenton-type reactions (described below) and ensuing oxidative stress.

Ferritin (FT) is a globular 450 kDa protein made up of 24 subunits of heavy and light chains with a molecular weight of 21 kDa and 19 kDa, respectively. Each FT molecule is capable of binding up to 4,500 iron atoms, stored as ferrihydrite crystals. There is, however, great variability in the iron content of FT, where the largest saturation rate is seen in liver, spleen and bone marrow, which are the major iron-storing organs [83]. Once taken up by FT, ferrous, redox-active iron (Fe^{2+}) is rapidly detoxified through oxidation by ferroxidase into its ferric, non redox-active form (Fe^{3+}), thereby preventing it from participating in oxidative reactions [84]. The mechanisms of iron-mobilization from FT have been much debated. Some advocate a direct release into the cytosol when iron is required for cellular processes [85, 86], while others claim it to be set loose following disassembly of FT within proteasomes [87, 88]. However, there is now substantial evidence for the hypothesis that autophagy of iron-containing FT with subsequent intralysosomal degradation constitutes the main route by which iron is liberated [89-92].

Additionally, since iron-rich mitochondria and metalloproteins are autophagocytosed and digested inside lysosomes, the lysosomal iron levels are higher than in any other type of organelle [93]. Due to the acidic environment within the lysosome, as well as the presence of reducing agents (e.g. glutathione and cysteine), much of the released iron is converted into a redox-active state. While most of this low mass-iron is rapidly transported back to the cytosol for reutilization, some remains loosely bound within the lysosome. Under conditions of oxidative stress, this intralysosomal ferrous iron may catalyze LF formation and/or permeabilization of lysosomal membranes with ensuing cell damage or death [81, 94].

With increasing age, there is an accumulation of iron in the human retina [95]. Interestingly, many observations have indicated an involvement of iron overload in the pathogenesis of AMD. For example, the levels of the iron carrier protein transferrin are up-regulated in AMD patients compared to healthy controls [96] and RPE cells of AMD-affected eyes show an excess of both chelatable and non-chelatable iron [97]. Hereditary iron overload diseases such as hemochromatosis, aceruloplasminemia and Friedreich's ataxia all exhibit retinal degenerations with some AMD-resembling features [98], which has also been demonstrated in a mouse model with RPE iron overload due to deficiency of the iron exporters ceruloplasmin and hephaestin [99].

Oxidative stress

Ever since being defined in the 1980's by Helmut Sies [100], oxidative stress has gained increasing recognition and is now considered to be a major pathogenic factor in many age-related diseases such as Alzheimer's disease [101], atherosclerosis [102] and cancer development [103] among others. In the eye, oxidative stress contributes to the development of many common chronic ophthalmic disorders including AMD, cataract, open-angle glaucoma, diabetic retinopathy, uveitis and ocular surface disorders [104]. The term 'oxidative stress' refers to an imbalance between the cell's anti-oxidant defense system and the intracellular amount of harmful reactive oxygen species (ROS), which are always present in the cell to some degree. Oxidative stress can be induced by many stimuli and conditions. Depending on cell type, environment and age, it may arise from endogenously produced ROS, or be inflicted upon the cell from external sources, such as cigarette smoke, pollutants or irradiation [105, 106]. In most cases, the oxidatively damaged cellular components are repaired or degraded and replaced with newly synthesized ones (see Autophagy-section above). However, as age progresses, this equilibrium shifts towards the pro-oxidative side of the scale. In post-mitotic cells, this largely depends on a decline in autophagic clearance and build-up of cellular garbage, including LF, which then in turn further amplifies ROS production [74]. While limited amounts of oxidative stress often stimulate cell replication, a little more will result in DNA damage, growth arrest and reparative autophagy. Finally, as will be more thoroughly discussed below, moderate or advanced oxidative stress may result in permeabilization of lysosomes with release of their content to the cytosol and subsequent apoptosis or necrosis [66].

Oxygen metabolism

Oxygen is of vital importance for almost all organisms since it is required for driving the energy production taking place inside the mitochondria. In this process, known as the electron transport chain, oxygen is reduced to water after a series of redox reactions where electrons are transferred through protein complexes in the inner mitochondrial membrane. The resulting membrane potential generates a flow of protons through the membrane-bound enzyme ATP synthase, powering the phosphorylation of ADP to ATP, which is the main energy currency of cell metabolism [107]. The absolute majority of oxygen entering the mitochondria is combusted to H₂O in a controlled manner inside the protein complexes. However, due to unavoidable leakage of electrons from other redox centers in the respiratory chain, a small fraction of the oxygen is only partially reduced, leading to the formation of potentially harmful ROS [72].

Reactive oxygen species and free radicals

Reactive oxygen species (ROS) constitute a diverse group of relatively unstable molecules with two common features: They are derived from oxygen (O_2) and are very prone to interact with other molecules due to their potent oxidizing properties. ROS are generally classified in two groups – non-radical and radical species (also known as “free radicals”). Free radicals are characterized by the presence of one or more unpaired electrons in their outer orbital. Because of these odd electrons, they are much more reactive than non-radical ROS, and usually have very short half-lives [108].

Although there are many kinds of differently generated ROS, only the ones relevant for the present work will be discussed herein. These include superoxide anion, hydrogen peroxide, hydroxyl radicals and singlet oxygen. As shown in Figure 4, the reduction of O_2 to H_2O occurs in a stepwise manner where electrons are transferred one at a time. In the first step, a superoxide anion ($O_2^{\cdot-}$) is formed, which is the most abundantly present intracellular ROS. Even though it classifies as a free radical, it is not the most potent one and does not possess enough reactivity to cause harm to other macromolecules apart from some sensitive enzymes [104]. On the other hand, it is a precursor to other, more aggressive ROS, and also acts as a reducing agent for ferric iron (Fe^{3+}) into its redox-active state (Fe^{2+}) [109]. The main source for $O_2^{\cdot-}$ formation is the accidental escape of electrons from the electron transport chain in mitochondria. It is estimated that about 1% of the oxygen used in the mitochondria leaks out in the form of superoxide radicals. In older individuals, however, this proportion is larger [47].

Figure 4. *The stepwise reduction of oxygen in the last step of mitochondrial respiration.* Electrons are accepted one at a time, the first step generating $O_2^{\cdot-}$, which then dismutates into H_2O_2 . Addition of another electron and a proton (H^+), produces a hydroxyl radical ($HO^{\cdot}$). In the final step, water is formed.

Usually, $O_2^{\bullet -}$ rapidly dismutates into hydrogen peroxide (H_2O_2), either spontaneously or through the enzymatic action of superoxide dismutase (SOD). H_2O_2 is a non-radical ROS which is uncharged, allowing it to diffuse easily throughout the different cellular compartments. Although most of it quickly gets degraded and transformed into water, mainly by the enzymatical action of catalases and peroxidases [72], a low, physiological level of H_2O_2 is always present, serving an important function as signaling molecule in the regulation of cytosolic redox-activity [110]. However, under conditions of oxidative stress when the antioxidant defense system fails to counteract the ROS actions, H_2O_2 may go through homolytical cleaving, resulting in the formation of a hydroxid anion and a highly reactive hydroxyl radical ($HO^{\bullet}$). This decomposition is catalyzed by redox-active iron in the Fenton-type reaction:

Since $O_2^{\bullet -}$ reduces Fe^{3+} back to Fe^{2+} , thereby preparing it for a new round of Fenton chemistry, the net reaction (known as the Haber-Weiss summary reaction) is as follows:

$HO^{\bullet}$ has a half-life of only a nanosecond (10^{-9} s) and is extremely dangerous to all types of molecules in the cell, such as amino acids, sugars, fatty acids, DNA and phospholipids. It is by far the most reactive of the oxygen-derived radicals and will instantaneously after its formation attack and damage whatever structure that is closest by. Hence, whenever iron and H_2O_2 meet at the same place within the cell, the consequences for the surrounding molecules might be

disastrous [106]. Other transition metals, such as copper, are also capable of catalyzing Fenton chemistry. However, since “free” copper, unlike redox-active iron, is virtually non-existent within cells, iron is by far the most important player in this context [111].

Besides the oxidative machinery of mitochondria, there are several other sources for ROS formation within the cell. For example, the NADPH oxidase system in macrophages, in which enzymatically generated superoxide anions and H_2O_2 partakes in the oxidative burst reaction aimed at killing invading microorganisms [112, 113]. Furthermore, $\text{HO}^\bullet$ may also form without the involvement of iron-catalyzation as a result of radiolytic cleavage of water [114] or dissemination of peroxynitrite, which is generated by a reaction between superoxide and nitric oxide inside lysosomes [72]. The beta-oxidation of fatty acids in peroxisomes also contributes to the production of H_2O_2 and $\text{O}_2^{\bullet-}$. Due to their continuous processing of phagocytosed, lipid-containing POS, this mechanism of ROS-generation is probably of particular importance in the RPE, as is the presence of NADPH oxidase in the phagosome [115].

Another particularly destructive oxygen metabolite is singlet oxygen ($^1\text{O}_2$), which is formed through photosensitization reactions. When a molecule with photosensitizing properties (e.g. lipofuscin, riboflavin, retinal) absorbs light of a particular wavelength, it gets converted into an excited state. This increase in energy level can be transferred to an adjacent oxygen molecule, thereby creating singlet oxygen while the photosensitizer returns to its ground state. Similarly to $\text{HO}^\bullet$, singlet oxygen also is highly aggressive and immediately seeks to react with membranes or other cellular components. In medicine, the generation of singlet oxygen through illumination of a photosensitizer is used in the treatment of several proliferative conditions where the deleterious effects of this radical is desirable, including skin cancer, acne and, prior to the anti-VEGF era, also certain variants of neovascular AMD [116, 117].

Lipid peroxidation

As harmful as ROS-mediated damage to DNA and proteins might be, the greatest threat against cellular integrity is lipid peroxidation. It is a complex process, defined as the oxidative deterioration of polyunsaturated fatty acids. Since PUFAs contain multiple double bonds, they are more susceptible to such oxidation than the more saturated ones. The phospholipid layers that constitute the membranes surrounding cells and organelles are rich in PUFA side chains, making them a vulnerable target for lipid peroxidation.

The initial step of the peroxidation process occurs when a potent free radical, such as HO[•] or singlet oxygen, is generated in close proximity to a PUFA-containing structure, for instance a cellular membrane or phagocytosed photoreceptor disks. Due to their high reactivity, the radical immediately removes a hydrogen atom from the PUFA, leading to the formation of water and a peroxy radical. This radical is itself capable of abstracting a hydrogen from another fatty acid, thereby propagating an autocatalytic chain reaction of oxidations, where the peroxy radical turns into a lipid hydro-peroxidase while, simultaneously, a new peroxy radical is generated [118]. Since the reaction between a radical and a non-radical always leads to the formation of another radical, the only way to discontinue this process is when the radical either gets trapped by an anti-oxidant scavenger (e.g. vitamin E), or reacts with another radical to produce a non-radical species [111]. If not terminated fast enough, membranes under attack will be irreparably damaged with ensuing leakage of ions, proteins and other enclosed components.

As mentioned previously, iron can contribute to the lipid peroxidation process through Fenton-mediated HO[•] formation [106]. It may, however, also directly catalyze the decomposition and fragmentation of the generated lipid hydroperoxides, leading to even more radical production and a further amplification of the oxidative chain reaction. Apart from the more direct membrane-damaging effects of lipid peroxidation, its end-products (such as malonaldehyde) also make up some of the building blocks for lipofuscin generation [119].

Lipofuscin

The progressive accumulation of lipofuscin (LF) within the lysosomes of post-mitotic cells is a hallmark of ageing and was described already in 1842 [120]. LF (also known as ceroid or age pigment) is a badly defined polymer built up of protein residues linked together by aldehyde bridges obtained by oxidation of fatty acids. LF has specific chemical and physical properties, but its exact composition varies depending on its source of origin. In addition to proteins, lipids and carbohydrates, this yellowish-brown, autofluorescent compound also contains significant amounts of metals, particularly iron [121]. Although clearly age-related, the mechanisms behind LF formation were not clarified until the 1990's, when Brunk and colleagues were able to elucidate the relationship between oxidative stress, autophagy and lipofuscinogenesis [122].

LF is mainly derived from autophagocytosed macromolecules and organelles that enter the lysosomal compartment for degradation. In the case of RPE cells, the constant influx of PUFAs from phagocytosed POS also acts as a major

contributor in LF formation. As previously pointed out, most of the material entering the acidic lysosomes is degraded by hydrolytic enzymes and returned to the cytosol for re-utilization in anabolic processes. However, some of the molecules will be subjected to peroxidation and polymerization as a result of iron-catalyzed formation of $\text{HO}^\bullet$ from H_2O_2 , which easily enters the lysosomes through diffusion, or is generated intralysosomally in worn-out mitochondria undergoing degradation [72]. These oxidatively modified LF compounds are resistant to the decomposing actions of lysosomal enzymes and will hence build up over time, especially in non-dividing cells [123].

Although LF accumulation in RPE cells is clearly related to AMD development, the exact mechanisms behind this correlation are not known in detail. However, it has been shown that LF build-up hampers both the hetero- and autophagocytic activity of RPE cells, hence decreasing their capacity for degradation of ingested POS material as well as intracellular macromolecules and organelles. This, together with other LF-mediated effects such as protein misfolding and inhibition of mitochondrial respiration, will lead to increased RPE cell damage [26, 119, 124-126]. Moreover, LF sensitizes cells to photo-oxidation [127] and makes lysosomes more susceptible to oxidative injury with ensuing cell death due to release of lysosomal degrading enzymes into the cytosol [128]. Since LF is rich in iron, which catalyzes $\text{HO}^\bullet$ formation, lysosomes loaded with LF may be particularly vulnerable to oxidative stress.

From an oxidative point of view, the environment in which the RPE resides is rather unfavorable. These cells are subjected to life-long exposure to intense light irradiation, a very high oxygen tension, a high metabolic activity as well as increasing amounts of intracellular iron, while daily performing a phagocytic task that is unparalleled in any other post-mitotic cell type. Considering this, it is remarkable that this single cell layer somehow usually manages to evade significant LF accumulation until late in life.

Protective anti-oxidant mechanisms

In order to safeguard their survival, evolution has provided organisms with extensive and elaborate defense systems against oxidative damage. This includes preventive measures to inhibit both the generation and action of ROS, as well as various reparative functions to restore the oxidatively injured structures once harm has been inflicted. The latter mechanism, for instance reparative autophagy and remodeling of damaged proteins, has been mentioned previously and will not be discussed in further detail here.

Enzymatic defense

1. **Superoxide dismutase (SOD)** is a metalloenzyme that accelerates the spontaneous dismutation of $O_2^{\bullet -}$ to H_2O_2 a 1000-fold [129]. Since most $O_2^{\bullet -}$ is formed within mitochondria as a by-product of cellular respiration, SOD is most abundant in this location. However, it is also present in the cytosol where it dismutates $O_2^{\bullet -}$ generated from other sources, such as NADPH oxidase. Increased synthesis of SOD is induced under conditions of oxidative stress [130].
2. **Catalase** is mainly found in peroxisomes where it efficiently catalyzes the decomposition of H_2O_2 , which is generated as a result of β -oxidation of fatty acids. The action of catalase is very potent, one single molecule being capable of converting around 6 million H_2O_2 molecules into water and oxygen gas each minute [106]. Interestingly, catalase levels have been found to be six times higher in RPE cells than in any other ocular tissue, but are significantly decreased in aged or AMD-affected eyes [131].
3. **Glutathione peroxidase**, a selenoenzyme found in the cytosol, catalyzes H_2O_2 degradation into water by using glutathione as a reducing agent [132]. At minor concentrations, most of the generated H_2O_2 in the cell is handled by this enzyme, which is considered to be the major source of protection for low levels of oxidative stress [106].

Free radical scavengers

Once formed, free radicals may be caught or quenched by free radical scavengers (FRS), which then transform them into less aggressive compounds. The water-soluble FRS, such as ascorbic acid (vitamin C) and glutathione, are located in the cytosol where they act as reducing or scavenging agents of singlet oxygen, superoxide and hydroxyl radicals [106]. The most common liposoluble FRS include α -tocopherol (vitamin E) and carotenoids (β -carotene, lutein, zeaxanthin, lycopenes). Being lipophilic, these are bound in cellular membranes, mainly lysosomes and mitochondria. α -tocopherol is one of the most powerful antioxidants in the human body, due to its capability of breaking the autocatalytic chain reaction of lipid peroxidation [133, 134]. The carotenoids act in a similar manner by scavenging peroxy radicals, but they can also quench singlet oxygen [135]. Lutein and zeaxanthin, sometimes referred to as ‘macular pigment’, are ubiquitously present in the macular area where, apart from radical scavenging, their two primary functions are to improve image quality by reducing scattering of incoming light, as well as to absorb potentially harmful blue light [136].

Furthermore, RPE *in vivo* is rich in melanin granules. Melanin primarily acts as an absorbent of incoming photons that have passed the photoreceptor layer, thereby preventing intraocular light scattering that otherwise may reduce visual acuity. In addition, it has also been shown to possess anti-oxidant properties by scavenging free radicals and, possibly, also due to chelation of transition metals such as iron and copper [137, 138].

Iron chelators

Although iron is of vital importance in several life-sustaining processes, such as cellular respiration and oxygen transport in hemoglobin, it also poses a threat because of its capacity to catalyze the generation of HO[•]. Hence, cells have developed ways to keep iron and other transition metals under strict control and bound in non-reactive forms. As pointed out in previous sections, the presence of cytosolic and mitochondrial ferritin (FT) is an extremely effective way to chelate and store iron in a non redox-active state, reducing it to very low levels in these locations [139]. However, apart from FT, several other endogenous intracellular proteins have also been shown to possess strong iron-binding properties, two of them being metallothionein (MT) and heat shock-protein-70 (HSP70) [140, 141]. Under normal conditions, FT is always present to some degree, whereas the levels of MT and HSP70 in unstressed cells are usually low. All three of these iron-binding compounds are so called phase II proteins (or “stress proteins”), meaning that their production is induced by different kinds of stressors.

- FT transcription is controlled by cytosolic iron-regulatory proteins that stimulate production of FT under conditions of increased oxidative stress or raised intracellular iron levels [142, 143].
- HSP70 synthesis goes up dramatically as a response to heat exposure, but also under conditions of oxidative stress and pH changes. In addition to its iron-chelating capabilities, HSP70 also reconstitutes misfolded proteins and prevents their aggregation [144].
- MT is up-regulated by oxidative stress, glucocorticoids and different heavy metals, such as zinc, copper and mercury [145]. It also functions as a free radical scavenger [146].

Since iron accumulation seems to play an important role in the pathogenesis of AMD, it is tempting to assume that addition of exogenous iron chelators to RPE cells would have a protective effect against oxidative stress mediated damage. A recent publication has reported beneficial results of oral treatment with the iron chelator deferiprone in a mouse model where it ameliorated oxidative stress and prevented iron overload-induced retinal degeneration [147]. Moreover, several studies on other cultured cell lines have also shown supplementation with iron chelators to make cells less sensitive to H_2O_2 exposure [148-151]. Systemic iron overload diseases, such as hemochromatosis, have successfully been treated with iron chelators for many years. However, if the iron accumulation is more local, as in AMD, general chelation therapy may be more questionable due to side effects, such as induced iron deficiency, with consequential anemia to name one.

Lysosomal membrane permeabilization

As pointed out above, H_2O_2 has the capacity to escape and diffuse from its main production sites (mitochondria and peroxisomes) and enter other cellular compartments, such as the lysosomal one, especially under conditions of oxidative stress, when the anti-oxidative defense systems get overwhelmed. Inside the lysosomes, none of the H_2O_2 -degrading enzymes are present. There is, however, plenty of free redox-active iron in lysosomes as a result of degradation of iron-containing organelles and macromolecules but also, if present, in lipofuscin. Additionally, the acidic pH and intralysosomal presence of reducing agents, such as cysteine, provides a hospitable environment for Fenton chemistry to take place. Consequently, the conditions for generation of toxic $HO^\bullet$ within lysosomes are optimal [66].

As described previously, a minor, continuous generation of free radicals within lysosomes, contributing to LF formation, is inevitable even under normal conditions. However, if the oxidative stress is enhanced, massive peroxidation of lipids in the lysosomal membrane may result in the lysosomes becoming leaky. Such lysosomal membrane permeabilization (LMP) with subsequent release of the contained proteolytic enzymes, many of which are partly active also at the more neutral pH of the cytosol, may induce cell death either via apoptosis or, in case of a more substantial leakage, lead directly to necrosis of the cell [152].

Apoptosis, or “programmed cell death”, is a very complex procedure that will only be briefly summarized in this context. Many stimuli can induce apoptosis through several pathways, the most important being the extrinsic and the intrinsic ones. The extrinsic pathway is triggered by external stimulation of death receptors on the plasma membrane, e.g. tumor necrosis factors, whereas the intrinsic apoptotic pathway is initiated by internal cell injury, with release of cytochrome *c* from permeabilized mitochondria. Both of these pathways end up with the activation of the effector protein caspase-3. Once activated, this proteolytic enzyme initiates the organized and controlled degradation of the cell [72, 153]. Morphologically, apoptosis is characterized by cell shrinkage, nuclear pyknosis, membrane blebbing and finally fragmentation of the cell into small vesicles, called apoptotic bodies, which are then phagocytosed by neighboring cells or macrophages [93].

Over the last decades, an increasing body of evidence has shown LMP to be an early event in many cases of apoptosis, mainly mediated through the action of released cathepsins [154-156]. These may be involved in the initial stages of both the extrinsic and the intrinsic pathways but may in some cases also directly activate caspases [93]. There appears to be a cross-talk between mitochondria and lysosomes under conditions of oxidative stress-induced apoptosis, where released lysosomal enzymes permeabilize mitochondrial membranes, leading to even more ROS production, which in turn further enhances LMP [72]. In cases where LMP is not the triggering event in the apoptotic process, it is still usually induced by several mechanisms in later stages of the apoptotic process, thereby amplifying the death signal even more [157].

Apoptotic cell death has several advantages for the organism and is crucial for its capability of eliminating cells undergoing malignant transformation. The controlled, “silent” manner in which damaged or diseased cells are removed by apoptosis is a lot less harmful for adjacent cells than the more violent necrotic cell death, where rupture of the plasma membrane and release of cellular contents to the surrounding tissues causes an inflammatory response [93].

Experimental models for AMD

There are many more or less available models for experimental AMD research, all of which have their advantages and drawbacks. In this thesis, we have utilized cultured, immortalized human RPE cells (ARPE-19), the most commonly used cell line for experiments aiming at investigating basic AMD mechanisms. They are commercially available, easy to culture and have retained most of their native characteristics [158]. However, some concerns have been raised as to whether the properties of the ARPE-19 cells may change following multiple passages, and that their active gene profile differs somewhat from the human genome [159]. Additionally, their rate of pigmentation is low.

Human fetal RPE (hfrPE) cells exhibit better coherence with native RPE, including melanogenesis [46], but have a low availability and generally only keep their properties for a limited number of passages. Additionally, many of these primary cells do not survive the isolation procedure or the stress of being moved from a physiological to an *in vitro* environment [160]. In recent years, the expanding field of stem cell research has also provided new possibilities for providing cultured cells exhibiting a highly differentiated RPE phenotype, including good pigmentation. However, these cells are more capable of polarizing and differentiating *in vivo* than under culture conditions [46].

RPE cells from rabbit and other species have been successfully used [126, 161] but, in addition to not being of human origin, they suffer the same drawbacks as hfrPE. Post-mortem RPE cells from human donors with or without AMD do, for obvious reasons, provide excellent conditions for investigating differences between diseased and normal eyes, but do unfortunately have a very low accessibility (at least in Sweden) and, much like other primary cells, tend to de-differentiate after only a few sub-cultivations.

In addition to cultured cells, there are a number of animal models for replicating pathological aspects of AMD. Commonly, genetically modified mice that exhibit some features resembling human AMD lesions are used, e.g. strains with accelerated senescence, silenced genes for superoxide dismutase or complement factor H, or, as mentioned previously, mice lacking the iron exporters ceruloplasmin and hephaestin [162]. Moreover, laser- or growth factor-induced choroidal neovascularization in murine or primate models have largely contributed to the knowledge and treatments of wet AMD [163].

Methods for exposure to oxidative stress

Chronic or acute oxidative stress may be inflicted upon cultured cells in many different ways. One variant is exposure to exogenously added H_2O_2 , either as a single bolus dose or in the form of continuous/repeated supplementations. Since H_2O_2 is quickly degraded by the cells, usually within an hour, prolonged oxidative stress is commonly inflicted by utilization of H_2O_2 -producing enzymes, i.e. glucose oxidase, which continuously generates H_2O_2 by oxidation of glucose in the culture medium, or by incubating cells under hyperoxic condition (40% O_2) [161, 164]. Bolus dose experiments with H_2O_2 are easier to perform, since the set-up of continuous steady-state exposure conditions have proven to be rather complex [165]. Other commonly used methods for administering oxidative stress are irradiation with blue light to generate singlet oxygen [127] or exposure to toxic compounds, such as components of cigarette smoke.

Importantly, some caution is advised when interpreting the results of oxidative stress-related experiments performed on cultured cells, since the short growth and exposure time of only days to weeks does not necessarily correlate to the conditions *in vivo*. This is obviously of particular concern in post-mitotic cells, such as the RPE, which usually withstand conditions of chronic oxidative stress for many years before any significant degenerative alterations are seen. In spite of these reservations, cultured RPE cells may still be an acceptable choice for experimental work on AMD pathogenesis, in particular since the animal models available do not closely resemble human AMD.

DCF

There are several commonly used methods to assess “general” oxidative stress in cultured cells. The dihydro-dichlorofluorescein diacetate ($\text{H}_2\text{DCF-DA}$) technique is one of the most frequently performed tests for this purpose [166-168]. Usually, flow cytometry or microplate readers are used in the experiments, and a careful morphological analysis is rarely performed. This, however, may cause serious misinterpretations.

$\text{H}_2\text{DCF-DA}$ is a non-fluorescent, lipophilic ester that, after passing the plasma membranes, is split by unspecific esterases intracellularly. One of the reaction products is the alcohol dihydro-dichlorofluorescein (H_2DCF) which is trapped within the cell due to its hydrophilic properties. H_2DCF may be oxidized into 2', 7'-dichlorofluorescein (DCF) by a process that is often considered to involve unspecified ROS. DCF is highly fluorescent upon illumination with blue light

and the magnitude of this fluorescence is often believed to reflect the level of “general” oxidative stress, without further defining what kind of ROS may give rise to the oxidation of H₂DCF [169, 170]. However, it is not always recognized that, being a hydrophilic molecule, H₂DCF also does not pass membranes surrounding cellular compartments, except for the outer, fenestrated mitochondrial ones. It is also not generally realized that oxidation of H₂DCF relies on either Fenton-type reactions or enzymatic oxidation by cytochrome c, and that hydrogen peroxide or superoxide themselves do not oxidize H₂DCF [171-173].

Consequently, oxidation of H₂DCF demands the presence of either cytochrome c or of both redox-active transition metals and hydrogen peroxide simultaneously. Redox-active metals exist mainly within lysosomes, while cytochrome c resides bound to the outer side of the inner mitochondrial membrane.

AIMS OF THE PRESENT STUDY

General aim:

To contribute to the understanding of the involvement of oxidative stress in the pathogenesis of AMD and the reasons why the post-mitotic RPE cells, in spite of living in one of the most oxidatively exposed environments of the body, usually are able to evade significant damage until late in life.

Specific aims:

- To investigate how human ARPE-19 cells handle acute oxidative stress compared to another cell line of professional scavengers (murine J774 cells), and if their susceptibility can be altered or alleviated when the cells are protected with an iron chelator (**paper I**).
- To evaluate the suitability of the commonly used H₂DCF-test for assessing general oxidative stress (**paper II**).
- To analyze and quantify the levels of total and intralysosomal iron in ARPE-19 and J774 cells, as well as their content of intracellular proteins with known iron-binding properties, such as metallothionein, HSP70 and ferritin (**paper III**).
- To determine whether up-regulation of the intracellular levels of metallothionein, HSP70 and/or ferritin in ARPE-19 cells further increases their resistance to H₂O₂ exposure and, contrarily, if down-regulation of these proteins makes them more sensitive (**paper IV**).
- To elucidate if other putative proteins with iron-chelating or otherwise anti-oxidative stress-related properties are present in larger quantities in ARPE-19 cells by utilizing a human cell stress array (**paper IV**)

MATERIALS & METHODS

This is an overview of the different methods used in this thesis. For further information and more specific details, the reader is referred to the methods sections of the appended studies.

Cells and culture condition (papers I-IV)

Three different cell lines were used in the present work. Since the major aim of the studies was to investigate how oxidative stress affects RPE cells, the commercially available ARPE-19 (human HPV16-immortalized retinal pigment epithelial) cells [158] were selected as our primary cell type, utilized in all four papers presented herein. In papers **I-III**, a murine cell line of macrophage-like professional scavenger cells (J774 cells), which, like ARPE-19 cells, are also lysosome-rich and well characterized, was chosen as a suitable reference cell type. Additionally, human immortalized HeLa cells were used as a second line of control in some experiments of paper **I**. All cells were grown in their recommended culture media in 75 cm² bottles, incubated at 37°C in humidified air with 5% CO₂ and were split twice a week.

Basic conditions for exposure to oxidative stress (papers I, II and IV)

Although seeding conditions, incubation time, cell confluence and treatments varied between different experimental settings and methods, the procedure in which the cells were exposed to oxidative stress was essentially the same for all experiments and is briefly summarized here. After removal of the culture medium, the adherent cells were rinsed with PBS. Thereafter, H₂O₂ at different concentrations in HBSS was added to the wells, followed by 30 min incubation at 37°C. Cells were then returned to standard culture conditions (in normal growth medium) for 6-8 h before being evaluated for morphological alterations or survival rate, either microscopically or in a plate-reader.

Exogenous iron chelation (paper I)

In some experiments, cells were subjected to H₂O₂ in the presence of the potent lipophilic iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) at a concentration of 100 µM. SIH has a high complex constant where two molecules are able to bind all six coordinates of an iron atom [174]. Its lipophilic properties allow it to easily traverse cellular membranes, e.g. the lysosomal one, thereby accessing and binding free iron within all cellular compartments and preventing it from participating in Fenton reactions. After H₂O₂ exposure, SIH may be rinsed away from the cells, allowing them to rapidly regain their habitual level of

labile iron, needed for normal cellular metabolism. Experiments with prolonged iron starvation were also performed, in which SIH was added to the culture medium and left without replacement for up to 10 days. Cell survival and proliferation was then evaluated microscopically.

Degradation of hydrogen peroxide (paper I)

To verify that any divergences in oxidative stress-sensitivity between the cell lines were not due to differences in their capacity to degrade H_2O_2 , experiments were carried out where the rate of H_2O_2 clearance was determined. Briefly, ARPE-19 and J774 cells were seeded in equal numbers and supplemented with a bolus dose of 100 μM H_2O_2 in HBSS. Aliquots of 25 μl culture supernatant were then repeatedly sampled during a period of 60 min and analyzed for H_2O_2 concentration using the horseradish peroxidase-mediated H_2O_2 -dependent p-hydroxyphenylacetic acid (pHPA) oxidation technique [175].

Lysosomal membrane stability assay (papers I and II)

Lysosomal membrane permeabilization (LMP) is an early event in many cases of apoptosis and, hence, evaluation of lysosomal stability in relation to H_2O_2 exposure is of interest. For this purpose the acridine orange uptake technique was utilized. Acridine orange (AO) is a lysosomotropic, metachromatic fluorophore that, following uptake in the cell, accumulates in lysosomes where it is retained by proton trapping. In the acidic environment of the lysosome, the highly concentrated AO emits red fluorescence when excited with blue light, whereas AO located in the more neutral pH of the nucleus and cytosol will fluoresce in green following such illumination. When the membrane of an AO-containing lysosome permeabilizes, AO is released to the cytosol resulting in an increased green fluorescence (AO relocation method). This method is useful when measuring the shift from red lysosomal to green cytosolic fluorescence early after lysosomal damage has been induced by oxidative stress or other means, e.g. exposure to a lysosomal detergent (**paper II**). The AO uptake method, on the other hand, is performed 6 h after H_2O_2 treatment. The cells are incubated with AO, which then concentrates in still functioning lysosomes. Many red granules (reflecting intact lysosomes) and a low green cytosolic fluorescence indicate a low degree of LMP, while few red granules and a higher green fluorescence suggest that many lysosomes have ruptured (**papers I and II**). [148, 149]. However, some caution is advised when evaluating these methods with a fluorescence microscope, since the energy difference between the exciting blue light and the emitted red fluorescence is large enough to generate singlet oxygen. Hence, AO also acts as a photosensitizer. Singlet oxygen attacking lysosomal membranes leads to LMP

within less than a minute [176]. Keeping exposure times to a minimum and focusing rapidly is of great importance to avoid misinterpretations.

Assessment of cell viability (papers I and IV)

The methods used for evaluating cell survival and proliferation are outlined below.

Morphological assessment (papers I and IV)

In paper **I**, cell numbers and typical morphological signs of apoptosis such as plasma membrane blebbing, nuclear pyknosis and formation of apoptotic bodies were evaluated 6-8 h after H₂O₂ exposure. Some cells were studied in their wells using inverted phase contrast microscopy, while others were grown on coverslips and depicted using the Nomarski optics of a Zeiss Axiovert microscope after being mounted in culture media on an object glass. The phase contrast pictures of paper **IV** were achieved in a somewhat different manner, whereby APRE-19 cells were grown to confluence in 35 mm glass bottom dishes, treated with H₂O₂ as described above and then documented without further mounting steps using the Zeiss microscope mentioned above.

MTT viability assay (paper IV)

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) viability assay reflects the mitochondrial metabolic activity of the cells and is one of the most commonly used methods for evaluating cell viability and proliferation [177-179]. Briefly, ARPE-19 cells grown in 96-well plates, with or without pre-treatments to up- or down-regulate MT, HSP70 or FT. They were then exposed to H₂O₂ as described above. Following 8 h of incubation at standard culture conditions, 0.5 mg/ml MTT (in medium) was added to the wells. In functioning mitochondria, the yellow MTT is converted into purple formazan, the amount of which is measured in a spectrophotometer following lysis of the cells 4 h after MTT addition. A high absorbance in the purple colour spectrum indicates the presence of many vital mitochondria and, consequently, viable cells.

Automated cell counting (paper IV)

Due to the large number of samples (performed in triplicates) in the last study, a protocol for automated counting of Hoechst-stained nuclei was used in order to minimize experimentator bias and to increase throughput. This method is a slightly modified version of a procedure initially developed by Germundsson et al. for counting corneal wing cells [180]. ARPE-19 cells were grown in 24-well plates, treated with different small interfering RNAs (siRNAs) and subsequently exposed to oxidative stress as described in separate sections. Eight hours after exposure to H₂O₂, cell nuclei were stained with 0.5 µg/ml Hoechst dye (in medium) in the dark for 30 min, washed twice and then depicted in HBSS, using the Zeiss fluorescence microscope. Image analysis and automated cell counting were performed using the free NIH ImageJ software. Following initial background subtraction and contrast enhancement, the image was transformed into black/white (“Make binary” function). To better delineate separate nuclei that, image-wise, may touch or overlap each other, the picture was then further processed using the ‘Watershed’ function. Separate particles with an area of 40 pixels or more were then automatically counted by the software. Two calculations were performed in each picture, one with nuclei touching the image edges included and one without. The mean value of these two measurements was then recorded. The Hoechst-mediated fluorescence from nuclei of apoptotic cells is much brighter than that of normal ones. Since only viable cells were to be included in the analysis, such apoptotic cells were manually counted and subtracted from the automatically recorded value in each picture.

Assessment of autophagic flux (paper I)

Immunodetection of LC3 (microtubule-associated protein 1 light chain 3) is a widely used method for evaluating the rate of autophagy in cultured cells [181]. LC3-I, one of the two detected sub-types of LC3, is located in the cytosol, whereas LC3-II is the lipidated form that is incorporated in the membrane of autophagosomes upon their formation. The amount of LC3-II is closely correlated to the number of formed autophagosomes within the cell and was, in the early days of this assay, believed to directly reflect the autophagic activity. However, the mere formation of autophagosomes does not necessarily mean that the autophagic pathway is completed, since the degradation of engulfed material occurs only after fusion with a lysosome. In addition, LC3-II is itself also degraded by autophagy, making the time point for the analysis an important factor. Hence, a more appropriate variable for measuring autophagy is the “autophagic flux”, which refers to the entire autophagic process including the delivery of cargo to the lysosomes.

The parameter of most relevance in this evaluation is the difference in the amount of LC3-II between samples where supplemented lysosomal inhibitors are absent or present, respectively. If levels of LC3-II are higher in cells with inhibited lysosomal activity, this is an indicator of autophagic flux, the magnitude of which can be determined by the ratio between LC3-II in samples with and without inhibitor ($\text{LC3-II}_{\text{inhibitor}}/\text{LC3-II}_{\text{no inhibitor}}$) [182]. In the present work, ARPE-19 and J774 cells were initially exposed to H_2O_2 for 30 min as described above. In order to avoid autophagic degradation of LC3-II, some cells were incubated for 4 h with the lysosomal enzyme inhibitors pepstatin A and E64d. Following 6–8 h at standard conditions, cells were harvested and lysed in preparation for western blotting.

Determination of iron content and distribution (paper III)

Possible differences between ARPE-19 and J774 cells with regard to their total iron content were evaluated by atomic absorption spectroscopy. Cell suspensions from both cell lines were prepared and lysed, followed by iron measurement in a polarized Zeeman Atomic Absorption Spectrophotometer. The total iron content was calculated from a standard curve and corrected for cell number and total protein content. Since one of the aims was to investigate whether the resistance to oxidative stress exhibited by ARPE-19 cells may be related to unusually low concentrations of intralysosomal “loosely bound” iron, the sensitive cytochemical sulphide-silver method was performed on both cell lines. This is an improved variant of a technique developed by Timm et al. in the 1950’s to visualize intracellular heavy metals [183] that has been adapted to detect iron that is loosely bound, yet not necessarily redox-active. The procedure is rather complex and is described in more detail in paper III.

Up-regulation of MT, HSP70 and FT (papers III and IV)

As previously reported, supplementation with zinc and iron to cells induces transcription of the iron-binding proteins MT and FT, respectively, whereas the level of HSP70 is known to increase following heat exposure [121, 140, 184]. Hence, 24 h after seeding, cells were exposed to 100 μM ZnSO_4 or 500 μM FeCl_3 in growth medium in order to induce production of MT and FT, respectively. In medium, FeCl_3 forms insoluble iron phosphate which is then taken up by endocytosis. To up-regulate HSP70, other cells were subjected to “heat shock” (43°C, water bath) for 30 min, then supplemented with fresh medium and returned to standard culture conditions. 24 h after the above treatments, the now confluent cells were either exposed to H_2O_2 with subsequent viability assessment as described above (**paper IV**), or collected and prepared for protein detection using western blotting (**papers III and IV**).

Attenuation of protein expression by RNA interference (paper IV)

RNA interference (RNAi) is a commonly used technique which effectively decreases or knocks down the expression of specific target proteins. SiRNA constitute small pieces of double-stranded RNA that, after entering the cell, get integrated into a protein complex called RNAi-induced silencing complex (RISC). RISC localizes the correct target mRNA and promotes its degradation. This results in attenuated translation of the target protein. Although siRNA, to some degree, may be taken up directly by the cell, liposome- or polyamine-based transfection agents are usually utilized to increase the transfection efficiency. They form complexes with the siRNA and facilitate its transportation across the plasma membrane [185].

To down-regulate the expression of the three investigated iron-binding proteins, siRNAs targeting MT, HSP70, FT light chain and FT heavy chain were transfected into the cells using the siPORT amine transfection agent according to the manufacturer's protocol. Additionally, some cells were transfected with scrambled control siRNA as a reference, since the silencing procedure itself may be harmful to the cells. In brief, 12 h after seeding when cells had settled and adhered to the bottom surface of the wells, the transfection agent was mixed with one or more of the different siRNAs. Following 10 min incubation at room temperature the mixture was added to the well medium. Plates were then incubated at 37°C for 48 h before cells were either lysed in preparation for immunoblotting or exposed to oxidative stress as described above.

Western blots (papers III and IV)

Many methods for determining cellular levels of specific proteins exist, all with their different benefits and disadvantages. Western blotting, also known as immunoblotting, is probably the most frequently used technique for this purpose. By running tissue homogenates or cell lysates through a gel (denaturing SDS-PAGE electrophoresis), proteins are separated according to size and are then transferred to a nitrocellulose or PVDF membrane (blotting). After blocking with 5% fat-free milk to saturate the non-specific binding sites on the membrane, the membrane is incubated with antibodies targeting the investigated protein. These primary antibodies are then in turn detected by secondary horse-radish peroxidase-conjugated antibodies that, following exposure to a chemiluminescent substrate is visualized with a digital image analyzer or photographic film. The intensity and size of the specific protein bands reflect the amount of protein within the sample and can be further analyzed and quantified using densitometry. To ensure that equal amounts of total protein was initially added to each well of the gel, the membrane is often stripped of antibodies (but not proteins) and reprobed

with a new primary antibody targeting one of the cells “house-keeping” proteins, such as actin, β -tubulin or GAPDH. These proteins are usually abundantly expressed “house-keeping” proteins and can be used to normalize the level of the investigated protein.

For a more detailed description of antibody dilution, gel type, transfer procedure, washing steps and other experimental parameters, please see the methods section of papers **III** and **IV**.

Human cell stress array (paper IV)

In order to investigate the possibility of ARPE-19 cells expressing high levels of proteins with anti-oxidant properties other than MT, HSP70 and FT, an array kit aimed at identifying a selection of 26 different human cell stress-related proteins was used according to the manufacturer's instructions. Briefly, lysates from untreated ARPE-19 cells were prepared and mixed with a cocktail of biotinylated detection antibodies provided within the kit. Following overnight incubation at 4°C with the stress array, membranes were washed to remove unbound material and chemiluminescent detection reagents were then applied. Each capture spot produces a signal that corresponds to the amount of protein bound, which is visualized using an image analyzer (as in western blotting).

Experiments investigating mechanisms for DCF- fluorescence (paper II)

ARPE-19 and J774 cells, grown on coverslips, were exposed to 10 μ M H₂DCF-DA for 30 min and then studied by confocal laser scanning microscopy following mounting in HBSS with or without H₂O₂. Some cells were pre-treated with H₂O₂ for 30 min 6 h prior to incubation with H₂DCF-DA in order to induce LMP and consequential apoptosis. To induce LMP by other means than oxidative stress, separate experiments were carried out in which ARPE-19 cells were exposed to AO for 15 min and then mounted in 100 μ M of the lysosomotropic detergent *O*-methylserine dodecylamide hydrochloride (MSDH), which rapidly induces LMP. The samples were then followed over a 15 min period of time and the AO-fluorescence was evaluated. The same experiment was then performed again with H₂DCF present but without AO. This was a way to correlate the degree of LMP to the DCF-induced fluorescence intensity.

Mitochondria were visualized by exposing the cells for 15 min to 100 nM of the mitochondria-specific fluorochrome TMRE (tetramethylrhodamine ethyl ester). To verify that H₂DCF oxidation is dependent on the presence of redox-active iron, cells were, in addition to H₂DCF-DA as above, also exposed to either

500 μ M ferric ammonium citrate (FAC) to increase the amount of available iron, or to 100 μ M of the iron chelator CP22 to reduce it. In order to even further prove its iron-dependent oxidation, H₂DCF was exposed to FeCl₃, cysteine and hydrogen peroxide in separate test tube experiments without cells.

Statistical analysis

In paper **II**, the intensity of the DCF-induced fluorescence was determined using the free NIH software ImageJ. Each cell was manually delineated and, following calculation of mean fluorescence, analyzed for statistically significant differences compared to controls using independent t-test. This test was also used in paper **III**, where comparisons of protein levels were made between untreated ARPE-19 and J774 cells. In paper **IV**, One-way ANOVA with post-hoc Bonferroni analysis was used to compare more than two groups. All experiments in papers **III** and **IV** where statistical significance was calculated were carried out in triplicates. Results were presented as means $\pm$ SD. P-values <0.05 were considered significant. Statistical analysis was performed using Microsoft Excel (**papers II and III**) and SigmaStat 3.5 (**paper IV**).

RESULTS

Paper I

ARPE-19 retinal pigment epithelial cells are highly resistant to oxidative stress and exercise strict control over their lysosomal redox-active iron.

As mentioned in the introduction, LF formation and oxidative stress-induced apoptosis – considered to be possible pathogenic factors in AMD development – are dependent on iron-mediated formation of hydroxyl radicals. This process mainly takes place within the lysosomes, in which most of the cell's redox-active iron is located.

In the first paper of the thesis, we sought to evaluate how ARPE-19 cells (an immortalized cell line derived from human RPE cells), which are supposed to have retained most of their normal properties, react to oxidative stress in terms of survival and lysosomal stability. We were also interested in whether the presence of an iron chelator might affect this response. As controls, we selected the murine macrophage-like J774 cells, since these, like the RPE cells, are lysosome-rich professional scavengers.

ARPE-19 cells are extremely resistant to oxidative stress and were shown to be capable of withstanding more than 150 times higher concentrations of hydrogen peroxide as compared to control cells (J774) without significant permeabilization of lysosomal membranes with subsequent release of lytic enzymes and ensuing apoptosis occurring (Figure 5). Another type of HPV-immortalized cells, HeLa cells, was also tested to rule out the possibility that the high resistance of the ARPE-19 cells might be a function of HPV immortalization. The HeLa cells exhibited significant signs of apoptosis already following 100 μM H_2O_2 and the majority of cells did not survive treatment with 1 mM H_2O_2 . This indicates that the immortalization per se is not the reason for the large observed differences in sensitivity to oxidative stress. A superior capacity for degradation of H_2O_2 in the ARPE-19 cells could possibly have explained their remarkable resistance to oxidative damage. However, both ARPE-19 and J774 cells were shown to be equally effective in this respect.

Addition of the iron chelator SIH to the culture medium prior to H_2O_2 exposure resulted in an added protective effect on cell survival rate as well as on the lysosomal stability in both ARPE-19 and J774 cells (assessed with the acridine orange uptake method). Importantly, even at the extremely high concentration of 20 mM H_2O_2 , which killed about 80% of the ARPE-19 cells, SIH gave an almost full protection. Furthermore, ARPE-19 cells were able to survive, and even replicate under prolonged exposure to high concentrations of SIH (which usually kills cells rapidly due to iron starvation). Immunodetection of LC3 revealed a higher degree of basal autophagic flux in ARPE-19 cells compared to J774 cells (western blot).

Figure 5. (A and B)

ARPE-19 cells are substantially more resistant to oxidative stress in the form of a single bolus dose of H_2O_2 than J774 cells. However, both cell types are protected by the presence of the iron chelator SIH during the stress period. Apoptotic and post-apoptotic necrotic cell death were evaluated by phase contrast microscopy 6–8 h following H_2O_2 exposure. Interestingly, even at the extremely high hydrogen peroxide concentration of 20 mM, SIH almost fully protected the ARPE-19 cells.

Means $\pm$ SD. $n = 3$.

Paper II

What does the commonly used DCF test for oxidative stress really show?

Experiments were performed in order to investigate our hypothesis that H₂DCF generally is too uncritically used when evaluating oxidative stress. The results are presented below.

ARPE-19 and J774 cells exposed to H₂DCF-DA both showed a weak mitochondrial-like fluorescence pattern, which was confirmed by an identical fluorescence pattern in cells treated with the red-fluorescent mitochondrial marker TMRE. Spontaneously apoptotic cells, which are always present in early cultures, displayed a strong cytosolic DCF-induced fluorescence that was almost 9 times stronger than that of control cells. Such intense fluorescence was also seen in cells in which apoptosis was induced by pre-treatment with H₂O₂ 6 h prior to incubation with H₂DCF-DA. Following exposure to the iron compound FAC, DCF fluorescence was also significantly increased, whereas iron chelation with CP22 decreased it. Cells mounted directly in H₂O₂ only showed a very slight increase in fluorescence, again indicating that this compound alone is not sufficient to induce any substantial oxidation of H₂DCF.

However, when ARPE-19 cells were exposed to the lysosomotropic detergent MSDH after pre-exposure to H₂DCF-DA as above, they displayed a time-dependent strong increase in cytosolic fluorescence that paralleled a decrease in the number of intact AO-containing lysosomes (Figure 6). Test tube experiments proved both ionic iron and cytochrome c to be potent catalysts of H₂DCF oxidation, while H₂O₂ by itself did not significantly oxidize H₂DCF.

Figure 6 (A and B). Lysosomal rupture induced without oxidative stress greatly enhances cytosolic DCF-mediated fluorescence in ARPE-19 cells. (A) Cells incubated with H_2DCF -DA for 30 min were mounted on an object glass in $100\ \mu M$ of the lysosomotropic detergent MSDH. Note the time-dependent increase of green DCF-induced fluorescence in (A) which correlates well with the decrease in intact (red) lysosomes seen in (B) where the degree of LMP was determined with the AO relocation method.

Paper III

Autophagy of iron-binding proteins may contribute to the oxidative stress resistance of ARPE-19 cells.

Since redox-active iron catalyzes the formation of hydroxyl radicals through the Fenton reaction, a possible, however unlikely, reason for the resistance to oxidative stress exhibited by the ARPE-19 cells, might be that their iron content is lower than that of J774 cells. Another possibility is that they keep their iron bound to proteins in a non redox-active form. Initially, experiments comparing total iron were performed. Atomic absorption spectroscopy of lysates from both cell lines showed that, in relation to their different size (ARPE-19 cells are bigger than J774 cells), both ARPE-19 and J774 cells seem to contain similar amounts of total iron.

To visualize the level of intralysosomal iron, the sulphide-silver method was utilized. This technique detects loosely bound (but not necessarily redox-active) iron. Both cell types displayed a clear lysosomal-type granular staining pattern, indicating that lysosomes are the main intracellular location for low-mass iron (Figure 7). The ARPE-19 cells did not seem to contain less intralysosomal iron than J774 cells. If any difference, it was rather the other way around.

Figure 7. J774 (**A**) and ARPE-19 cells (**B**) appear to contain comparable amounts of intra-lysosomal low-mass iron (sulphide-silver method). Note the lysosomal staining pattern. Due to morphological differences (ARPE-19 cells are bigger and flat, while J774 cells are more spherical), the background staining of the cytosol is stronger in (**A**) than in (**B**).

Next, intracellular levels of the iron-binding proteins MT, HSP70 and FT were investigated using western blotting. Lysates from untreated cells of both lines were analyzed. It was found that all three proteins were present to a much larger extent in ARPE-19. Their basal levels of MT, HSP70 and FT were 30, 22 and 4 times, respectively, higher than those seen in J774 cells, in which the protein bands for HSP70 and MT were hardly detectable. However, if the expression of MT, HSP70 and FT was up-regulated by different stimuli (see methods section), the levels of all three proteins readily increased, although to much higher levels in the ARPE-19 cells, especially regarding FT and MT (Figure 8).

Figure 8. ARPE-19 cells have higher basal levels of FT (A), MT (B) and HSP70 (C) than J774 cells. Following appropriate induction, ARPE-19 cells also have a better capacity for up-regulation of these proteins.

Paper IV

Attenuation of iron-binding proteins in ARPE-19 cells reduces their resistance to oxidative stress.

Initially, experiments aiming at verifying the previously described oxidative stress resistance of ARPE-19 cells were performed. This time, however, the MTT viability assay was utilized in addition to morphological evaluation with phase contrast micrographs. As expected, the MTT assay confirmed that these cells were able to tolerate up to 5 mM H_2O_2 without being significantly affected, and even at 10 mM their survival rate was reduced by only 37%. The morphological appearance of confluent cells from parallel experiments, treated and oxidatively stressed the same way, correlated well with the MTT results (Figure 9).

Figure 9. Confluent ARPE-19 cells tolerate a single high bolus dose of hydrogen peroxide. Any significant impact on cell survival was seen only after exposure to 8 mM H_2O_2 or more.

Further up-regulation of the already high basal levels of MT, HSP70 and FT did not significantly increase the resistance to H_2O_2 treatment. Pre-treatment with zinc (to induce MT production), however, resulted in a decreased viability when the cells were exposed to very high concentrations of H_2O_2 (8 mM and 10 mM).

The effects of siRNA-mediated down-regulation of MT, HSP70, FT light chain (FTL) and/or FT heavy chain (FTH) expression on the cells susceptibility to H_2O_2 exposure were then evaluated using the MTT viability assay and an automated cell counting protocol. ARPE-19 cells treated with scrambled control siRNA (CS) that did not alter the protein levels were compared with cells that had received one or more of the selective siRNAs targeting the investigated proteins.

Western blots verified that the selected siRNAs effectively reduced the expression of their target proteins. Interestingly, attenuation of FTH resulted in a marked compensatory up-regulation of FTL (Figure 10). Hence, in the viability assays, siRNAs for both FT chains were given as a combination to depress FT expression more efficiently.

Figure 10. Pre-treatment with selective siRNAs decreased the levels of the target proteins MT, HSP70, FTL and FTH. Untreated control (C) cells and cells treated with scrambled control siRNA (CS) were used as reference. Note the compensatory up-regulation of FTL when FTH levels were depressed. Representative blots are shown.

Combined siRNA-mediated attenuation of both FT chains (H and L), or simultaneous down-regulation of all three proteins, made the cells significantly more sensitive to treatment with high concentrations of H_2O_2 (10 mM) (Figure 11). Similarly, cells with reduced MT levels were also sensitized, albeit only in the cell count experiments. The interpretation is more difficult in cells with down-regulated HSP70 levels, since already the cells that received no H_2O_2 treatment differed significantly from the controls.

Additionally, it was noted that down-regulation of FTL + FTH, or simultaneous treatment with all four siRNAs to attenuate the levels of all of the three investigated proteins, resulted in an increased absorbance value measured with the MTT assay in cells that were not exposed to H_2O_2 . No such increase could be seen in the corresponding cell counting experiments. This observation is probably related to a higher induced metabolic activity rather than to an increased rate of cell proliferation.

Figure 11 (A and B). SiRNA-mediated down-regulation of FTL+FTH, or of all three target proteins simultaneously, resulted in an increased sensitivity to high concentrations of H₂O₂ measured with MTT viability assay (A) and an automated cell counting procedure (B). Cells with attenuated levels of MT showed a significant sensitization only with the method used in (B). Regarding down-regulation of HSP70, the observed sensitization appears to be related to other factors than oxidative stress, since already the cells that received HSP70-siRNA, but no H₂O₂-treatment (blue bar), were clearly affected compared to C-siRNA (blue bar) in both (A) and (B).

Finally, lysates from untreated, confluent ARPE-19 cells were tested with an array of 26 different stress-related proteins in order to evaluate whether more such proteins, other than MT, HSP70 and FT, are abundantly expressed. Apart from the already described high content of HSP70 (which was verified), three other proteins appeared to be present in larger quantities; namely carbonic anhydrase IX (CA-9), thioredoxin-1 (TRX-1) and superoxide dismutase 2 (SOD2) (Figure 12).

Figure 12. A human cell stress array revealed apparently high levels of several other stress-related proteins in ARPE-19 cells. Each investigated protein is represented by duplicate dots.

DISCUSSION

It is well established that cellular sensitivity to oxidative stress is largely related to the content of redox-active iron within the lysosomal compartment [186, 187]. Iron catalyzes the formation of highly reactive hydroxyl radicals which contribute to the formation of the age-pigment lipofuscin and, if abundantly present, also may cause permeabilization of lysosomal membranes with ensuing cell death [93]. Oxidative stress-related damage to the retinal pigment epithelium (RPE) is a major pathogenic factor in the development of age-related macular degeneration (AMD) [188]. In spite of the exposed environment in which the RPE reside, with high ambient oxygen, extensive illumination and a substantial burden of phagocytosing the discarded, lipid-rich tips of the photoreceptor outer segments (POS), it is remarkable that these post-mitotic cells usually stay functional until old age, with a surprisingly slow rate of lipofuscin (LF) accumulation. This thesis has focused on investigating possible explanations for this remarkable capability.

Initially, we found that ARPE-19 cells, which are supposed to behave in a similar fashion as normal RPE cells, are extremely resistant to oxidative stress administered as a single bolus dose of H_2O_2 . The cells were able to tolerate concentrations of up to 15 mM before oxidative stress-induced lysosomal rupture and related apoptosis occurred (**paper I**). The cell lines that were used as a reference (J774 and HeLa cells) behaved completely differently, and were severely affected at H_2O_2 concentrations of only 100 μM . Hence, the resistance exhibited by the ARPE-19 cells was more than a 150-fold higher. Later experiments, conducted in **paper II**, verified the previous findings, with induction of apoptosis following 15 mM H_2O_2 in ARPE-19 cells, whereas the much more sensitive J774 cells lost their lysosomal integrity and died after being subjected to merely 100 μM H_2O_2 . However, in **paper IV**, the resistance of the ARPE-19 cells was somewhat less pronounced and the cells started showing a decreased survival rate already after exposure to 8 mM H_2O_2 . Nonetheless, it must be recognized that also 8 mM is an extremely high H_2O_2 concentration that would rapidly kill most other normal cells. This observed difference in susceptibility between the different experiments might be explained by several factors: In **paper I** and **II**, H_2O_2 was administered to sub-confluent cells only 12 h after seeding when several anti-oxidant mechanisms may still be up-regulated as a response to the sub-cultivation process.

The viability experiments in paper **IV**, on the other hand, were performed on 60 h old, confluent cells, possibly affecting their H_2O_2 sensitivity. Moreover, other variations in the experimental settings, such as different brands and surface properties of the various kinds of utilized culture wells (glass-bottomed or plastic), might also influence the outcome. Methodological differences are probably the reason why some other publications [189, 190] have found ARPE-19 cells to be much less resistant to oxidative stress, while the results of others are more in agreement with our findings [137, 191].

The observed tolerance to high concentrations of H_2O_2 indicates that the lysosomal content of iron available for redox reactions must be minute in ARPE-19 cells. However, since their already high resistance was even further enhanced in the presence of the potent iron chelator SIH, lysosomal redox-active iron cannot be completely absent. The protective effect of intralysosomal iron chelation against oxidative stress has been described previously [148, 149], albeit most commonly in cells that are much more sensitive than ARPE-19 cells. Due to the longevity and exposed location *in vivo* of RPE cells, it was anticipated that ARPE-19 cells would be more resistant to oxidative stress than J774 cells, but the magnitude of the actual difference was quite unexpected and raised many questions regarding what mechanisms could lie behind these findings. Since both cell lines were shown to be equally effective in degrading H_2O_2 (**paper I**), the difference rather had to be related to how they handle and store their redox-active iron.

When analyzed with atomic absorption spectroscopy, both cell lines were shown to contain comparable amounts of total cellular iron (**paper III**). This was not surprising, since all cells need a certain amount of iron for their basic metabolic processes. Neither did the presence of loosely bound iron within lysosomes seem to differ between J774 and ARPE-19 cells as demonstrated with the cytochemical sulphide-silver method (**paper III**). Apparently, the lysosomes of ARPE-19 cells have the capacity to withstand oxidative stress-mediated destabilization in spite of containing substantial amounts of iron and, seemingly, do not have any particular mechanism for rapidly exporting such iron from the lysosomal compartment to the cytosol.

To our reasoning, the resistance of ARPE-19 cells to H_2O_2 exposure might then be explained either by a low autophagic degradation of iron-containing cellular components in lysosomes, or by the presence of endogenous intralysosomal iron chelators that bind iron in a non-redox active form. Since immunoblotting of LC3 revealed that ARPE-19 cells have a high basal autophagic flux (**paper I**),

which has also been implied by others [26, 42], the latter possibility appeared to be more plausible.

Hence, the intracellular basal levels of three proteins with known iron-binding properties, namely MT, HSP70 and FT, were investigated and found to be much more abundantly present in ARPE-19 than in J774 cells (III). Numerous reports have shown that up-regulation or addition of these proteins promotes cell survival under various conditions of oxidative stress in several cultured cell lines. This protective effect seems to be largely the result of lysosomal membrane stabilization [140, 187, 192-199]. Contrarily, attenuation of FT or HSP70 expression seems to sensitize cells to oxidative stress [192, 200]. In addition, many studies have implied that autophagy with subsequent decomposition in the lysosomal compartment is the normal way for turnover of these stress proteins [78, 82, 88, 89, 141, 201-203].

Consequently, a continuous autophagic influx of MT, HSP70, FT and, perhaps, other iron-binding proteins into lysosomes would result in a temporary chelation of intralysosomal redox-active iron prior to their degradation. If the rate of such an influx is large enough, most of the intra-lysosomal iron would be steadily kept from catalyzing hydroxyl radical formation in the presence of H_2O_2 . The high basal content of these iron-binding proteins in ARPE-19 cells, combined with their high autophagic activity, make up the optimal conditions for this kind of protective mechanism. Possibly, this may explain the observed insensitivity to treatment with high concentrations of H_2O_2 and may also, under more physiological conditions of low-grade oxidative stress, also be the reason why substantial LF build-up in the RPE normally is not seen until older age (Figure 13). Consistent with this theory, there is strong evidence that decreased autophagic flux is associated with RPE damage and AMD development [75, 77, 78].

Figure 13. Hypothetical mechanisms behind increased LF formation in AMD. Intralysosomal LF forms as a result of Fe-catalyzed peroxidation of auto- and heterophagocytosed material (e. g. POS). When redox-active iron is kept low by a high steady-state autophagic influx of iron-binding proteins, little LF will be formed. If autophagy is reduced (as in AMD), more redox-active iron will be available for catalyzing hydroxyl radical generation with ensuing LF formation.

MT, HSP70 and FT could all be further up-regulated in both cell lines following appropriate stimulation with zinc (for MT), heat exposure (for HSP70) and iron (for FT). However, the level to which these proteins increased was much higher in the ARPE-19 cells (**paper III**). Interestingly, even though the up-regulation was substantial, it did not result in further protection against oxidative stress in the ARPE-19 cells (**paper IV**), most probably because the levels of these proteins are very high to begin with. Another possible explanation for this finding is, in the case of FT induction with $FeCl_3$, that the added extra iron by itself might render the cells more sensitive to H_2O_2 exposure, thereby equalizing the protective effect of an increased FT content. In these experiments, the only significant difference seen compared to untreated controls was a reduced survival rate in cells that had received pretreatment with zinc in order to induce MT production. It is, however, unlikely that the reason for this finding is the up-regulated levels of MT per se, since it has been shown by others that induced MT

expression (by plasmid transfection) protects RPE cells from oxidative stress-related damage [196]. Instead, the observed sensitization to H_2O_2 treatment is probably related to other intracellular effects resulting from the rather unphysiological treatment with 100 μM ZnSO_4 for 24 h. It has been suggested that too high levels of free Zn^{2+} have a negative impact on mitochondrial function and amplifies their production of reactive oxygen species [204]. This is of particular interest since the MTT test that was used in these experiments mainly measures the function of mitochondria. On the other hand, zinc supplementation (with subsequent induction of MT expression) has been shown to exert a protective effect against many types of oxidative stress as well as slowing down AMD progression in vivo [20, 140, 205-207]. Moreover, it has been shown that RPE cells in AMD-affected eyes contain reduced amounts of zinc compared to healthy controls [98, 208] and that pigmented rats with zinc deficiency have an increased accumulation of lipofuscin [209].

In order to further elucidate the importance of iron-binding proteins in the resistance to oxidative stress exhibited by ARPE-19 cells, siRNA-mediated attenuation of MT, HSP70 and both chains of FT (FTH and FTL) were then conducted (**paper IV**). It was found that down-regulation of MT or FT, or all three proteins simultaneously, sensitized the cells to treatment with high concentrations of H_2O_2 (10 mM) compared to controls. However, regarding cells with reduced amount of MT, this difference was only significant in one of the two viability methods used. It is also noteworthy that treatment with HSP70-siRNA seemed to inhibit cell proliferation significantly even when no oxidative stress was applied. This makes it more difficult to draw any conclusions regarding their response to H_2O_2 treatment compared to the controls. Since most other cell lines have a much lower basal expression of HSP70 than ARPE-19 cells and still manage to proliferate just fine, this finding is somewhat confusing.

Furthermore, when exposed to lesser concentrations of H_2O_2 than 10 mM, no significant differences between controls and cells with attenuated protein levels could be seen. Possibly, this might be explained by compensatory up-regulation of other protective mechanisms. Another reason may be that the siRNA treatment is not 100% effective in depleting the target proteins. The remaining amount might then still be sufficient to keep the intra-lysosomal iron safely bound in a non redox-active form. It needs to be emphasized that there are many pitfalls to consider when performing experiments with siRNA-mediated down-regulation of proteins. The treatment itself may be harmful to the cells, either by total depletion of a target protein needed for vital cell functions, or as a result of toxicity of the transfection agent. Therefore, in order to avoid such confounding factors

related to the siRNA exposure, it is important that comparisons are made between cells treated with scrambled control siRNA and cells exposed to selective siRNAs targeting the desired proteins.

One might wonder why the ARPE-19 cells were not even more sensitized to H₂O₂ exposure when the levels of our three investigated proteins were decreased. As mentioned above, several other cell lines have been rendered much more susceptible to oxidative stress following similar down-regulations. Since cells rarely rely on one single defense systems to keep their vital functions intact, there are probably other protective mechanisms involved. In fact, when ARPE-19 cell lysate was tested in a human cell stress array with 26 proteins, several stress-related proteins were found to be highly expressed (**paper IV**). Apart from confirming the already reported high basal content of HSP70, an abundance of carbonic anhydrase IX (CA-9), thioredoxin-1 (TRX-1) and superoxide dismutase 2 (SOD2) was seen. The membrane-bound protein CA-9 has a major role in the regulation of H⁺ flux and exercises a protective effect on cells, mainly under hypoxic conditions. It is commonly expressed in many tumors, but usually much less so in most normal tissues [210]. TRX-1 and SOD2 both possess well-characterized anti-oxidative properties [211, 212]. However, it must be taken into account that the utilized array primarily is designed to compare different cell lines or relative changes of protein levels occurring after various treatments or exposures. Hence, no precise quantification was possible in the present experimental setting.

Another important factor to be considered when relating our findings to AMD pathogenesis is that ARPE-19 cells are virtually unpigmented *in vitro*, particularly when grown for shorter time periods. RPE cells *in vivo*, on the other hand, are rich in melanin pigment granules (melanosomes). Melanin has been shown to possess both radical scavenging and iron-chelating properties [137, 213]. Additionally, cultured RPE cells rich in melanin are better protected against LF formation than melanin-poor ones [214]. The greater number of melanosomes found in the RPE of darkly pigmented individuals may explain the higher incidence of AMD in the Caucasian population [19]. Interestingly, since melanosomes may fuse with lysosomes, it is plausible that melanin might contribute to intralysosomal iron chelation *in vivo*.

Apart from oxidative stress-related damage, increasing evidence has shown inflammatory and immunological aspects, involving activation of the complement system and of inflammasomes, to be of importance in AMD pathogenesis [15, 42]. Recently, several reports have described linking bridges between oxida-

tive stress-related features, such as LF accumulation, and these inflammatory events. The complement system, one of our defense mechanisms against bacterial infections, can be activated in an alternative route by other stimuli than bacteria, e.g. LF, A2E (a product of retinal), oxidative stress and smoking. In such cases, the RPE cell itself may be attacked and even killed [215-218]. Furthermore, SNPs (single nucleotide polymorphisms) in certain complement factors may lead to the same result. Complement factor H is an inhibitory factor, preventing such damage. SNPs in the factor H gene result in loss of this inhibition, which will promote RPE damage and development of AMD [11, 219, 220].

Several publications have reported on other links between LF accumulation and inflammatory events. For example, it has been shown that permeabilization of lysosomes in ARPE-19 cells, achieved either by LF-mediated photo-oxidative membrane disruption or by using a lysosomotropic detergent, resulted in activation of NLRP3 inflammasomes with ensuing release of pro-inflammatory cytokines [221, 222]. Another recent report describes a more direct connection between iron and the inflammatory response seen in AMD, where iron supplementation of ARPE-19 cells resulted in accumulation of toxic Alu RNA which also is known to cause priming of the NLRP3 inflammasome [223]. Raised levels of Alu RNA have been found in the RPE of patients with advanced dry AMD [58]. Hence, iron accumulation, known to occur in the ageing retina, and even more so in AMD-affected eyes, is probably a key factor in both oxidative stress-related and inflammation-linked RPE cell damage and degeneration.

In paper **III**, the properties of the commonly used DCF-test for oxidative stress were investigated. H_2DCF oxidation seems to rely both on iron-mediated Fenton-type reactions and enzymatic oxidation by cytochrome c in the presence of hydrogen peroxide. In normal cells, the amount of redox-active iron in the cytosol is minute, since most of it is trapped within the lysosomal compartment, to which H_2DCF , being a hydrophilic molecule, has no access. Cytochrome c, on the other hand, resides mainly in the inter-membraneous space of mitochondria, into which H_2DCF easily can pass through the fenestrations of the outer membrane. There it encounters small amounts of endogenous H_2O_2 , thus explaining the mitochondrial fluorescence pattern seen in normal cells.

In apoptotic cells with permeabilized lysosomes, however, much stronger cytosolic fluorescence was seen, probably due to LMP-related relocation of labile iron. This was also confirmed by the pronounced, time-dependent increase in DCF-induced fluorescence that arose when LMP was induced without oxidative stress, using the lysosomotropic detergent MSDH. A similar increase in DCF

fluorescence was also seen when cells with intact lysosomes were exposed to redox-active iron (FAC). LMP is an upstream or potentiating event in apoptosis that, apart from releasing redox-active iron, also results in leakage of lytic enzymes. This causes mitochondrial membrane permeabilization (MMP) with ensuing relocation of cytochrome c to the cytosol [66, 81]. Possibly, this further increases the strong cytosolic fluorescence of apoptotic cells. A clear understanding of the factors that determine DCF-induced fluorescence is required in order to appreciate what DCF-induced fluorescence really means. Presently, we suggest that it should be considered to reflect LMP and MMP with subsequent relocation to the cytosol of redox-active iron and cytochrome c as well as release of iron from cytosolic ferritin by lysosomal proteases rather than being the result of some incompletely defined “ROS” or “general oxidative stress”.

CONCLUSIONS

To summarize, the results of this thesis show that ARPE-19 cells are extremely resistant to oxidative stress. Unlike most other cell types, these cells can tolerate exposure to a single bolus dose of H_2O_2 of up to 15 mM before lysosomal rupture with ensuing cell death occurs. In the presence of a potent exogenous iron chelator, the cells are able to survive exposure to even higher H_2O_2 concentrations. Compared to another cell line of lysosome-rich professional scavengers (J774 cells), the resistance to oxidative stress exhibited by the ARPE-19 cells is more than 150 times higher. This difference is neither explained by a superior capacity for degradation of H_2O_2 , nor by a lower content of total or intralysosomal iron. However, the ARPE-19 cells have a higher autophagic activity as well as a much larger content of several iron-binding proteins than the J774 cells.

Based on our findings, it seems that the extraordinary capability of ARPE-19 cell to handle oxidative stress is related to a continuous and pronounced autophagic influx of such iron-binding proteins into the lysosomes. Prior to being degraded, these proteins will keep the majority of the intralysosomal iron bound in a non redox-active form, thereby preventing it from catalyzing the generation of highly toxic hydroxyl radicals. When the levels of these proteins are depressed in ARPE-19-cells their resistance to high concentrations of H_2O_2 decreases, which further strengthens this reasoning. Keeping a tight control over lysosomal iron will prevent extensive lipofuscin formation and, hence, preserve a continued well-functioning autophagic turnover of worn-out cellular material which otherwise would build up in the cytosol and, eventually, promote cell death.

The mechanisms involved in the pathogenesis of AMD, which often results in loss of central vision, are elusive and still incompletely understood. However, it is possible that the progressive accumulation of iron known to occur in the aging RPE, combined with a decreased rate of autophagy, and perhaps also individual differences in the expression of endogenous iron-binding proteins, might partly explain why some individuals develop AMD while others do not. Hopefully, the results of this thesis and further research will contribute to finding new ways of combatting or at least ameliorating sight-threatening AMD in the future. This may possibly include treatment with iron chelators and/or selective up-regulation of iron-binding proteins (if insufficiently expressed) in the RPE.

SVENSK SAMMANFATTNING

Bakgrund

Macula, eller "gula fläcken", utgör den mest centrala delen av näthinnan (retina). Där är de ljuskänsliga fotoreceptorerna – stavarna och tapparna – som tätast sammanpackade vilket gör att den mest högupplösta synskärpan, den som används till bland annat läsning och ansiktsigenkänning, återfinns i maculaområdet. Fotoreceptorernas yttersegment är fyllda av membraner ("diskar") som omvandlar inkommande ljus till nervsignaler. Det pågår en ständig omsättning av fotoreceptorerna genom att nya diskar bildas vid basen av yttersegmenten, medan den uttjänta toppen dagligen "knipsas av". Det restmaterial som då bildas tas upp (fagocyteras) av det retinala pigmentepitelet (RPE) där det sedan bryts ned och återvinns.

Figur 14 (A och B). (A) visar en schematisk bild av näthinnans olika cellager. (B) är ett foto av den centrala näthinnan. Maculas läge är pilmarkerat.

RPE ligger strategiskt placerat mellan fotoreceptorerna och deras blodförsörjning, de koroidala kapillärerna. Eftersom RPE endast är ett cellager tjockt och aldrig förnyas under hela livet omsätter lysosomerna i dessa celler enorma mängder fagocyterat material från fotoreceptorerna. Man uppskattar att varje RPE-cell under en livstid bryter ned upp till 300 miljoner yttersegmentsdiskar. Denna tunga arbetsbörda, kombinerat med att näthinnan har mycket hög syrekonzentration och dessutom dagligen exponeras för stora mängder ljus, leder till att det i RPE-cellerna bildas potentiellt skadliga reaktiva syreföreningar. Om mängden av dessa överstiger vad cellens anti-oxidativa försvarssystem klarar av att hantera uppstår s.k. "oxidativ stress".

Åldersrelaterad maculadegeneration (AMD), även kallad åldersförändringar i gula fläcken, är en av de vanligaste orsakerna till svår synnedsättning hos äldre individer. Mer än 50 miljoner människor är drabbade i världen och förekomsten av AMD förväntas stiga dramatiskt de kommande decennierna på grund av att vi blir allt fler och allt äldre. Sjukdomen leder till förlust av den centrala synskärpan och lättsynen. Det perifera synfältet, ledsynen, är däremot oftast bevarad. Mekanismerna bakom AMD-utveckling är mångfacetterade och inte helt klarlagda, men mycket tyder på att oxidativ stress är en viktig bidragande faktor. Det är allmänt accepterat att utvecklingen av AMD börjar i RPE, som i senare stadier av sjukdomen så småningom går under. Detta leder till att fotoreceptorerna, som är beroende av ett fungerande RPE, i sin tur långsamt förtvinar och orsakar irreversibel synförlust.

Med ökande ålder sker en ansamling av det icke-nedbrytningsbara ålderspigmentet lipofuscin i RPE-cellerna. Lysosomerna i lipofuscinfyllda celler har en försämrad förmåga att helt bryta ner alla fagocyterade yttersegment, med påföljden att icke-degraderat material stöts ut och ger upphov till gulaktiga inlagringar bakom RPE, s.k. ”drusen”, som är ett tidigt steg i utvecklingen av AMD.

Vidare är det känt att järn med åren ansamlas i näthinnan och RPE, speciellt hos patienter med AMD. Redox-aktivt järn, som frisätts i lysosomerna vid nedbrytning av järninnehållande cellorganeller och proteiner, är mycket skadligt för cellerna. Detta beror på att redox-aktivt järn kraftigt ökar graden av oxidativ stress genom att katalysera (förstärka) bildningen av extremt reaktiva hydroxylradikaler ($\text{HO}^\bullet$) från väteperoxid (H_2O_2) genom den s.k. Fenton-reaktionen.

$\text{HO}^\bullet$ är reaktiva syreföreningar som hör till gruppen ”fria radikaler” och de bidrar dels till lipofuscin-bildningen, men kan också orsaka direkt skada på lysosomernas membran med påföljande läckage av nedbrytande enzymer till övriga delar av cellen. Om detta läckage blir tillräckligt stort går cellen under. Således är det mycket viktigt för alla celler – och kanske RPE-celler i synnerhet då dessa ska fungera en hel livstid – att hålla det redox-aktiva järnet bundet i en ofarlig form.

Syfte, material och metoder

Hänvisning till de olika delarbetena sker med romerska siffror inom parantes.

Det primära syftet med denna avhandling var att bidra till förståelsen om hur oxidativ stress är involverat i uppkomstmekanismerna vid AMD, samt att undersöka möjliga orsaker till varför RPE-celler, trots att de befinner sig i en av kroppens mest utsatta miljöer, ändå oftast lyckas överleva upp till hög ålder innan signifikant lipofuscin-bildning och påföljande cellskada uppkommer.

Vi har analyserat hur odlade immortaliserade humana RPE-celler (ARPE-19) hanterar oxidativ stress i form av bolusdoser med H_2O_2 . Dessa resultat har sedan jämförts med en annan cellinje (J774) som också har en liknande fagocytisk kapacitet som ARPE-19-celler och är rika på lysosomer (**I**). Vidare har vi undersökt om cellernas känslighet mot oxidativ stress påverkas om man tillför en järn-chelator (som binder upp redox-aktivt järn i en ofarlig form) (**I**). Eventuella skillnader i järninnehåll, såväl i lysosomerna som i cellen som helhet, utvärderades också i de båda cellinjerna (**II**), samt deras förmåga att bryta ned H_2O_2 (**I**).

Med western blot-teknik har vi även analyserat skillnader i basala och uppreglerade nivåer av tre proteiner med kända järnbindande egenskaper, nämligen metallothionein (MT), heat shock-protein 70 (HSP70), och ferritin (FT) (**III**), samt undersökt huruvida inducerad upp- eller nedreglering av dessa förändrar ARPE-19-cellernas känslighet mot oxidativ stress (**IV**). Då det är möjligt att fler stress-relaterade och järnbindande proteiner, utöver de ovan nämnda, är rikligt uttryckta i ARPE-19-celler undersökes även detta med en särskild "cell stress array" (**IV**).

Det finns många tester för att utvärdera om celler är utsatta för oxidativ stress. Ett av de mest frekvent använda är den s.k. H_2DCF -tekniken. När H_2DCF oxideras till DCF uppstår grön fluorescens. Hittills har man ofta ansett att fluorescensintensiteten speglar den "generella" oxidativa påverkan i cellen. Då vi inte delar denna uppfattning har vi i delstudie **II** närmare försökt belysa de verkliga orsakerna till DCF-inducerad fluorescens och vad denna egentligen visar.

Resultat och diskussion

ARPE-19-celler befanns vara extremt motståndskraftiga mot oxidativ stress och klarade av att hantera en koncentration av tillsatt väteperoxid som var 150 gånger högre än vad J774-cellerna kunde utstå innan nämnvärd skada på lysosomerna med påföljande celledöd uppkom. Denna okänslighet visade sig dock kunna förstärkas ytterligare när ARPE-19-cellerna exponerades för H_2O_2 under skydd av en potent järn-chelator som oskadliggör redox-aktivt järn (**I**). Då en möjlig förklaring till denna stora skillnad i känslighet mellan cellinjerna skulle kunna vara en ökad förmåga hos ARPE-19 att snabbt bryta ned den tillförda väteperoxiden undersöktes även detta. Båda celltyperna visade sig dock vara lika effektiva i att degradera H_2O_2 (**I**). Vi fann heller ingen skillnad i järninnehåll mellan ARPE-19- och J774-celler, vare sig i deras lysosomer eller i cellen som helhet (**III**).

Eftersom redox-aktivt järn i lysosomerna katalyserar bildning av hydroxylradikaler blev då vår hypotes att ARPE-19-celler måste ha en unik förmåga att lagra och omsätta järninnehållande molekyler så att mängden järn i som är i redox-aktiv form hålls på ett minimum. Därför undersöktes och jämfördes nivåerna av de tre järnbindande proteinerna MT, HSP70 och FT i de båda cellinjerna. Alla tre visade sig vara betydligt mer rikligt förekommande i ARPE-19-cellerna, och kunde dessutom uppregleras till högre nivåer än i J774-cellerna. Sådan uppreglering ledde dock inte till en ökad resistens mot väteperoxid-behandling, sannolikt på grund av att nivåerna redan från början är så pass höga att ytterligare ökning inte ger någon effekt. När uttrycket av MT, HSP70 och FT inhiberades i ARPE-19-cellerna blev dessa dock betydligt mer känsliga för H_2O_2 -behandling, främst vid högre koncentrationer (**IV**). Detta fynd indikerar att de höga basalnivåerna av dessa proteiner, åtminstone delvis, har en roll i den observerade motståndskraften mot oxidativ stress.

I delstudie **I** fann vi även att ARPE-19-celler har en hög aktivitet av autofagocytos, d.v.s. den process där cellens uttjänta proteiner och organeller tas upp och bryts ned av lysosomerna så att grundelementen sedan kan återanvändas i uppbyggnaden av nya strukturer och proteiner.

Utöver de ovan diskuterade järnbindande proteinerna befanns ytterligare tre stressrelaterade proteiner vara rikligt förekommande, nämligen karbanhydras IX, superoxid-dismutas-2 samt thioredoxin-1.

Experimenten i delstudie **II** som syftade till att belysa de verkliga mekanismerna bakom den frekvent använda H_2DCF -metoden visade att signifikant DCF-inducerad fluorescens uppkommer först när lysosomernas och/eller mitokondriernas membran skadats. Snarare än att mäta en odefinierad ”generell oxidativ stress”, vilket tidigare varit den allmänna uppfattningen, reflekterar således DCF-fluorescensen mängden frisläppt redox-aktivt järn (från permeabiliserade lysosomer) eller cytokrom *c* (från skadade mitokondrier).

Den huvudsakliga slutsatsen av de presenterade resultaten är att den remarquabla resistens mot oxidativ stress som ARPE-19-cellerna uppvisar skulle kunna bero på ett högt kontinuerligt inflöde till lysosomerna (via autofagocytos) av rikligt förekommande järnbindande proteiner såsom MT, HSP70 och FT. Dessa bryts så småningom ned av degraderande enzymer i lysosomen, men kan dessförinnan tillfälligt binda upp majoriteten av det lysosomala redox-aktiva järnet och förhindrar det därmed från att katalysera bildningen av reaktiva hydroxylradikaler. Skador på lysosomens membran med påföljande celldöd kan då undvikas, trots att koncentrationen av tillförd väteperoxid är mycket hög.

Det är möjligt att individuella variationer i graden av järnackumulering och autofagocytos, samt skillnader i uttrycket av järnbindande proteiner, delvis kan förklara varför vissa individer utvecklar AMD förhållandevis tidigt och andra inte drabbas alls. Förhoppningsvis kan våra resultat utgöra ytterligare en pusselbit i förståelsen av de komplexa mekanismer som ligger bakom utvecklingen av denna vanliga synhandikappande sjukdom. Möjligen kan tillförsel av järnchelatorer, eller inducerad uppreglering av kroppsegna järnbindande proteiner (om dessa är lågt uttryckta) i RPE, vara ett sätt att i framtiden kunna förebygga uppkomst och progress av AMD.

*Gold is for the mistress – silver for the maid –
Copper for the craftsman cunning at his trade.
“Good” said the Baron, sitting in his hall,
“But Iron – Cold Iron – is master of them all”.*

Rudyard Kipling, 1910

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to:

Christina Frennesson, my head supervisor, for sharing with me your profound knowledge of age-related macular degeneration, for your kindness, encouragement, commitment and never-ending support throughout this whole process.

Sven Erik Nilsson, my supervisor, for your extraordinary enthusiasm and brilliant scientific mind, for always encouraging me and providing me with numerous references as well as a top of the line snow blower.

Tino Kurz, my supervisor, for being a true scientist with remarkable competence in both theoretical and practical matters, and for taking on the difficult task of patiently teaching me most of what I know about experimental research. Luckily, we share the same circadian rhythm, which made the sometimes late evenings at the lab a lot easier and more fun.

Ulf Brunk, my former supervisor, for supporting me with his vast experience and knowledge in this research field, for having plausible explanations for nearly everything and for an endless supply of jokes.

Per Fagerholm, for helpful and encouraging advice and for providing moral and financial support.

Johan Germundsson, my colleague, sidekick, roommate and good friend, for your loyalty, sense of humor and capability of always making me focus on what is essential.

Therese Gustafsson, for keeping the cells alive in my absence and helping me with some of the experiments.

Neil Lagali, for assistance with parts of the statistics and for showing me how to perform the automated cell counting procedure.

Monica Johnsson, for your invaluable, instant support with both big and small issues (including, but not limited to, the countless times I got locked out of my room). It is a privilege having you across the hallway!

Per Lagman, for patient and skilled assistance with the layout and design of this thesis as well as of several previous posters.

Henrik Andersson, for providing many useful advices in the blotting lab.

Peter Jakobsson, for helping me win the battle against unruly Word templates.

Beatrice Bourghardt Peebo, for your positivity and moral support.

Sven Jarkman, for allowing me time away from the clinic to perform the experimental work and writing.

Colleagues and friends at the Eye Clinic in Linköping, for helpful advice, guidance and support throughout my clinical career.

The nurses of the glaucoma section, for putting up with my sometimes long leaves of mental and physical absence without complaining (not too loudly, anyway).

All my friends, for great fun in the past and more to come in the future. Hopefully, I will now have some more time to show you my appreciation.

Christer and Elisabeth Erlandsson, for invaluable practical help and for being the kindest and most accepting parents-in-law anyone could ever ask for.

All my extra family members: Håkan, Kerstin, Sara, Jonas, Victoria and Peter, for all your encouragement and help.

My brother and neighbor Erik, for many happy moments during our child- and adulthood, for musical collaborations and for sharing my dream of one day becoming a real farmer.

My parents Per-Inge and Birgitta, for always believing in me and for guiding, helping and backing me up in all my choices and projects throughout life.

My fabulous children Filip, Ella and Maja, for your unconditional love and for being my greatest achievements in life.

Lina, my wonderful wife, great love and best friend, for incredible patience, tolerance, support, time and for just putting up with me. This would not have been possible without you.

And finally,

The sheep, horse and cats back at the small but ever growing farm, for bringing me down to earth every once in a while and for teaching me the importance of carrying hay.

Important financial contributions to this thesis were made by:

Crown Princess Margareta's Foundation for the Visually Handicapped

Edvin Jordans Foundation for Ophthalmological Research

The Eye Foundation (Ögonfonden)

County Council of Östergötland

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