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Doctor of Philosophy

The study for molecular mechanisms of human retinal pigment epithelial cell dysfunction

The Graduate school of the University of Ulsan

Department of Biological science

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The study for molecular mechanisms of human retinal pigment epithelial cell dysfunction

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of human retinal pigment epithelial cell dysfunction

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Overview

The function and pathology of retinal pigment epithelial cells in retinal degeneration

Abbreviations

RPE	Retinal pigment epithelium
PR	Photoreceptors
POS	Photoreceptor outer segment
BRB	Blood-retinal barrier
AMD	Age-related macular degeneration
GA	Geographic atrophy
CNV	Choroidal neovascularization
DR	Diabetic retinopathy
DME	Diabetic macular edema
PDR	Proliferative diabetic retinopathy
NDPR	Non proliferative diabetic retinopathy
RP	Retinitis pigmentosa
CME	Cystoid macular edema

Introduction

1. RPE (Retinal pigment epithelium)

The basal membrane of the retinal pigment epithelium (RPE), a simple layer of cuboidal cells, is strategically situated between the photoreceptor cells and the choroid to mediate a number of activities which are essential for proper development, survival and differentiation of the retina [1]. The RPE closely interacts with photoreceptor outer segment. Importantly, retinal is constantly exchanged between photoreceptors and the RPE. There is a complicated metabolic exchange between the retina and choroid with the RPE as key player [2]. To maintain the photoreceptor excitability, retinal is transported to the RPE reisomerized to 11-cis-retinal and transported back to photoreceptors [3]. This process is known as the visual cycle of retinal. In the recycling of pigment during the visual cycle, the shed discs of the photoreceptor outer segments are phagocytosed by the RPE cells. Moreover, The RPE absorbs the light energy focused by the lens on the retina and transports ions, water, and metabolic end products from the subretinal space to the blood [3]. The RPE takes up nutrients such as glucose, retinol, and fatty acids from the blood and delivers these nutrients to photoreceptors [4]. In addition, the RPE is able to secrete a variety of growth factors helping to maintain the structural integrity choriocapillaris endothelium and photoreceptors. Furthermore, the secretory activity of the RPE plays an important role in establishing the immune privilege of the eye by secreting immunosuppressive factors (Figure 1) [5]. However, dysregulated growth factor secretion can also be involved in the pathogenesis of retinal diseases [6]. With these complex different functions, the RPE is essential for visual function. A failure of any one of these functions can lead to degeneration of the retina, loss of visual function, and blindness [7].

2. RPE related retinal diseases

The retina, lining the back of the eye, is a light-sensitive tissue made up of several neuronal layers that convert light stimuli into electrical impulses. Photoreceptors, which convert these light inputs, are in contact with a specific epithelial layer, the retinal pigment epithelium (RPE), which provides a trophic support and maintains photoreceptors homeostasis [3, 8]. The relationship between the RPE and photoreceptors cells is crucial to sight; a number of defects altering the functions of this RPE layer lead to some forms of photoreceptors degeneration [9]. The loss of photoreceptors, due to their malfunctions or to a primary dysfunction or death of RPE cells, might impact the vision of affected patients and in some cases ultimately lead to retinal degeneration and blindness (Figure 2) [10].

2.1. Age-related macular degeneration (AMD)

Age-related disease, macular degeneration, is leading cause of untreatable blindness in people of +50 years [11, 12]. The macula at the center of the human retina includes the cone-rich fovea that is essential for high acuity vision; photoreceptor degeneration and RPE dysfunction in the macula, therefore, results in impaired central vision and age-related macular degeneration (AMD) [13]. AMD represents the leading cause of blindness in Western countries [14]. The elderly population is at risk with 12% of people older than 80 years being affected [15, 16]. As the life expectancy increases worldwide, AMD is becoming a global burden. AMD, a multifactorial disease, are advanced age, smoking, and genetic predispositions related to the alternative complement pathway [17, 18]. The disease usually begins in one eye but becomes quickly bilateral in 80% of patients. RPE cells appear to be altered in AMD. RPE cells are lost with age, the decline being approximately 2.3% of total RPE per decade of life (Figure 3) [1, 2]. Accumulation of deposits close to RPE cells, inflammatory modulation, and oxidative damage seem to be at the origin of RPE alterations [19]. These age-related changes appear to be strongly associated with the pathogenesis of AMD. AMD could be classified into 2 types: the wet (neovascular) and dry (geographic) forms. In advanced disease, atrophic AMD, also known as advanced dry AMD or geographic atrophy (GA). GA is characterized by areas where RPE cells are lost, leading to degeneration of photoreceptors [20]. Dry AMD patients tend to have minimal symptoms in the earlier

stages; visual function loss occurs more often if the condition advances to geographic atrophy. Dry AMD accounts for 80–90% of cases and tends to progress slowly. In 10–20% of people, dry AMD progresses to the wet type. Patients with wet AMD additionally involves the formation of abnormal blood vessels, is susceptible to leakage, and damages photoreceptors and RPE cells. These abnormal vessels are relatively fragile and prone to become permeabilised, which may lead to the accumulation of hemorrhages [21]. These blood vessels eventually cause irreversible damage to the photoreceptors and RPE cells in the macular area. Furthermore, affecting the central vision. There is no curative treatment for the dry form of AMD [22, 23].

2.2. Diabetic retinopathy (DR)

Diabetic retinopathy (DR) is one of the most severe complications of diabetes and a leading cause of blindness in working age adults. There are over 128 million diabetics worldwide and approximately 28% of those patients have some symptoms of DR [24]. The earliest changes leading to diabetic retinopathy include disorder of blood- narrowing of the retinal arteries associated with reduced retinal blood flow; dysfunction of the neurons of the inner retina [25]. Later stages by changes in visual function; dysfunction of blood-retinal barrier (BRB) leading to the leaking of blood content to the retina. Later, the basement membrane of the retinal blood vessels thickens, capillaries degenerate and lose cells, particularly breakdown of the RPE barrier. RPE cells are the most important cells involved in DR. As the blood content leaked through the RPE barrier causes excessive water influx to the retina, the breakdown of the RPE barrier is likely to play a causative role in the development of some forms of diabetic macular edema (DME), a major cause of vision loss in DR (Figure 4) [26]. DR could be classified into 2 types: the proliferative diabetic retinopathy (PDR) and Nonproliferative diabetic retinopathy (NPDR) forms. As the disease progresses, severe nonproliferative diabetic retinopathy enters an advanced or proliferative (PDR) stage, where blood vessels proliferate/grow. Further, Nonproliferative diabetic retinopathy shows up as cotton wool spots, or microvascular abnormalities or as superficial retinal hemorrhages [27]. Even so, the advanced proliferative diabetic retinopathy (PDR)

can remain asymptomatic for a very long time, and so should be monitored closely with regular checkups.

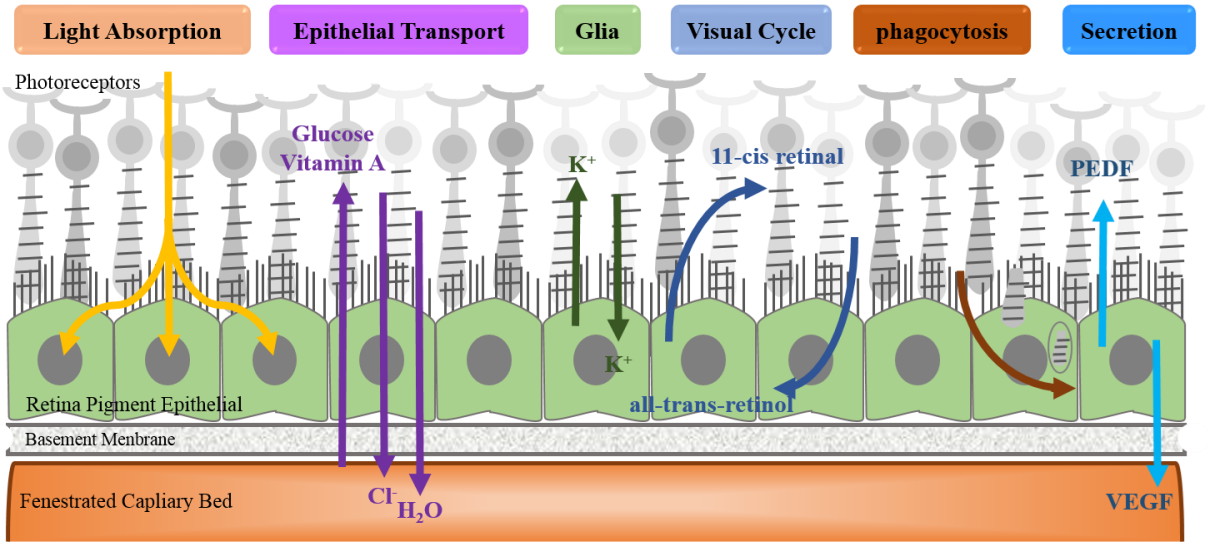

Figure 1. Schematic representation of the outer retina and the support functions of RPE [5].

The retinal pigment epithelium (RPE) comprises a monolayer of polarized pigmented epithelial cells interposed between the photoreceptors (PR) and the fenestrated choroid capillaries. One RPE cell provides support for 30–50 adjacent PR. (1) Light absorption; (2) Epithelial transport; (3) Spatial buffering of ions; (4) Visual cycle; (5) Phagocytosis of photoreceptor outer segment (POS) membranes; (6) Secretion, essential for the regeneration of PR, that compensates for the highly oxidative environment of the retina. All of these RPE functions are essential for retinal homeostasis.

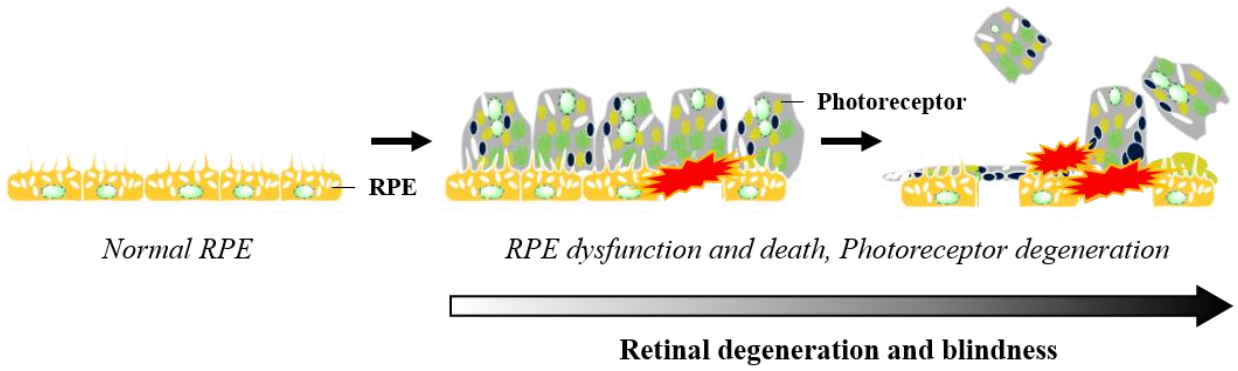

Figure 2. The RPE is critical for retinal homeostasis [31].

Retinal pigment epithelium (RPE) are specialized, multifunctional cells in the retina. It performs numerous functions that are indispensable for the health of the photoreceptors and for vision; but damage to the RPE and photoreceptors are also the initiating factor that eventually leads to vision loss in macular degenerations.

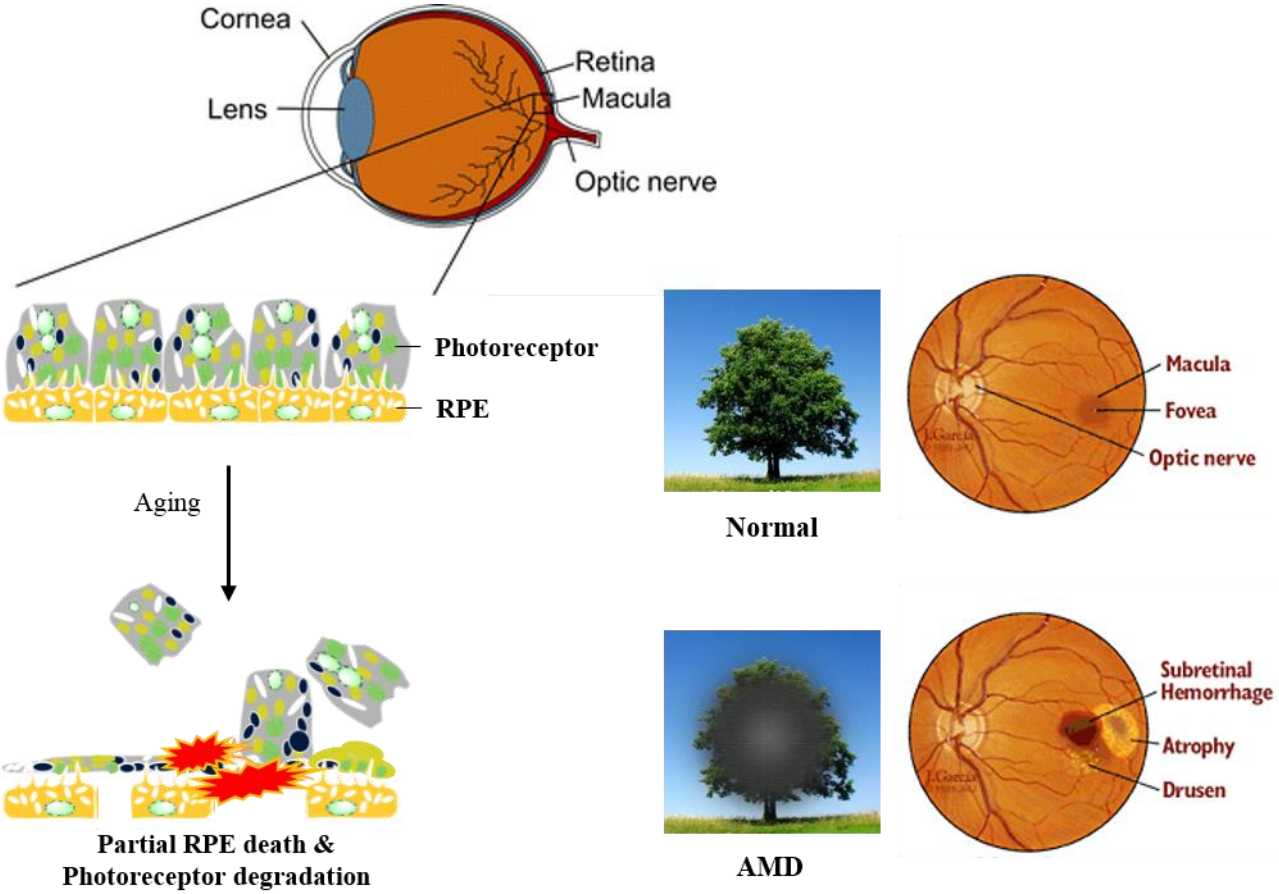

Figure 3. Pathophysiology of age-related macular degeneration (AMD) [31, 32].

Age-related macular degeneration (AMD) affects the macula, a specific part of the central retina. It involves extensive death of retinal pigment epithelium (RPE) cells and photoreceptors and leads to a progressive loss of central vision and eventually blindness.

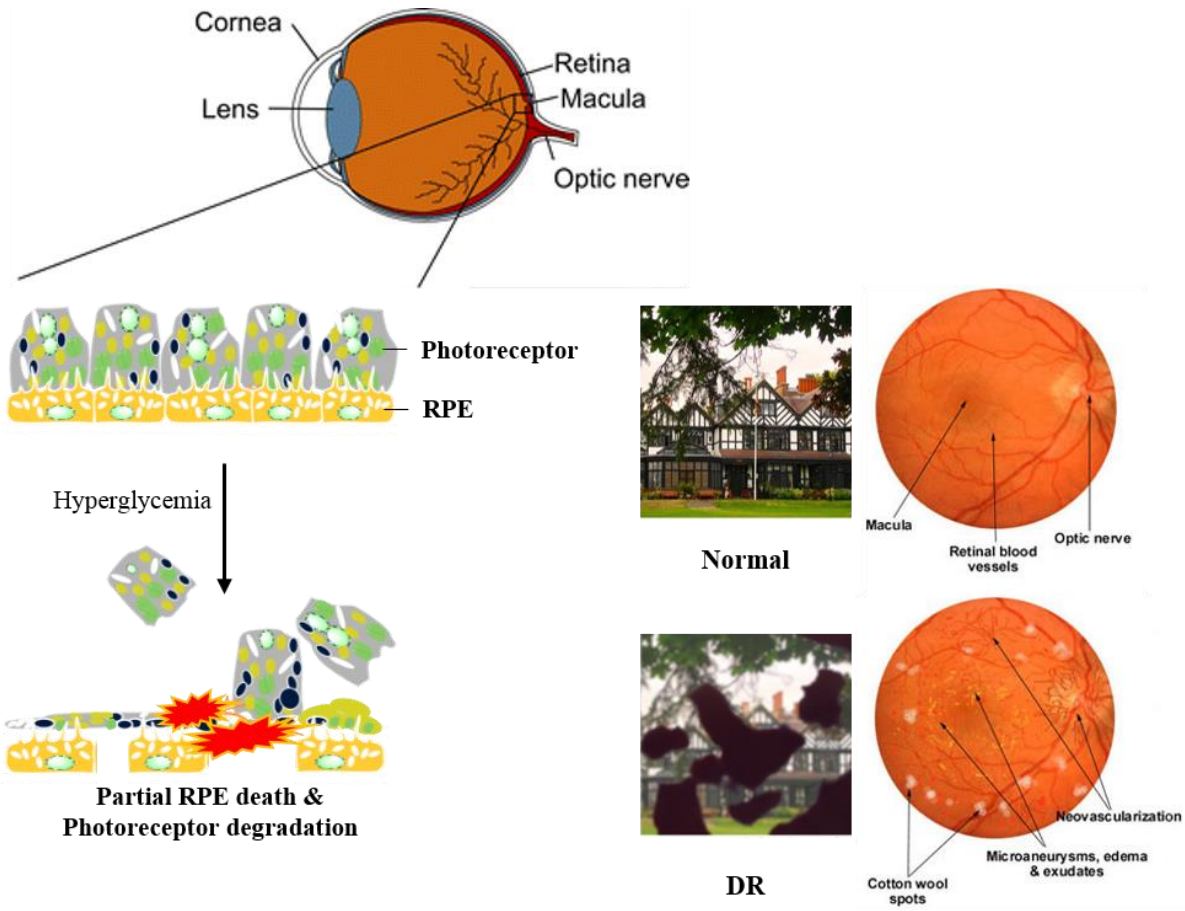

Figure 4. Pathophysiology of diabetic retinopathy (DR) [31, 33].

Hyperglycemia instigates biochemical changes leading to retinal pigment epithelium (RPE) dysfunction which increases vascular permeability followed by macular edema and retinal neovascularization. Growth of abnormal blood vessels in the eyes of diabetics lead to blindness.

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Part 1.

The role of PTX3 under sodium iodate ($NaIO_3$)-induced oxidative stress in human retinal pigment epithelial cells.

Abbreviations

RPE	Retinal pigment epithelium
AMD	Age-related macular degeneration
DR	Diabetic retinopathy
CRP	C-reactive protein
SAP	Serum amyloid p component
PRP	Pattern recognition receptor
PTX3	Pentraxin 3
TNF-α	Tumor necrosis factor-alpha
CFI	Complement factor I
CFH	Complement factor H
APOE	Apolipoprotein E
TLR4	Toll-like receptor 4
ROS	Reactive oxygen species
SOD	Superoxide dismutase
CAT	Catalase
GPX	Glutathione peroxidase
GR	Gutathione reductase
GSH	Glutathione
ALS	Amyotrophic lateral sclerosis
PD	Parkinson's disease
AD	Alzheimer's disease
HD	Huntington disease

Abstract

The retinal pigment epithelium (RPE), a monolayer located between the photoreceptors and the choroid, is essential for survival of the retina, including maintaining the overlying photoreceptors, mediating the uptake of nutrients, ions, and water, and phagocytizing the shed photoreceptor outer segment [1]. Several retinal degenerative diseases, including age-related macular degeneration (AMD), are closely related with RPE dysfunction [2]. Due to intense illumination from focal light, high oxygen tension in the macular area, and phagocytosis of photoreceptor outer segments, RPE cells are specifically sensitive to oxidative stress [3]. As a result, the RPE is constantly damaged by oxidative stress. Although the exact pathogenesis of AMD remains largely unknown, oxidative stress-induced RPE damage is believed to be one of the primary causes [4, 5]. Therefore, cellular oxidative stress plays an important role in RPE cell death during aging and the development of AMD, the primary cause of blindness in elderly persons [6]. Therefore, understanding the mechanisms of RPE cell dysfunction under oxidative stress conditions is critical for developing new therapies for AMD.

Pentraxins are soluble, pattern recognition receptors, within a family of proteins that contain a pentraxin domain with pentraxin signature (HxCxS/TWxS) in their carboxy-terminal region [7, 8]. Pentraxins are a superfamily of conserved proteins, characterized by a cyclic, multimeric structure and a conserved C-terminal domain. Based on the primary structure, pentraxins are divided into two groups, termed short and long pentraxins. Classic pentraxins, such as C-reactive protein (CRP) and serum amyloid P, are acute phase proteins that are rapidly activated in response to inflammation. Pentraxin 3 (PTX3; also called tumor necrosis factor-alpha [TNF-α]-stimulated gene 14) is the prototypic long pentraxin, which shares similarity with the classic pentraxin in the C-terminal domain but has an unrelated N-terminal sequence. Further, PTX3 is an essential component of the innate immune system [9]. It is rapidly produced and released by several cell types, including RPE cells, such as inflammatory

signals, and plays a non-redundant role in controlling inflammation.

Previously, I reported that PTX3 is expressed and secreted in response to either pro-inflammatory mediators, such as IL-1β and TNF-α, or endoplasmic reticulum stress inducer, tunicamycin, in human RPE cells. I described that plasma PTX3 levels were elevated in patients with neovascular AMD [10]. However, the expression and molecular mechanisms of PTX3 in response to $NaIO_3$-induced oxidative stress have not been investigated in RPE cells. In the present study, I demonstrated that NaIO3, a known oxidative toxic agent, induced the expression of PTX3, with AKT and ROS signaling pathways playing a role in the molecular mechanism. Moreover, oxidative stress-induced PTX3 is involved in the oxidative stress response and the expression of AMD-related genes, including complement factor I (*CFI*), complement factor H (*CFH*), apolipoprotein E (*APOE*), and toll-like receptor 4 (*TLR4*), in human RPE cells and accelerated RPE cell death. Taken together these results suggesting that critical insights into the pathologic effects of PTX3 during oxidative stress in the early development of age-related macular degeneration.

Introduction

1. Oxidative stress

Reactive oxygen species (ROS) are generated by living cells as normal cellular metabolism and environmental factors. Under excessive stress conditions, cells will produce numerous ROS, and the living organisms eventually evolve series of response mechanisms to adapt to the ROS exposure as well as utilize it as the signaling molecules [11]. Excessive ROS formation can disrupt the redox homeostasis, lead to the oxidative stress and ROS-mediated damage of the important organelles and biomolecules such as DNA and proteins as well as the injuries implicated in neurodegeneration, cancer, cardiovascular disease, cataracts and aging (Figure 1) [12]. Oxidative stress is considered to be an important factor to promote cell death in response to a variety of signals and pathophysiological situations (Figure 2) [13]. Also ROS molecules would trigger necrosis or autophagy, which are the switches of cell survival and death [14]. Cells harbor the comprehensive defense system against excessive ROS exposure. Antioxidants can attenuate the damaging (harmful) effects of ROS in vitro and delay many events that contribute to cellular aging. The balance between ROS and antioxidants is optimal, as both extremes, oxidative and antioxidative stress, are damaging [15]. Therefore, there is a need for accurate determination of individual's oxidative stress levels before prescribing the supplement antioxidants.

1.1. Endogenous and exogenous reactive oxygen species

ROS form as products under normal physiological conditions due to the partial reduction of molecular oxygen. ROS comprise both free radical and nonfree radical oxygen containing molecules such as hydrogen peroxide (H_2O_2), superoxide ($O2^-$), singlet oxygen ($^1O_2$), and the hydroxyl radical (OH^-) [16]. They have inherent chemical properties that confer reactivity to different biological targets.

Endogenous ROS arise in many ways, as a product of the respiratory chain in mitochondria and peroxisomes, in endoplasmic reticulum and enzymatic reactions, as a result of the leading to increased oxidative stress. Exogenous ROS triggers that induce oxidative stress that have direct or indirect effects on responses in the various cell types [17]. Cigarette Smoke, Ozone Exposure, ionizing and nonionizing radiations, as well as foods and drugs can all contribute to oxidative stress. Heavy metals such as lead, arsenic, mercury, chromium, and cadmium; organic solvents; and pesticides are common exogenous sources of ROS [18]. The generation of endogenous or exogenous ROS can be easily measured from plasma, blood, or bronchoalveolar lavage samples as biomarkers of oxidation by standardized assays.

1.2. Antioxidants (Enzymatic antioxidants, Nonenzymatic antioxidants)

Oxidation reactions are crucial for aerobic life, but uncontrolled ROS production leads to damage of cellular macromolecules such as DNA, lipids, protein and other small antioxidant molecules [12]. Although free radicals are continuously generated, the body is equipped to defend against the harmful effects of ROS with the help of antioxidants, collectively called the antioxidant defense system [19]. The role of antioxidants is to remove free radicals from the system, to protect the cells against their toxic effects and to contribute to disease prevention. The major enzymatic scavengers responsible for the protection of ROS generation and oxidation are Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX) and glutathione reductase (GR). SOD and catalase is the body's primary defense as it prevents further formation of free radicals [20]. Nonenzymatic (exogenous) antioxidants include low-molecular-weight compounds, such as vitamins (vitamins C and E), β-carotene, uric acid, and glutathione (GSH), flavonoids, omega-3 and omega-6 fatty acids group [21]. Exogenous antioxidant supplementation is increasingly used to fight against oxidative stress. A lot of research is being undertaken to identify new plant resources which have no or low side effects and potent antioxidant activity [22]. The range of antioxidant defenses available within the cell and extracellularly should be adequate to protect against oxidative damage. However, the balance can be lost because of overproduction of free radicals, by exposure to sources that overwhelm the oxidant defenses, or by

inadequate intake of nutrients that contribute to the defense system.

2. Retinal diseases associated with oxidative stress

Oxidative stress plays a crucial role in the development and progression of many general neurodegenerative pathologies such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington disease (HD), protein misfolding diseases and ophthalmological diseases [23]. Oxidative stress and neurodegeneration are also involved in several eye diseases, including AMD, glaucoma, DR and retinal vein occlusion. Aging, gene abnormalities, and excess exposure to exogenous oxidative stressors (e.g., a light exposure, cigarette Smoke) induce oxidative stress in the eye [24]. The retina resides in an environment that is primed for the generation of ROS and resultant oxidative damage. Also, an excess amount of ROS can lead to functional and morphological impairments in RPE. The RPE is particularly metabolically active, highly oxygenated, and vulnerable to oxidative damage because it is exposed to photosensitizers such as antioxidants [25]. Numerous studies have suggested that the cumulative amount of damage increases with age due to impairments in the DNA repair system along with intensified oxidative stress and decreased antioxidant defense. Moreover, the much less effective recovery systems for mitochondrial DNA damage can cause oxidative stress and the accumulation of the resulting aberrant products [26]. The result is retinal dysfunction and cell loss leading to visual impairment. Oxidative stress has long been considered a major influence on the RPE in AMD pathophysiology. It appears that these age-related oxidative changes are a hallmark of early AMD which, in combination with hereditary susceptibility and other retinal modifiers, can progress to the pathology and visual morbidity associated with advanced AMD (Figure 3) [27].

3. Pentraxin 3 (PTX3)

3.1. Pentraxin suferfamily

Pentraxins (PTXs) form a superfamily of multifunctional conserved proteins that constitute the

prototypic components of the humoral arm of the innate immune system. The pentraxin superfamily is characterized by a cyclic multimeric structure and by the presence in their carboxyl-terminal of a ~200 amino acid domain involving a highly conserved motif of 8-amino-acid sequence, which has been named the pentraxin signature (HxCxS/TWxS, where x is any amino acid) [28]. Based on the primary structure, pentraxins are divided into short pentraxins, including CRP and SAP, and long pentraxins, such as the prototype long pentraxin PTX3 (Figure 4). The classical short pentraxins CRP and SAP are acute-phase proteins that are rapidly activated in response to inflammation. CRP and SAP play important roles in innate immune response by regulating the complement system, recognizing pathogens, and interacting with Fcγ receptors (FcγRs), thus favoring cytokine secretion and phagocytosis of microorganisms by immune cells [29]. Long pentraxins have an unrelated, long amino-terminal domain coupled to the carboxyl-terminal pentraxin domain and differ, with respect to short pentraxins, in their gene organization, chromosomal localization, cellular source, and in inducing stimuli and ligand-recognition ability [30].

3.2. Regulation of pentraxin 3 production

Pentraxin 3 (PTX3; also called tumor necrosis factor-alpha [TNF-α]-stimulated gene 14) was the first long pentraxin identified, localized to chromosome 3 in humans and mice and comprises three exons encoding the leader signal peptide, the N-terminal domain (amino acids 18–179), and the C-terminal domain (amino acids 179–381) featuring the pentraxin signature [31]. PTX3 acts as a soluble pattern recognition receptor (PRR) playing a non-redundant role in innate immunity against selected pathogens. The expression of PTX3 is mainly induced by inflammatory stimuli such as inflammatory cytokines [tumor necrosis factor alpha (TNFα) and IL-1β] and damage-associated molecular patterns (DAMPs). PTX3 is produced by different cell types, including DCs, monocytes, macrophages, epithelial cells, ECs, fibroblasts, and adipocytes, in response to primary pro-inflammatory stimuli (e.g., TNFα, IL-1β), TLR agonists and microbial moieties (lipopolysaccharide), is an essential mediator of innate resistance to selected pathogens of fungal, bacterial and viral origin, and is involved in regulation

of inflammation, tissue remodeling, and cancer (Figure 5) [32].

3.3. Pentraxin 3 in retinal diseases

AMD has been associated with both systemic and ocular alterations of the immune system. AMD is characterized by complement driven inflammation in the central retina (macula) leading to accumulation of particulate material (termed drusen), cellular damage and the associated loss of central vision [33]. The complement system is an important regulator of the inflammatory response. The Complement dysregulation has been implicated in AMD pathophysiology. Complement factor H (CFH) is a soluble regulator of the alternative pathway of complement and dysfunction of CFH due to several single nucleotide polymorphisms in the CFH gene has been implicated in the pathogenesis of AMD [34]. PTX3, an essential component of the innate immunity system that plays a non-redundant role in controlling inflammation, regulates complement by interacting with complement components. PTX3 activates the classical and the lectin complement pathways through interaction with C1q or the ficolins on cell surfaces, but it also mediates down regulation of alternative complement pathway amplification and activation by attracting CFH (Figure 6) [35]. Based on the retinal expression of PTX3, the known interaction between PTX3 and CFH and the strong association between CFH polymorphisms and AMD. In addition, CFH is PTX3, which is produced locally in the RPE. The RPE cell contributes to the immune privileged status of the eye as part of the blood–retinal barrier and by the secretion of immunosuppressive factors inside the eye [36].

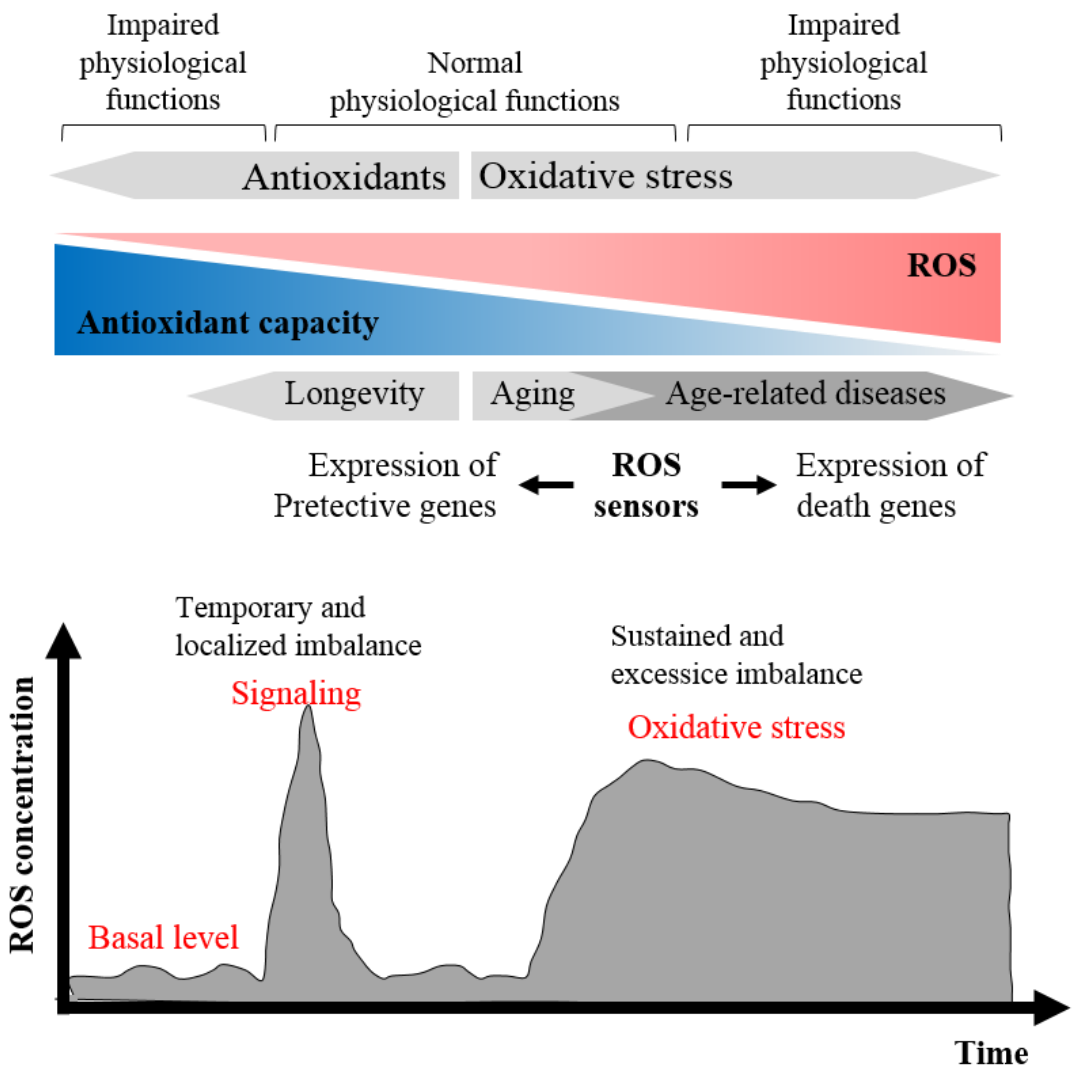

Figure 1. The balance between antioxidant capacity and ROS [12].

ROS have important functions in normal cell behavior, but also in disease. Thus, high and very low levels of ROS within cells lead to impaired cellular functions. However, ROS levels at which cells perform normal physiological functions can lead to the damage of macromolecules. Increased generation of ROS or decreased antioxidant capacity promotes aging and age-related disease. On the other hand, decreased oxidative stress or increased antioxidant capacity promote longevity. ROS sensor systems detect changes in intracellular oxidative stress and promote cell death or protection.

Oxidant	Reaction Equation
Superoxide anion	$NADPH + 2O_2 \leftrightarrow NADP^+ + 2O_2^{-\cdot} + H^+$
	$2O_2^{-\cdot} + H^+ \rightarrow O_2 + H_2O_2$
Hydrogen peroxide	$Hypoxanthine + H_2O + O_2 \Leftrightarrow xanthine + H_2O_2$
	$Xanthine + H_2O + O_2 \Leftrightarrow uric\ acid + H_2O_2$
Hydroxyl radical	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$
Hypochlorous acid	$H_2O_2 + Cl^- \rightarrow HOCl + H_2O$
Peroxyl radicals	$R^\bullet + O_2 \rightarrow ROO^\bullet$
Hydroperoxyl radical	$O_2^- + H_2O \Leftrightarrow HOO^\cdot + OH^-$

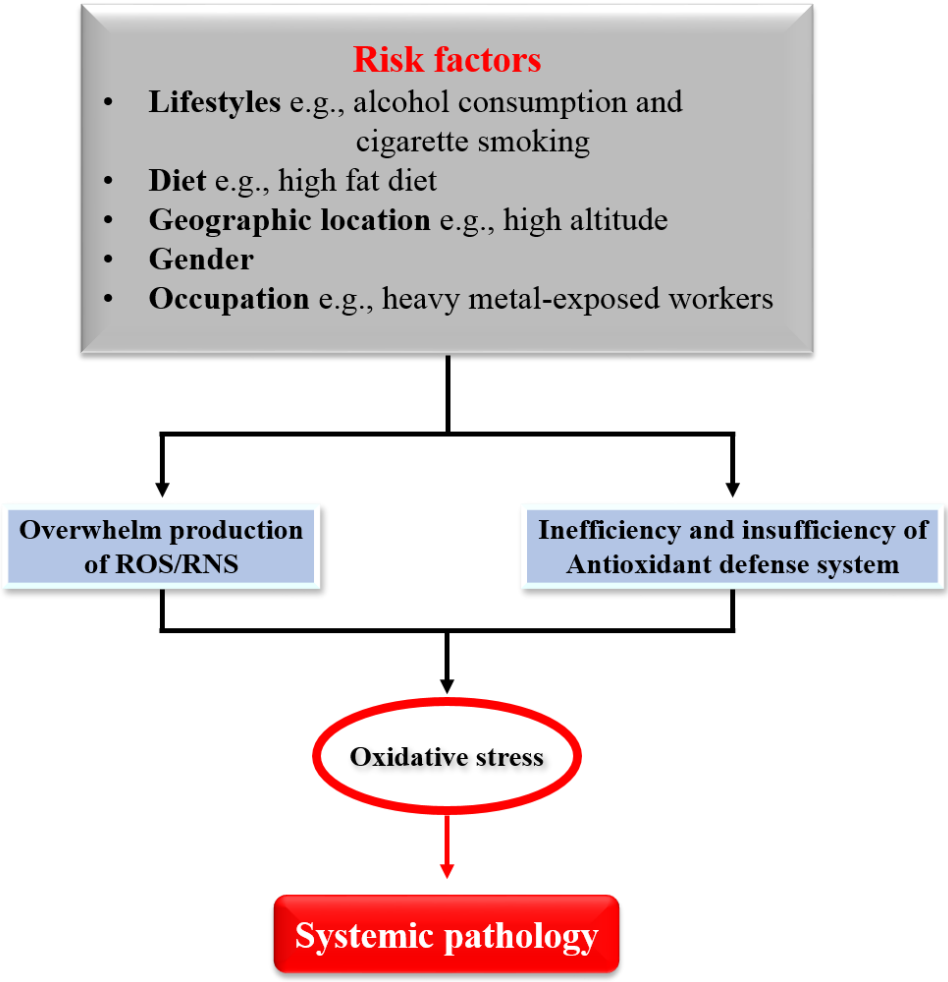

Figure 2. Risk factors related with oxidative stress-induced pathologies [13].

Risk factors which are related to oxidative stress-induced pathologies include alcohol consumption, cigarette smoking, diet, gender, geographic.

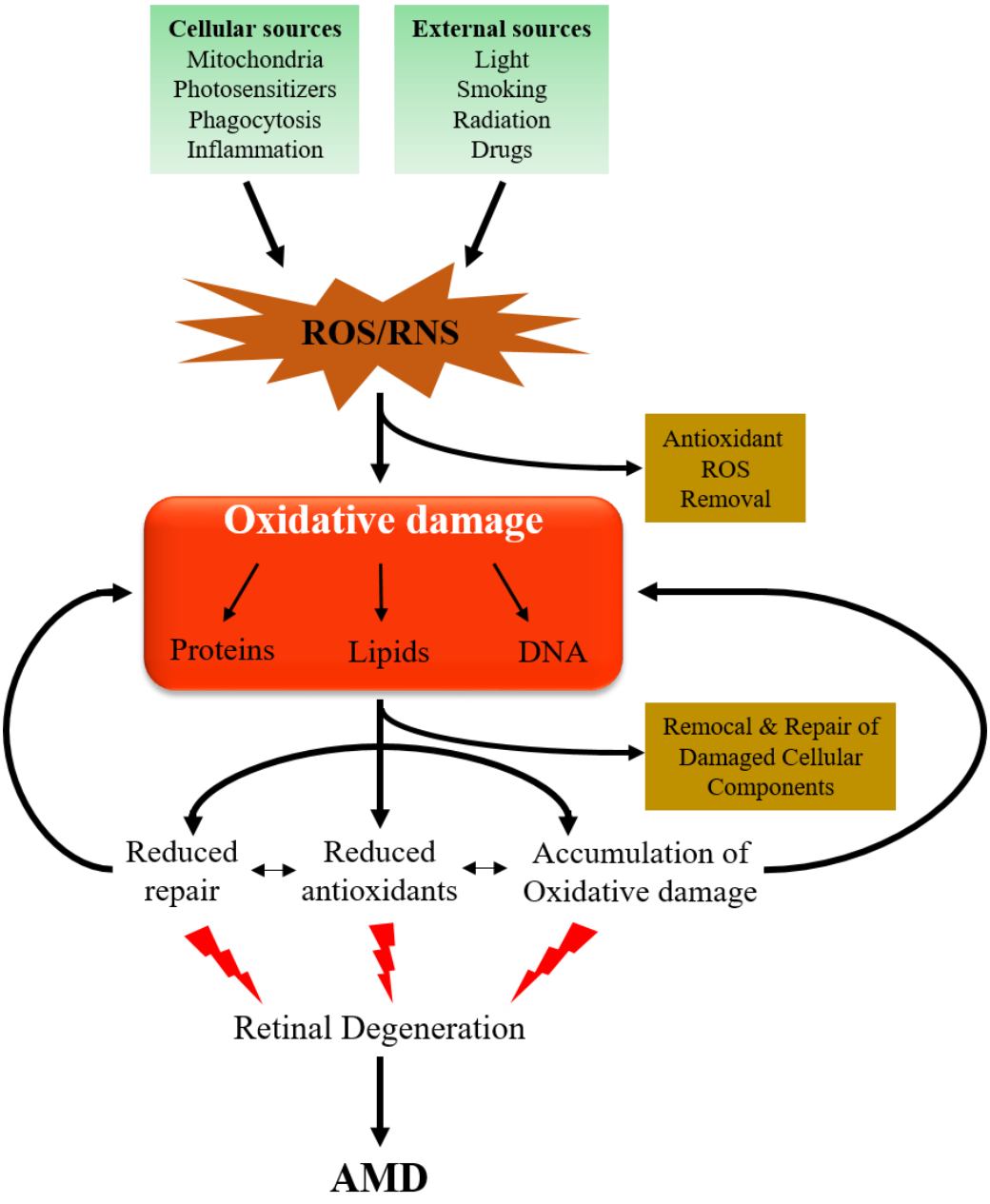

Figure 2. A diagram depicting the potential pathways leading from oxidative stress to retinal degeneration and age-related macular degeneration [27].

Lifelong accumulation of chronic oxidative damage will lead to dysfunction in retinal cells and increase their susceptibility to exogenous and endogenous insults eventually culminating in loss of visual function and cell death

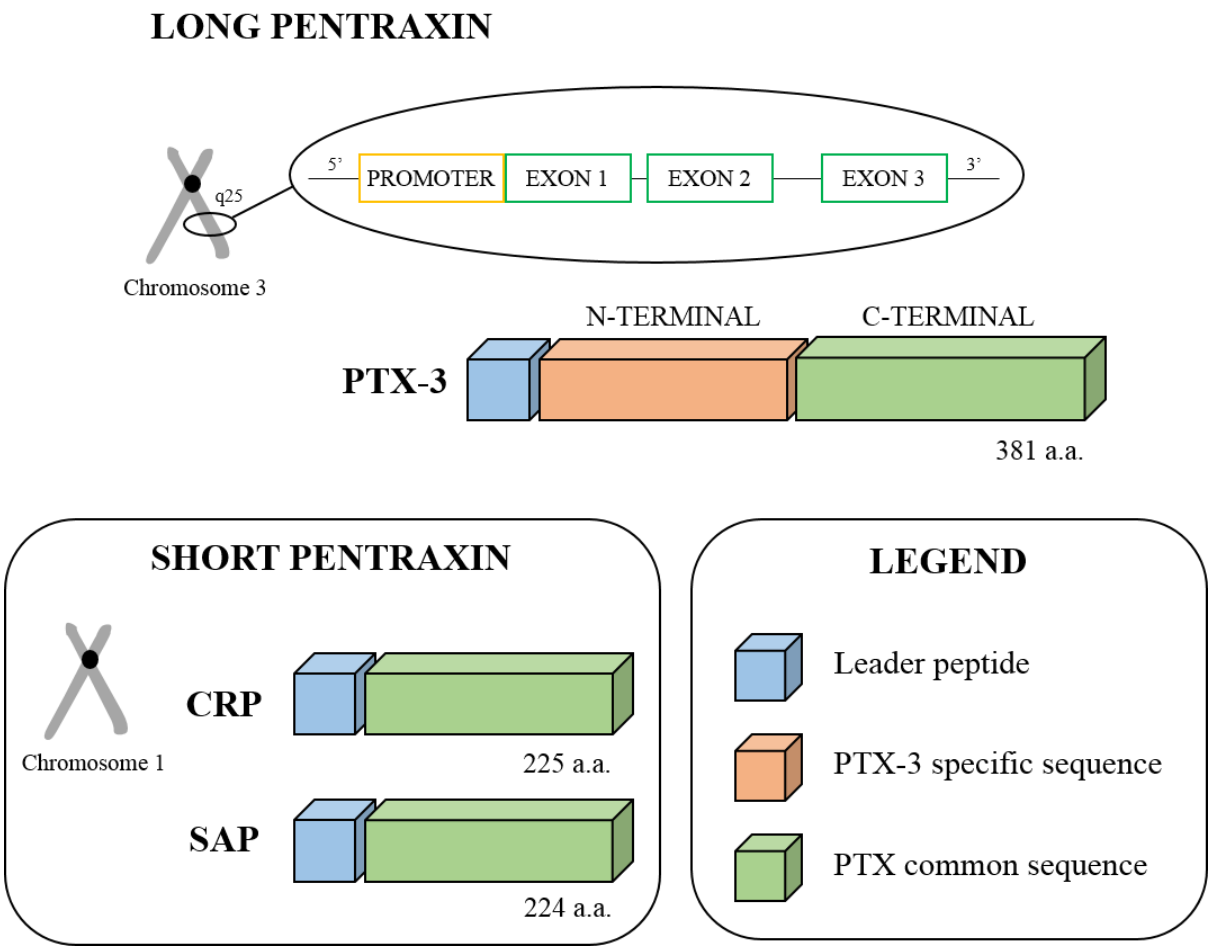

Figure 3. Pentraxin superfamily proteins [29].

Pentraxins (PTXs) are characterized by a conserved C-terminal region including the "PTX signature" sequence. Depending on their length, PTXs are classified in short and long ones. The first described short PTXs are C-reactive protein (CRP) and serum amyloid P component (SAP); their genes are both located in chromosome 1. Differently, the PTX-3 gene is located in chromosome 3 and formed by a promoter region and three exons.

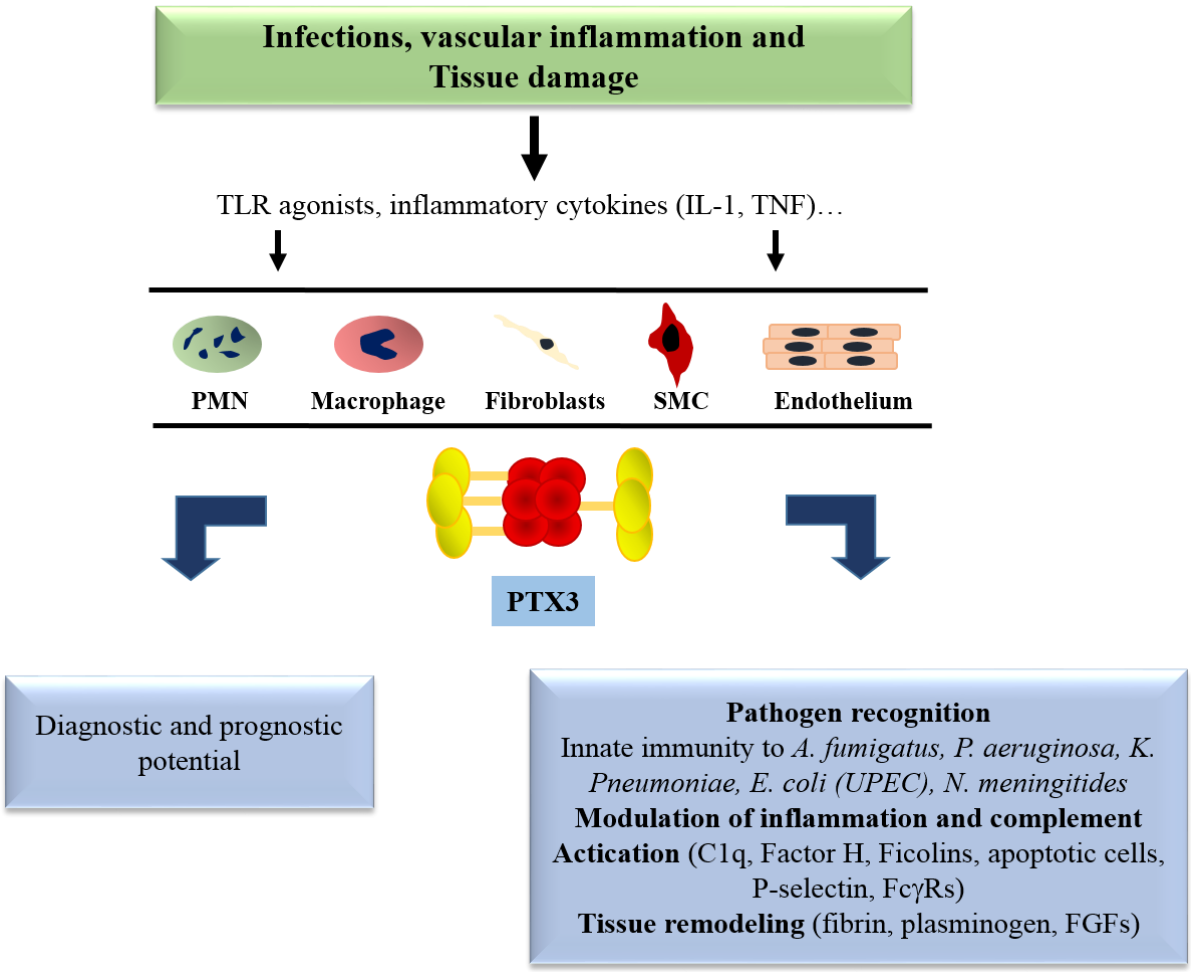

Figure 4. Schematic view of the functional roles of pentraxin 3 [32].

After injury or infection, proinflammatory cytokines and microbial moieties induce PTX3 production by neutrophils (PMNs), macrophages (MFs), and mesenchymal cells [fibroblasts, endothelium, smooth muscle cells (SMCs)]. Once released, PTX3 becomes a potential diagnostic and prognostic marker of inflammation and tissue damage. By interacting with various microbial or endogenous ligands, it is an important player in innate immunity and the regulation of inflammation as well as tissue remodeling and repair.

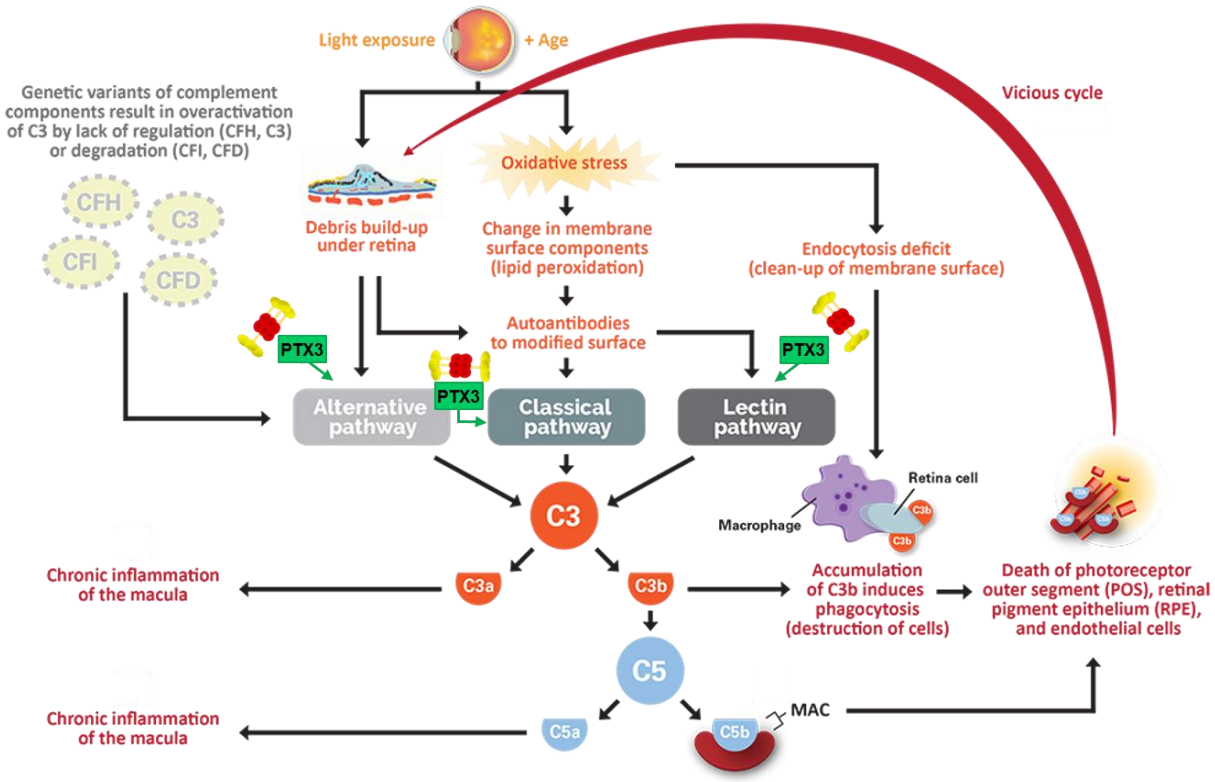

Figure 5. Schematic representation of pentraxins 3 involvement in regulation of complement activity in age-related macular degeneration [35].

The PTX3 have been found to interact with molecules of the complement system, indicating that they can use complement for their effector functions. Complement activation is triggered by the interaction of microbes with recognition molecules. The complement system is an important part of the immune system. Genetic and environmental risk factors can cause the body to lose control over the complement system, resulting in an overactivation (dysregulation) of the immune system. This dysregulation has been proposed as the cause of Age-related macular degeneration (AMD).

Results

Oxidative stress induces PTX3 expression

In general, oxidative stress is a well-known stimulus for retinal pigment epithelial (RPE) dysfunction in aging and the development of AMD [6, 37, 38]. Previously described observations suggest that PTX3 expression was enhanced in response to either pro-inflammatory mediators or endoplasmic reticulum stress inducers in human RPE cells. Specifically, plasma PTX3 levels were elevated in patients with neovascular AMD [10, 39]. However, the expression and biological functions of PTX3 under oxidative stress conditions in RPE cells have not yet been studied. To determine whether PTX3 is produced under oxidative stress in H-RPE cells (the human retinal pigment epithelial primary cells) and ARPE-19 cells (the human retinal pigment epithelial cell line) were stimulated with vehicle and oxidative stress inducer, $NaIO_3$, (500 μM, 100 μM) respectively. I analyzed both mRNA and protein expression levels of PTX3 in H-RPE and ARPE-19 cells in response to oxidative stress. I isolated total RNA from human primary H-RPE and ARPE-19 cells after treatment with $NaIO_3$ for the indicated doses (50 nM -2.5 mM) at 24 hours (Figure 1A and 1B). Using quantitative real time (RT)-PCR, PTX3 mRNA expression increased in H-RPE (500 μM $NaIO_3$ treatment) and ARPE-19 cells (100 μM $NaIO_3$ treatment). mRNA levels of PTX3 were 2.23±0.03-fold fold higher in H-RPE cells and 3.01±0.01-fold fold higher in ARPE-19 cells in the presence of 500 μM and 100 μM $NaIO_3$, respectively, compared with the vehicle treatment. Additionally, PTX3 mRNA levels began to increase and reached maximum expression 24 hours after $NaIO_3$ treatment in H-RPE (500 μM) and ARPE-19 cells (100 μM) (Figure 1C and 1D). These results showed that $NaIO_3$ upregulates the transcriptional level of PTX3 in retinal pigment epithelial cells. I next investigated PTX3 protein levels using ELISA methods after collecting supernatants post-$NaIO_3$ administration in H-RPE and ARPE-19 cells (Figure 2). Similar to the data of

Figure 1, the protein levels of PTX3 were upregulated with administration of various doses of $NaIO_3$ for 48 hours in H-RPE (500 μM) and ARPE-19 cells (100 μM) (Figure 2A and 2B). The protein levels of PTX3 were markedly increased 24 and 48 hours after $NaIO_3$ treatment compared with the vehicle treatment in H-RPE (500 μM) and ARPE-19 cells (100 μM) (Figure 2C and 2D). These data suggest that $NaIO_3$ promoted oxidative stress, which resulted in increased PTX3 mRNA and protein expression in human retinal pigment epithelia cells.

Oxidative stress activates PTX3 expression via PI3K/AKT signaling pathway

To identify the signaling molecules involved in regulating PTX3 expression by $NaIO_3$, I isolated protein from H-RPE cells at various time points after $NaIO_3$ (500 μM) administration. NaIO3 did not have a significant effect on overall unphosphorylated AKT, ERK, JNK, p38, and IκBα. The phosphorylation and expression of the signaling molecules over time were slightly altered by $NaIO_3$ administration, however, phosphorylation of AKT at Thr308 and Ser473, and phosphorylated ERK were increased by $NaIO_3$ in H-RPE cells (Figure 3A). Although phosphorylation of ERK was increased, phosphorylation of p38, JNK, and IκBα were weak in response to $NaIO_3$. I then assessed which signaling pathway(s) were responsible for stimulating PTX3 production upon $NaIO_3$ exposure in H-RPE cells. I used specific inhibitors of LY294002 (PI3 kinase inhibitor), NAC (cytosolic ROS scavenger), U0126 (MEK1/2 inhibitor), SB203580 (p38 MAP kinase inhibitor), SP600125 (JNK MAP kinase inhibitor), and Bay 11–7082 (NF-κB inhibitor), respectively [40, 41, 42, 43]. The H-RPE cells were treated with LY294002 (5 μM), U0126 (1 μM), SB203580 (10 μM), SP600125 (5 μM), and Bay 11–7082 (1 μM), in the presence or absence of $NaIO_3$, and mRNA or protein levels of PTX3 were assessed 24 hours or 48 hours after administration. LY294002, U0126, and NAC blocked mRNA and protein levels of PTX3 in response to $NaIO_3$ (Figure 3B and 3C). However, SB203580 (10 μM), SP600125 (5 μM), and Bay 11–7082 (1 μM) exerted no effect on PTX3 expression in the presence of $NaIO_3$ (Figure 3B and 3C). These data suggest that the ROS, AKT, and ERK signaling pathways may play a role in PTX3 production in response to $NaIO_3$ in human retinal pigment epithelial cells.

PTX3 enhances oxidative stress-induced antioxidant enzymes and ROS generation

To investigate the effects of PTX3 expression under $NaIO_3$-induced oxidative condition, I generated hPTX3 shRNA or control shRNA expressing ARPE-19 cells. To check down regulation of PTX3 expression in hPTX3 shRNA expressing ARPE-19 cells compared with control shRNA expressing ARPE-19 cells, total RNA and supernatants were harvested and $NaIO_3$-induced PTX3 mRNA and protein levels were analyzed hPTX3 shRNA or control shRNA expressing ARPE-19 cells. mRNA and protein levels of PTX3 were decreased in hPTX3 shRNA expressing ARPE-19 cells compared with control shRNA expressing ARPE-19 cells (Figure 4A and 4B). Oxidative stress is well known to induce RPE cell death in AMD [44, 45]. To further investigate the critical role of PTX3 in AMD pathogenesis, such as oxidative stress, RPE cell death, and AMD-associated gene expression, I examined mRNA levels of antioxidative enzymes in hPTX3 shRNA expressing ARPE-19 cells compared with control shRNA expressing ARPE-19 cells in response to $NaIO_3$. I harvested RNA from control or hPTX3 shRNA expressing ARPE-19 cells 24 hours after vehicle or $NaIO_3$ treatment. Thereafter, mRNA levels of antioxidant enzymes, such as glucose-6-phosphate dehydrogenase (G6PDH), catalase, glutathione S-reductase (GSR), glutathione peroxidase 1 (GPX1), superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2), were analyzed using quantitative real-time RT-PCR (Figure 4C-4H). mRNA levels of G6PD, catalase, and GSR increased in response to $NaIO_3$ in control shRNA expressing ARPE-19 cells, but not in hPTX3 shRNA expressing ARPE-19 cells (Figure 4C-4E). However, mRNA levels of GPX1, SOD1, and SOD2, did not increase in response to $NaIO_3$ in both shRNA expressing ARPE-19 cells (Figure 4F-4H).

PTX3 expression promotes oxidative stress-induced cell death

Cell viability was assessed to determine cellular response to $NaIO_3$. The cell viability of ARPE-19 cells decreased by 48.78±2.19% in response to 5 mM $NaIO_3$ (Figure 5A). Only NAC (cytosolic ROS scavenger) and LY294002 (PI3 kinase inhibitor) rescued the cell viability of ARPE-19 cells up to 94.29±4.71% and 79.40±2.98%, respectively, in response to 5 mM $NaIO_3$ (Figure 5B). To identify the role of PTX3 in $NaIO_3$-induced RPE cell death, I verified the viability 48 hours after 5 mM $NaIO_3$ administration in control or hPTX3 shRNA expressing ARPE-19 cells. While NAC and LY294002 rescued the cell viability in response to $NaIO_3$ in control shRNA expressing ARPE-19 cells, these inhibitors had no effects on the cell viability in response to NaIO3 in hPTX3 shRNA expressing ARPE-19 cells (Figure 5C).

PTX3 activates age-related macular degeneration marker genes

Thereafter, the effects of PTX3 expression on AMD-associated gene expression in response to NaIO3 using control or hPTX3 shRNA expressing ARPE-19 cells were assessed. mRNA levels of AMD-associated genes, including complement factor I (CFI), complement factor H (CFH), apolipoprotein E (APOE), and toll-like receptor 4 (TLR4) were enhanced 12 hours after $NaIO_3$ exposure to control shRNA expressing ARPE-19 cells, but not in hPTX3 shRNA expressing ARPE-19 cells (Figure 6). These results suggest that $NaIO_3$-induced PTX3 expression could lead to oxidative stress, cell death, and AMD-associated gene expression in RPE cells. Therefore, PTX3 production might play as a pathologic mediator under oxidative condition.

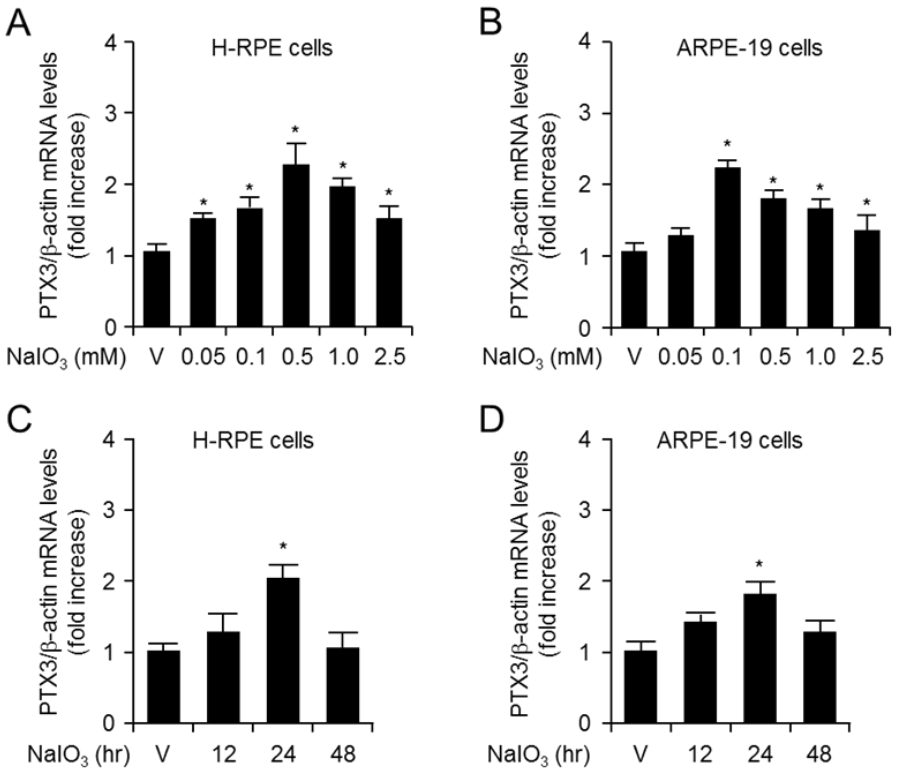

Figure 1. The expression of pentraxin 3 mRNA levels was enhanced after $NaIO_3$ administration in human RPE cells.

Primary human H-RPE cells (A) and ARPE-19 cells (B) were treated for 24 hours in various doses of $NaIO_3$. H-RPE cells were exposed to 500 μM (C) and ARPE-19 cells were exposed to 100 μM $NaIO_3$ (D), for the indicated time points. Second and third of the H-RPE cells were used. Values are presented as mean ± SD, n=3. *$p<0.05$, increased PTX3 mRNA expression after $NaIO_3$ administration vs vehicle (V)

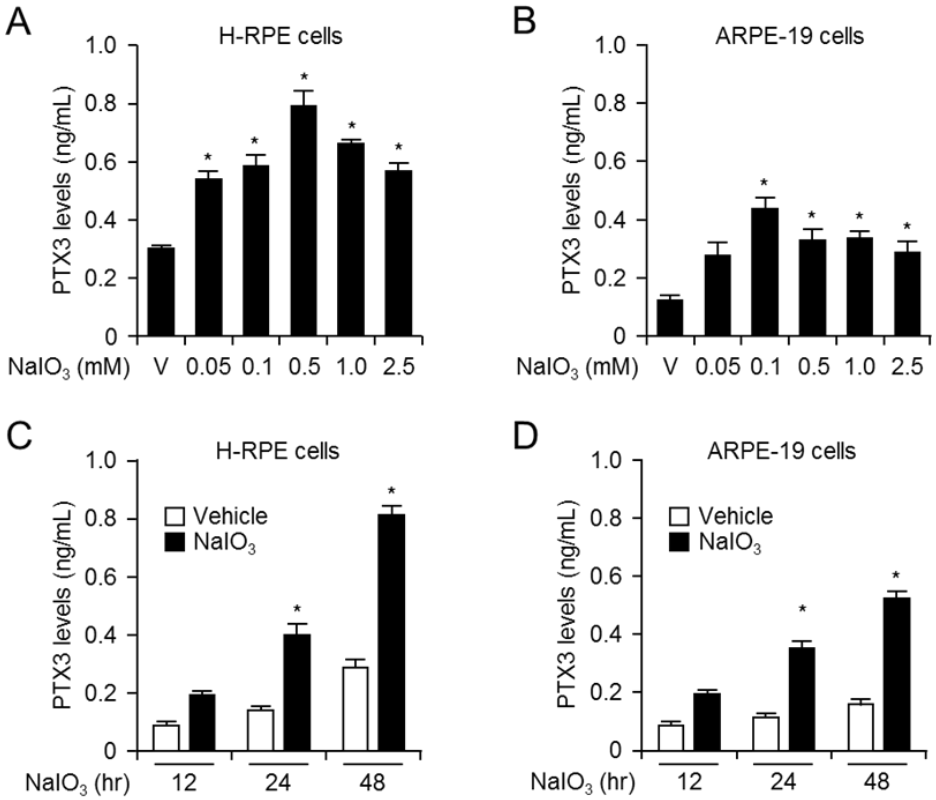

Figure 2. The protein levels of pentraxin 3 were enhanced after $NaIO_3$ administration in human RPE cells.

Primary human H-RPE cells (A) and ARPE-19 cells (B) were treated for 48 hours with vehicle or 500 μM $NaIO_3$ (H-RPE cells) (C) and 100 μM $NaIO_3$ (ARPE-19 cells) (D). Supernatants were harvested and analyzed for PTX3 production. Third and fourth passages of the H-RPE cells were used. Values are presented as mean ± SD, n=12. *$p<0.05$, increased PTX3 after $NaIO_3$ administration vs vehicle (V).

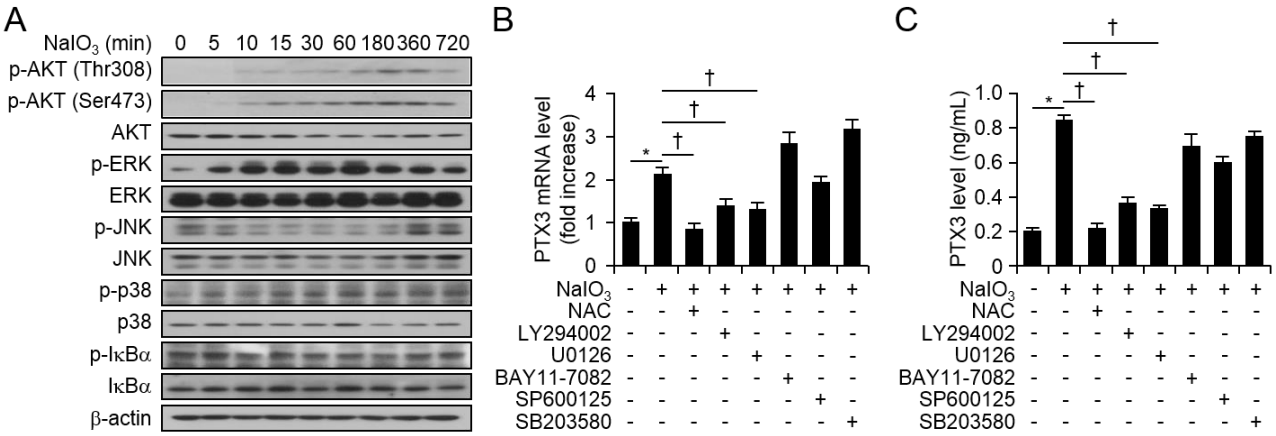

Figure 3. ROS and PI3 kinase signaling pathways are involved in pentraxin 3 induction by $NaIO_3$ in human RPE cells.

The levels of AKT, phosphorylated AKT (Ser473 and Thr308), total ERK, phosphorylated ERK, total JNK, phosphorylated JNK, total IκBα, phosphorylated IκBα, total p38, and phosphorylated p38 proteins were assessed using western blotting analysis (A). β-actin was used as a loading control. Experiments were performed at least three independent times. Total RNA was extracted from H-RPE cells 24 hours after 500 μM $NaIO_3$ with signaling inhibitor (1 μM BAY11-7082, 1 μM U0126, 10 μM SB203580, 5 μM SP600125, 5 μM LY2940002, or 5 mM NAC), administration. Quantitative real-time RT-PCR was performed to assess mRNA levels of PTX3. For all real-time PCR analyses, mouse β-actin was used as a control for normalization. Expression levels of each mRNA are divided by expression of β-actin and shown as a ratio of each mRNA/β-actin. Values are presented as mean ± SD, n=3 (B). Supernatants were harvested from H-RPE cells 48 hours after $NaIO_3$ administration with signaling inhibitors (C). Supernatants were harvested and measured for PTX3 production using human PTX3 ELISA kit. Third and fifth passages of the H-RPE cells were used. Values are presented as mean ± SD, n=12. *$p<0.05$, increased PTX3 after $NaIO_3$ administration vs vehicle (V). †$p<0.05$, decreased PTX3 in response of NaIO3 plus signaling inhibitor vs $NaIO_3$ alone.

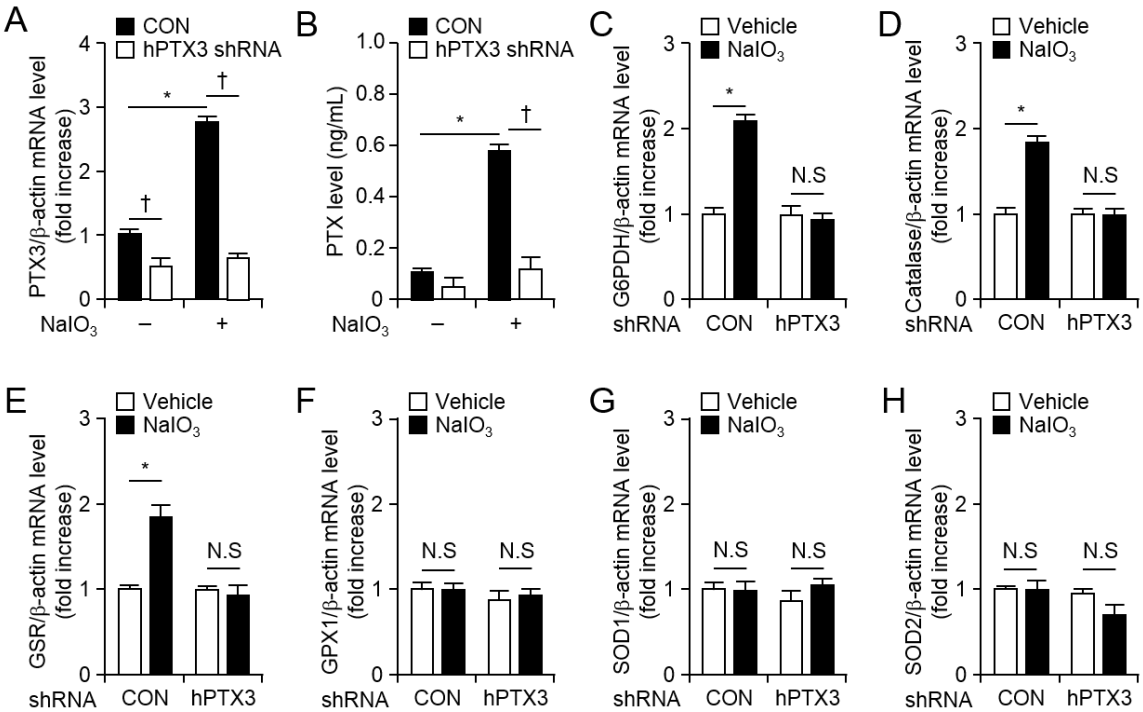

Figure 4. mRNA levels of antioxidant enzymes were decreased in hPTX3 shRNA expressing ARPE-19 cells in response to $NaIO_3$.

Total RNA was extracted from control or PTX3 shRNA expressing ARPE-19 cells 24 hours after 100 μM $NaIO_3$. mRNA expression (A) and protein levels (B) of PTX3 were analyzed. mRNA levels of G6PDH (C), CAT (D), GSR (E), GPX (F), SOD1 (G), and SOD2 (H) were analyzed by quantitative real-time RT-PCR. Human β-actin was used as a control for normalization. Expression levels of each mRNA are divided by expression of β-actin and shown as a ratio of each mRNA/β-actin. Fifth and sixth passages of the H-RPE cells were used. Values are presented as mean ± SD, n=3. †$p<0.05$, decreased mRNA levels of antioxidant enzyme after NaIO3 administration vs vehicle. *$p<0.05$, increased mRNA levels of antioxidant enzyme after $NaIO_3$ administration vs vehicle. N.S. indicates non-significance.

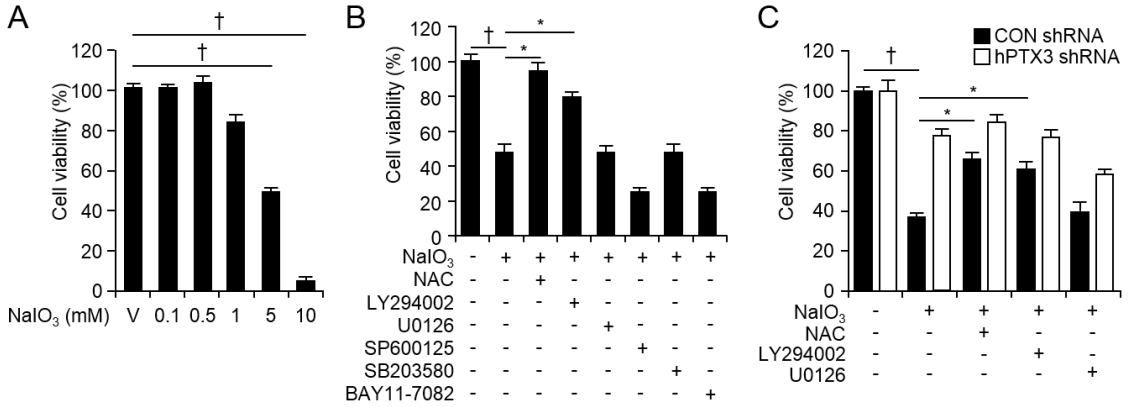

Figure 5. Cell viability was enhanced in hPTX3 shRNA expressing ARPE-19 cells in response to $NaIO_3$.

The cell viability was analyzed 48 hours after various doses of $NaIO_3$ administration in ARPE-19 cells (A). †$p<0.05$, decreased the cell viability after $NaIO_3$ administration vs vehicle (V). The cell viability was analyzed in response to $NaIO_3$ or $NaIO_3$ plus signaling inhibitors in ARPE-19 cells (B). †$p<0.05$, decreased the cell viability after $NaIO_3$ administration vs vehicle (V). *$p<0.05$, rescued the cell viability in response to $NaIO_3$ plus signaling inhibitors vs $NaIO_3$ alone. The cell viability was analyzed in response to $NaIO_3$ or $NaIO_3$ plus signaling inhibitors in control or hPTX3 shRNA expressing ARPE-19 cells (C). Fourth and fifth passages of the H-RPE cells were used. †$p<0.05$, decreased the cell viability after $NaIO_3$ administration vs vehicle (V). *$p<0.05$, rescued the cell viability in response to $NaIO_3$ plus signaling inhibitors vs $NaIO_3$ alone. Values are presented as mean ± SD, n=12.

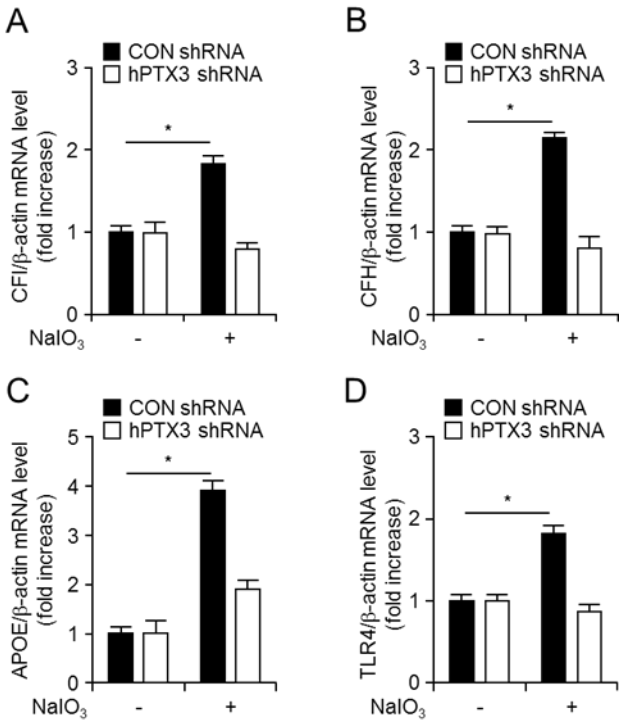

Figure 6. mRNA levels of AMD-associated genes were suppressed in hPTX3 shRNA expressing ARPE-19 cells in response to $NaIO_3$.

Total RNA was extracted from control or hPTX3 shRNA expressing ARPE-19 cells 12 hours after 100 μM $NaIO_3$ administration. mRNA levels of CFI (A), CFH (B), APOE (C), and TLR4 (D) were analyzed by quantitative real-time RT-PCR. Human β-actin was used as a control for normalization. Expression levels of each mRNA are divided by expression of β-actin and shown as a ratio of each mRNA/β-actin. Fourth passage of the H-RPE cells was used. Values are presented as mean ± SD, n=3. *$p<0.05$, increased mRNA levels of genes after $NaIO_3$ administration vs vehicle.

Discussion

AMD is a major cause of legal blindness in the elderly in developed countries. Furthermore, millions of AMD patients lose their sight each year, as there is no effective treatment for dry AMD [46, 47]. AMD is associated with several risk factors, and many of them are linked to increased oxidative stress. Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of oxygen reactive species (ROS) in cells and tissues. Oxidative stress is a major factor in retinal pigment epithelium (RPE) cell injury that leads to AMD-related pathological changes [48]. $NaIO_3$ (sodium iodate), oxidative stress inducer, is an oxidative toxic agent and its selective RPE cell damage allows it to be used as a reproducible AMD model in vitro and in vivo. Despite several publications using this model to describe cell death and molecular events underlying oxidative stress-induced cellular responses mimicking the pathogenesis of AMD, cell viability control remains unclear in RPE cells. Although the role of PTX3 in many diseases is controversial, PTX3 is considered an inflammatory marker in many inflammatory diseases, including vascular disease. Previously, I demonstrated the expression of PTX3 in response to inflammatory stimuli and ER stress inducers [49]. I reported that plasma PTX3 levels were elevated in patients with neovascular AMD [10]. However, the expression and effects of PTX3 in $NaIO_3$-induced signaling pathways and cell viability have not been elucidated in RPE cells, yet. In this study, I showed that $NaIO_3$ induces mRNA and protein levels of PTX3 in H-RPE and ARPE-19 cells. The expression and functions of PTX3 have been described in RPE cells, including our previous studies. Our research has indicated the expression and importance of PTX3 in RPE cells. Further, Nissen and colleagues have demonstrated that PTX3 acts as a ligand of complement factor H (CFH), and may participate in AMD immunopathogenesis [50]. Handa and colleagues have shown that PTX3 activity is induced by oxidative stress inducer, 4-hydroxynonenal (4-HNE), and acts

as an essential brake for complement and inflammasome activation in ARPE-19 cells [51]. However, I have observed different expression levels of inflammatory cytokines, IL-6, IL-1β, and TNFα, in the presence of $NaIO_3$ in PTX3 shRNA expressing ARPE-19 cells compared with control shRNA expressing ARPE-19 cells (data not shown). In recent study, they found that NaIO3 can induce cytosolic ROS but not mitochondrial ROS production and activate ERK, p38, JNK, and AKT signaling pathway [52]. Especially they described that cytosolic ROS-dependent p38 and JNK activation lead to cell death in NaIO3-treated ARPE-19 cells. On the other hand, they showed that cytosolic ROS-mediated autophagy and balance of mitochondrial dynamics contribute to cell survival, also. In their study, they suggested that NaIO3-induced ROS could simultaneously regulate multiple cellular events. Like their suggestion, NaIO3 induced activation of p38, JNK, EKR, and ROS signaling pathway in H-RPE cells. Among these signaling pathways, ROS, AKT, and ERK signaling pathways were involved in PTX3 expression in H-RPE cells, most of all, only ROS and PI3 kinase pathways were engaged in NaIO3-induced RPE cell death. Handa's group asserted that 4-HNE-induced PTX3 exerts protective effects against oxidative stress-induced complement and inflammasome activation [37]. $NaIO_3$-induced RPE cell death was rescued in PTX3 shRNA expressing ARPE-19 cells compared with control shRNA expressing ARPE-19 cells. More importantly, the expression of oxidative stress-induced antioxidant enzymes, G6PDH, catalase, and GSR, and AMD-associated genes, including complement factor I (CFI), complement factor H (CFH), apolipoprotein E (APOE), and toll-like receptor 4 (TLR4), were decreased in PTX3 shRNA expressing ARPE-19 cells. These data suggest that oxidative stress and a risk for AMD were reduced in PTX3 shRNA expressing ARPE-19 cells. In this study, I provide information regarding the critical role of PTX3 under oxidative stress conditions in the early stage of AMD development, especially the loss of RPE cells.

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Part 2.

The role of autophagy against ER stress-induced cell death in human retinal pigment epithelial cells.

Abbreviations

RPE	Retinal pigment epithelium
AMD	Age-related macular degeneration
DR	Diabetic retinopathy
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
GRP78	Glucose-regulated protein 78
IRE1	Inositol requiring element 1
PERK	Protein kinase RNA-like ER kinase
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
CHOP	C/EBP homologous protein
LC3	Microtubule-associated protein 1 light chain 3
PE	Phosphatidylethanolamine
4-PBA	4-phenylbutyric acid
CQ	Chloroquine
CFI	Complement factor I
CFH	Complement factor H
APOE	Apolipoprotein E
APOJ	Apolipoprotein J
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4

Abstract

Age-related macular degeneration (AMD), a progressive degenerative retinal disease, affects the retinal macula and results in loss of vision, and AMD is the leading cause of severe visual impairment and blindness among elderly people worldwide. AMD is characterized by the accumulation of discrete drusen deposits between the RPE and Bruch membrane and dysfunction of retinal pigment epithelial (RPE) cells. Since RPE, a postmitotic polarized cuboidal monolayer, is a controlling or preventing cell death in these conditions may help to prevent subsequent photoreceptor degeneration and vision loss. Plenty of evidence has revealed that the RPE dysfunction in AMD has been attributed to several pathological pathways including the accumulated effects of oxidative stress, toxic metabolites, and inflammation [1]. The pathogenesis of AMD remains unclear, and no effective treatment exists. Mitochondria and the endoplasmic reticulum which lie in close anatomic proximity to each other are targets of oxidative stress and endoplasmic reticulum (ER) stress, respectively, and contribute to the progression of AMD [2].

The ER stress, an accumulation of unfolded or misfolded proteins in the ER lumen, is triggered by loss of homeostasis in the ER, resulting in complex cellular response known as the unfolded protein response (UPR). Once the UPR is initiated cell undergoes various adaptive response the upregulation of chaperones, including glucose-regulated protein 78 (GRP78), which is normally bound to the luminal domain of three trans-membrane ER proteins: inositol requiring element 1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6) [3]. However, if this adaptive response fails to reestablish ER homeostasis, lead to a pro-apoptotic pathway [4]. ER stress induced apoptosis occurs through multiple mechanisms. One of the major signal transducers believed to be involved is C/EBP homologous protein (CHOP), depends on activation of capase-3.

Autophagy, A double-membrane structure, is essential in maintaining homeostasis, which requires protein degradation for energy needs, by removing damaged substrates for recycling [5]. As a result, autophagy is important response mechanism under different kinds of cellular stress, such as protein aggregate-induced stress and ER stress, generally promoting cell survival and cell death. The current consensus is that autophagy's role as regards cell death is primarily protective [6]. Autophagy removes damaged organelles and protein aggregates of RPE cells, which is a crucial function because these cells are exposed to celluar stress [7]. Studies have reported that autophagy occurs in RPE cells [8]. However, the role of autophagy in response to ER stress-induced cell death have not been investigated in RPE cells. . In the present study, I demonstrated that autophagy is activated by ER stress inducer has the protective effects by decreasing CHOP expression molecules in human RPE cells. Moreover, ER stress-induced autophagy is involved in the ROS production, particularly NAD(P)H oxidase, and the expression of AMD-related genes, including complement factor I (CFI), complement factor H (CFH), apolipoprotein E (APOE), apolipoprotein J (APOJ) toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4), in human RPE cells and accelerated PRE cell death. Taken together these results suggesting that critical insights into the protection effects of autophagy during ER stress in the early stages of AMD development.

Introduction

1. Autophagy

Autophagy (self-eating) is an evolutionarily conserved pathway occurring in eukaryotic cells. It is an important catabolic process that is used to deliver cytoplasmic molecules, including organelles and proteins, to the lysosome for degradation and recycling. It is induced under stress conditions such as absence of growth factors, low oxygen levels, or nutrient starvation assuring cell survival [9, 10]. Under the action of stress factors, basal autophagy may act as a pro-survival mechanism in an adverse environment. Autophagy is a highly regulated cell death process which cells use to recycle cellular contents for survival. The core autophagy-related (Atg) complexes in mammals are ULK1 protein kinases, Atg9-WIPI1 and Vps34-Beclin1 class III PI3-kinase complexes, and the Atg12 and microtubule-associated protein 1 light chain 3 (LC3) conjugation systems. PI(3)-binding proteins, PI3-phosphatases and Rab proteins also contribute significantly to autophagy. Several molecular markers of autophagy have been studied to date but the conversion of LC3-I to LC3-II via phosphatidylethanolamine (PE) conjugation has been accepted as the gold standard for autophagosome formation. p62/SQSTM1 is also important since it is a substrate for LC3 which facilitates selective degradation during autophagy. (Figure 1) [11, 12]. Although controversial, mitochondria and plasma membrane could also supply membranes for the formation of the autophagosomes under different conditions [13]. The elongating isolation membrane surrounds cargo that is ultimately enclosed in the double membrane autophagosome. Autophagosomes are represent the functional and structural hallmark of autophagy. Proteins that play a role in the formation of autophagosomes have been discussed to harbor the potential to visualize autophagy in tissue samples. The LC3B, in its cytosolic LC3B-I form is transformed to the lipidated LC3B-II while forming an integral part of the membranes

of the autophagosomes. p62/sequestosome 1 (SQSTM1) interacts with LC3B, targeting ubiquitinated substrates to autophagosomes (Figure 2) [14]. Once the autophagosome is formed, it fuses with lysosomes to form autolysosomes in which the cargo is degraded by lysosomal hydrolases. At this stage lysosomes must reform so that subsequent autophagy may occur [15]. Autophagy is also implicated in various diseases, including cancer, neurodegenerative diseases, pathogen invasion, and muscle and liver disorders. In most of these situations, autophagy has both beneficial and harmful effects [16].

2. Retinal diseases associated with autophagy

Autophagy plays an important role in cell survival, development, differentiation and intracellular homeostasis. Autophagy-related proteins are highly expressed in the retina, including in the ganglion cell layer, the inner nuclear layer, the outer nuclear layer and the RPE layer. The cells in these layers have a high energy demand and are susceptible to mitochondrial damage [17, 18]. Increasing evidence has shown that autophagy proteins are involved in retinal physiology and pathology and that defective autophagy contributes to retinal degeneration (Figure 3). Autophagy is responsible for degrading cellular components, and any autophagic defects will disrupt intracellular homeostasis and can cause specific retinal diseases. In retinal diseases, autophagy plays a dual role: promoting retinal cell survival and death. Autophagy at a normal level helps retinal cells defend themselves against harmful stress; however, excessive autophagy results in retinal deterioration [22].

2.1. Autophagy and AMD

The pathology of AMD is related to inflammation, oxidative stress, and defective lysosomal or proteasomal function, leading to the accumulation of abnormal intra- and extracellular products. The disease is characterized by the accumulation of drusen, which are abnormal extracellular deposits along the basal layer of the RPE, and drusen deposition is correlated with RPE damage. Autophagy clearly plays a protective role against diseases of the retina and RPE [17]. RPE cells are post-mitotic phagocytes that are non-self-renewing; therefore, the autophagy of these cells' intracellular components is essential

for normal cellular function. During the early stages of AMD, the autophagic level is elevated to compensate for damaged organelles and the results of oxidative stress. In human AMD samples and mouse AMD models, the levels of LC3, ATG9 and ATG7 are increased in the RPE and retinal layers [19]. However, in samples from advanced AMD, the levels of LC3, ATG9 and ATG7 are decreased, and the lack of autophagic activity causes the late-stage disease [22].

2.2. Autophagy and DR

Diabetic retinopathy (DR) is a serious complication of diabetes mellitus. It is a retinal microangiopathy, and damage to the pericytes on the retinal capillaries is evident during the early stages of DR. Retinal capillary occlusion resulting in elevated vascular permeability and ischaemia of the retina causes breaks in the blood-retina barrier and angiographic leakage [20]. The role of autophagy in DR is quite complicated. Autophagy promotes pericyte survival in early DR, whereas excessive autophagy causes excess stress and leads to necrosis. High blood glucose levels also trigger autophagy to prevent glucose-induced damage in the RPE. Moreover, a damaged blood-retina barrier releases cytoplasmic lipoproteins, and extra-vascular modified LDL promotes RPE damage through ER stress, oxidative stress, apoptosis and autophagy. In this case, autophagy is detrimental to DR treatment [21], suggesting that autophagy might be a potential target to treat DR [22].

3. Endoplasmic reticulum (ER) stress and unfolded protein response (UPR) signaling

The ER is the central intracellular organelle that contributes to the production and folding of cellular proteins and is involved in the maintenance of cellular homeostasis. The ER is responsible for protein translation, folding, synthesis, maturation and protein post-translational modification that only correctly folded proteins can reach their cell compartment. It also allow further transport of proteins to the Golgi apparatus and ultimately to vesicles for secretion or display on the plasma surface [23]. A specific ER stress pathway is activated when unfolded or misfolded proteins accumulate within the ER lumen, known as the unfolded protein response (UPR), a process named “ER-stress”. In cases where ER stress

cannot be reversed, cellular functions deteriorate, often leading to cell death. Accumulating evidence implicates ER stress-induced cellular dysfunction and cell death as major contributors to many diseases, making modulators of ER stress pathways potentially attractive targets for therapeutics discovery [23]. The UPR is orchestrated by three main sensors. These three main sensors of UPR that are initiated by distinct ER stress transducers located on the ER membrane: PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). In nonstressed cells, all three ER stress transducers are kept in an inactive state through binding to the chaperone glucose-regulated protein GRP78/BiP. Upon ER stress, excessive unfolded proteins accumulate in the ER lumen, resulting in the dissociation of GPR78 from the ER stress transducers, which triggers activation of the UPR branches. GPR78 has a crucial role in cell protection against ER stress: it is associated with the luminal domains of the UPR transducers, preventing PERK and IRE1 homo-dimerization, and impeding the translocation of ATF6 to the Golgi (Figure 4) [24].

3.1. The IRE1/XBP1 pathway

Inositol-requiring enzyme 1 (IRE1) similarly oligomerizes in ER membranes when released by Grp78. It was firstly identified as an ER transmembrane protein kinase is essential for signaling transduction from the ER to the nucleus. There are two different IRE1 proteins, both of which participate in the ER stress response or UPR. IRE1α is ubiquitously expressed while IRE1β is tissue-specific [25]. Activated IRE1α protein acquires the function as endogenous ribonuclease (RNase) and splices a 26-nucleotide intron from the mRNA of XBP1. The splicing results in a shift in the translational frame of the XBP1 gene, leading to the translation of a new protein, named spliced XBP1. The newly generated spliced XBP1 is an active transcription factor, which in turn induces diverse downstream genes, such as ER chaperones [26] and proteins involved in ER-associated protein degradation (ERAD). These proteins work together to restore the ER homeostasis and promote cell survival.

3.2. The PERK/eIF2α/ATF4/CHOP pathway

PKR-like ER kinase (PERK) is a serine/threonine protein kinase located on the ER membrane. Like IRE1, PERK is activated by ER stress via dimerization and autophosphorylation upon the dissociation with Bip. Activated PERK phosphorylates its downstream target protein, eIF2α, thereby globally shutting off mRNA translation and reducing the protein load on the ER. However, certain mRNAs gain a selective advantage for translation under these conditions, including the mRNA encoding transcription factor activating transcription factor 4 (ATF4). The ATF4 protein is a member of the bZIP family of transcription factors, which regulates the promoters of several genes implicated in the UPR. In addition, ATF4 is a major inducer of C/EBP homologous protein (CHOP), which has been considered as a central mediator of ER stress-induced apoptosis. CHOP, also named as growth-arrest and DNA-damage-inducible gene 153 (GADD153), is a major stress-inducible proapoptotic gene in ER stress-induced apoptosis [27]. CHOP is expressed at a very low level under physiological conditions but its expression level significantly increases in the presence of severe or persistent ER stress. Notably, the induction of CHOP well correlates with the onset of ER stress-associated apoptosis (Figure 5) [28].

3.3. The ATF6 pathway

Besides IRE1 and ATF4, Release of Grp78 from the N-terminus of activating transcription factor 6 (ATF6) triggers a different mechanism of protein activation. ATF6 has been identified as another basic leucine zipper- (bZIP-) containing transcription factor induced by ER stress. Like IRE1 and PERK, ATF6 binds to Bip and remains in an inactive state in unstressed condition. In response to ER stress, the Bip/ATF6 complex is dissociated, resulting in the translocation of ATF6 from ER membrane to Golgi apparatus. In Golgi apparatus, ATF6 is cleaved by two proteases, serine protease site-1 protease (S1P) and the metalloprotease site-2 protease (S2P), releasing this transcription factor into the cytosol and allowing it to migrate into the nucleus to regulate gene expression [29]. The active ATF6 then moves to the nucleus and activates the ER stress response element- (ERSE-) related genes through binding their promoters. ATF6 also regulates other URP genes, such as XBP-1 and CHOP [24].

4. ER stress and autophagy

Cells respond to ER stress through the activation of UPR. However, severe ER stress can cause cell death, usually by activating intrinsic apoptosis. Moreover, in order to clear the ER from the accumulation of terminally misfolded protein aggregates that cannot be degraded by the proteasome, the UPR may upregulate the autophagy machinery [30]. Already in the 1980's, ultrastructural studies revealed that cells with autophagic vacuoles often had dilated ER [31]. The first link between protein aggregation in the ER and autophagy derives from data in mammalian cells showing that accumulation of a mutant form of a cell surface protein, decay accelerating factor, was associated with autophagy [32]. Autophagy is a major lysosomal pathway for the in bulk degradation of cytoplasmic materials. In spite of its role as a self-digestion mechanism, autophagy is mainly activated to protect against cell death. The activation of autophagy could results in the degradation of unfolded proteins and assisting cells to cope with stress load. However, just like in the case of the UPR, stimulation of autophagy can under certain circumstances be required to activate the cell death machinery. Although both the UPR and autophagy can function independently from each other, recent reports show that they may be interlinked and share the functional duality of exerting both a cytoprotective and cytocidial activity [30].

5. Retinal diseases associated with ER stress

Recently published literature has provided evidence that the UPR is involved in the development of retinal degeneration. The scope of these studies encompassed DR, glaucoma, light-induced retinal degeneration, AMD and inherited retinal degeneration (Figure 6) [24]. Retinal degeneration is progressive deterioration of the retinal cells, eventually culminating in their death. Together, these findings support a pivotal role of cell death in the pathogenesis of retinal diseases. Apoptosis, that is, programmed cell death, is the most common form of cell death in various cell types, including retinal cells. Apoptosis is tightly controlled by a variety of signaling pathways that either promote or inhibit the apoptotic cascades. The ER stress response has been recently proposed as a contributing factor to retinal degenerative disease [33]. The fact that UPR activation can induce retinal cell death in mice has been previously reported [34]. The primary goal of the UPR is to reestablish homeostasis while

maintaining a prosurvival signaling environment. However, if the ER capacity to deal with persistent stress is insufficient, the UPR-associated signaling shifts from a prosurvival to a proapoptotic cascade that eventually becomes dominant and leads to cell death. A study demonstrating UPR involvement in the cellular mechanism of DR was conducted in 2006 [35]. The authors demonstrated that glucose fluctuation activated UPR-specific enzymes in pericytes, cells that participate in the proliferation of DR. These studies were conducted in cultured pericytes. However, subsequent work in diabetic Akita mouse [36] and rat retinas [37] demonstrated the activation of the ER stress response, suggesting that it may be one of the earlier events in DR progression. In addition, ER stress could be an important mechanism in the pathogenesis of AMD and, with oxidative stress, could trigger inflammation and disease [38]. However, another study suggested that in addition to oxidative stress, impaired phagocytosis, elevation of polyunsaturated fatty acids, and exposure to light can provide ideal conditions for the development of retinal degeneration in the macular region. That said, in human RPE cells, oxidative stress leads to inhibition of proteosomal activity and aggresomal accumulation of ubiquitinated proteins, thus suggesting that prolonged oxidative stress is in turn capable of activating the ER stress response [39].

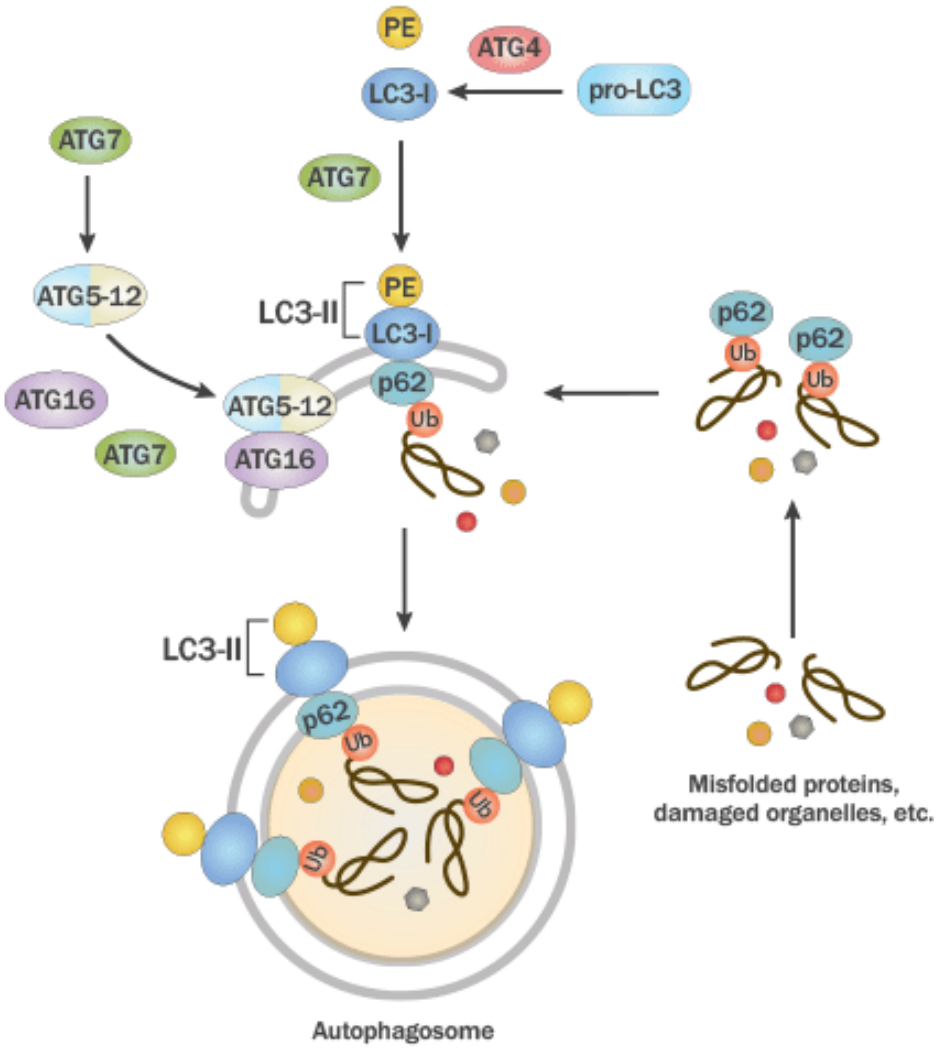

Figure 1. The major of autophagy signaling molecules [11].

Atg4 cleaves pro-LC3 to form LC3-I which then gets conjugated to PE (by Atg7) for the generation of LC3-II. The latter gets recruited to the autophagosomal membrane for helping membrane elongation. ATG7 also mediates ATG5-ATG12-ATG16 complex formation and the latter along with LC3-II is highly critical for autophagosome formation. Adaptor protein p62/SQSTM1 binds to ubiquitinated proteins and LC3-II for mediating autophagy via localizing into autophagic compartments, transporting ubiquitinated proteins and organelles for degradation.

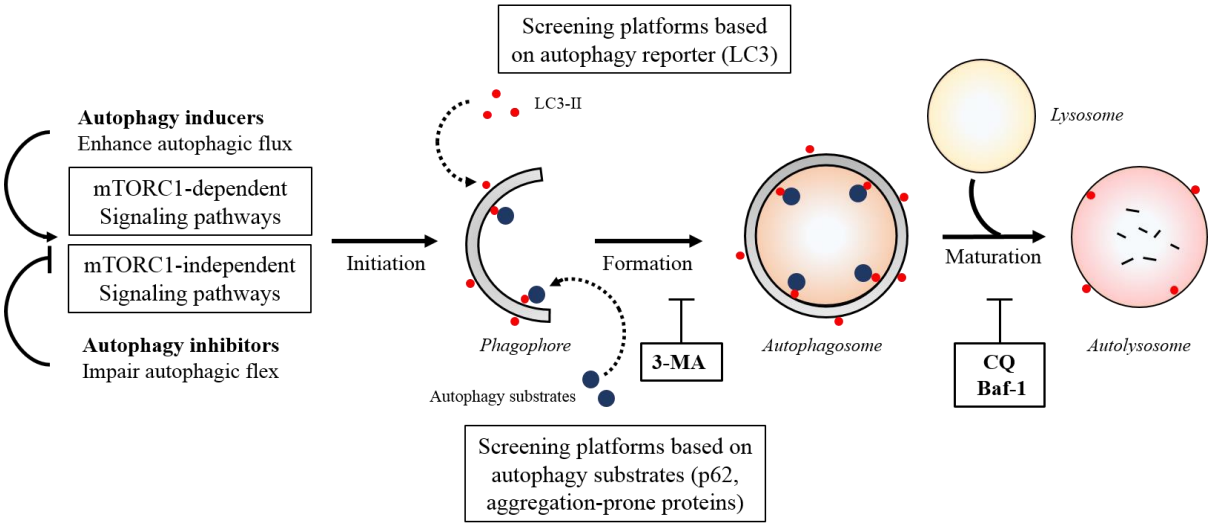

Figure 2. Dynamic turnover of cellular contents occurs through the process of autophagy [40].

This process initiates by the formation of phagophores that expand and engulf autophagy substrates to form autophagosomes, which then fuse with the lysosomes to form autolysosomes where the autophagic cargo is degraded. Autophagy inducers and inhibitors increase or decrease autophagosome formation, respectively, at the early stages of autophagy, whereas autophagy blockers prevent lysosomal degradation and/or autophagosome maturation at late stages of autophagy. Autophagic flux is thus enhanced by autophagy inducers but is retarded by autophagy inhibitors and blockers. Autophagy modulators are commonly based on the readouts of perturbations in autophagy reporters such as LC3-II, or autophagy substrate clearance such as aggregation-prone proteins or p62/SQSTM1.

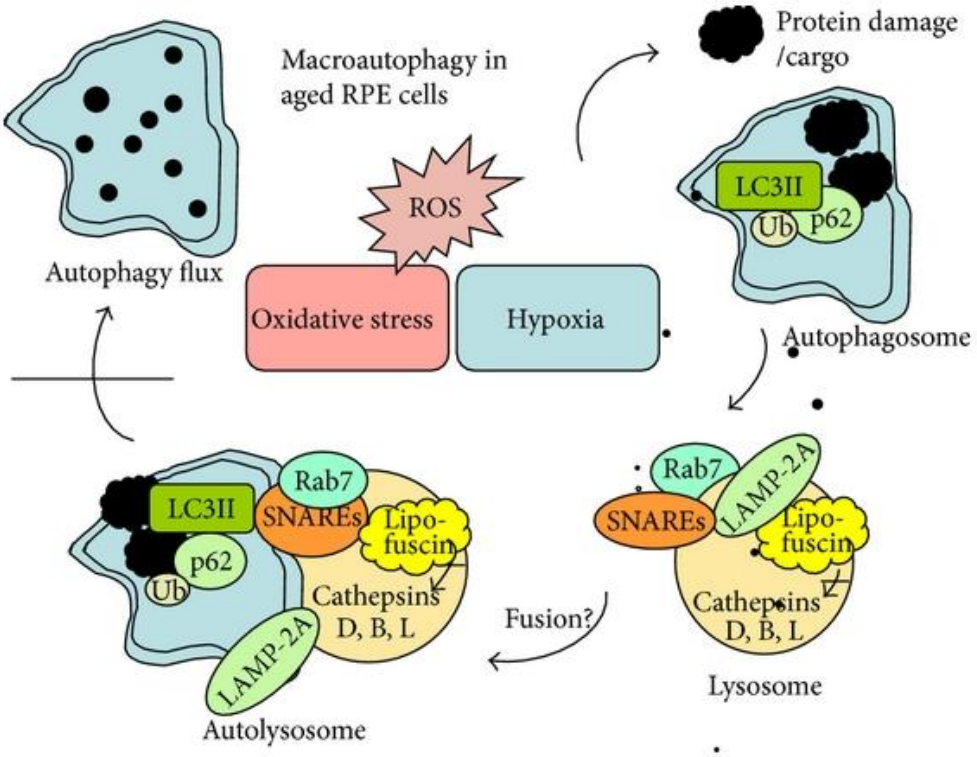

Figure 3. Schematic presentation of the macroautophagy process in aged RPE cells. [41]

Oxidative stress, ROS, and hypoxia lead to protein damages and aggregation that induces autophagy. The substrate (cargo) for autophagy is degraded by lysosomal acid hydrolases. Ubiquitin (Ub), LC3II, and p62 are complexed to the cargo and connect autophagy to the proteasomal clearance system. Macroautophagy is prevented in AMD, since lysosomal lipofuscin disturbs cathepsin activity and autophagy flux. Fusion mechanisms in the RPE cells are under investigation.

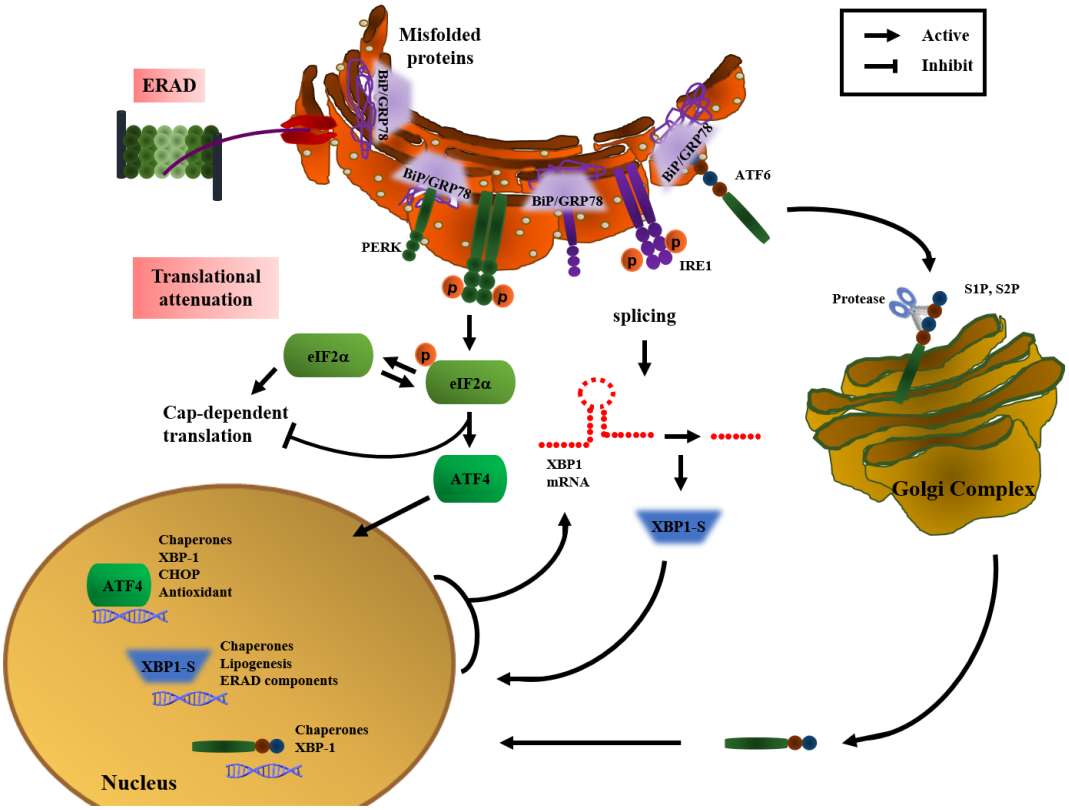

Figure 4. ER stress and the UPR [24, 42].

A number of conditions such as disturbed lipid homeostasis, disturbed calcium signaling, oxidative stress, inhibition of glycosylation, increased protein synthesis, and decreased ER-associated degradation can cause ER stress and activate the UPR. The UPR is mediated by three ER membrane-associated proteins, PERK, IRE1α, and ATF6α, to induce translational and transcriptional changes upon ER stress. PERK phosphorylates eIF2α to attenuate general protein translation and decrease protein efflux into the ER. Phosphorylated eIF2α also selectively stimulates ATF4 translation to induce transcriptional regulation of UPR genes. IRE1α cleaves XBP1 mRNA to a spliced form of XBP1 that translates XBP1s to up-regulate UPR genes encoding factors involved in ER protein folding and degradation. ATF6α traffics to Golgi for cleavage by S1P and S2P to release ATF6 that works synergistically or separately with XBP1s to regulate UPR gene expression.

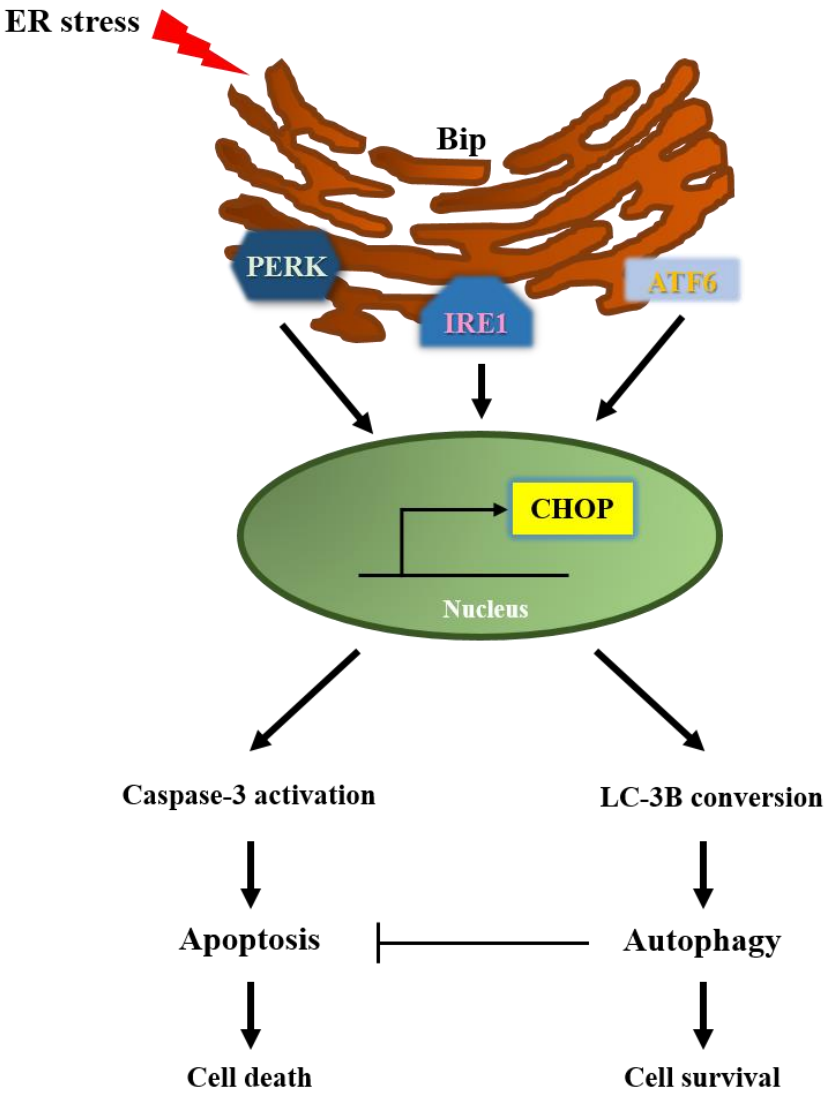

Figure 5. ER stress-mediated autophagy and apoptosis [28].

ER stress induced apoptosis through the CHOP-mediated apoptosis signaling pathway, which was protected by ER stress-mediated autophagy.

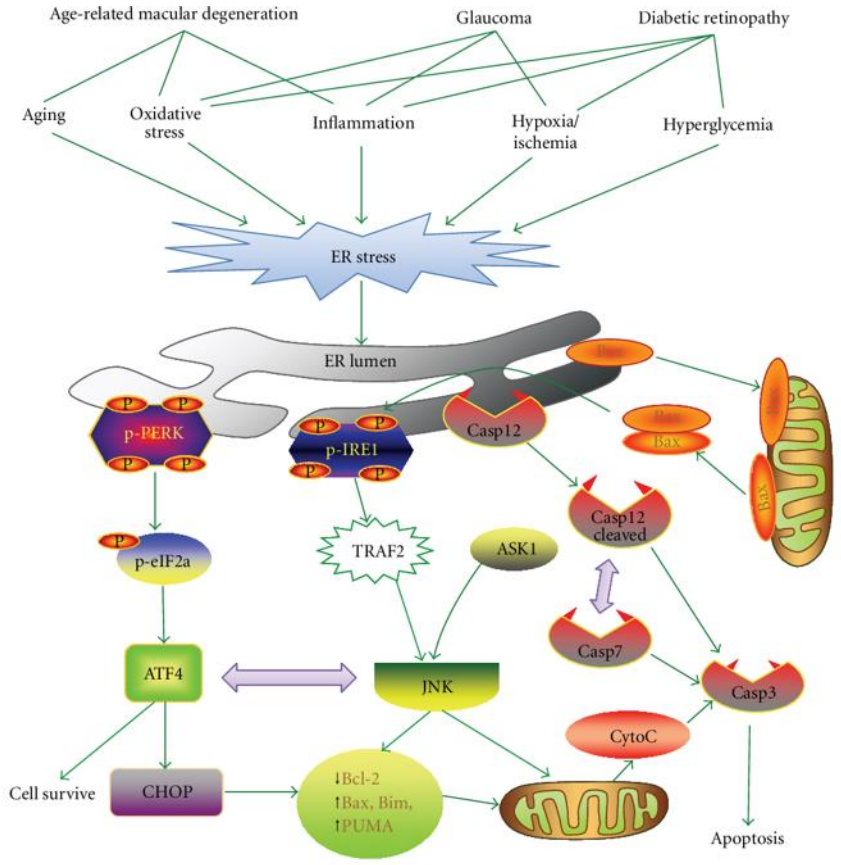

Figure 7. ER stress-associated apoptotic pathways in retinal diseases [24].

A variety of pathogenic factors in chronic retinal degenerative diseases (e.g., age-related macular degeneration, glaucomatous retinopathy and diabetic retinopathy), including aging, oxidative stress, hypoxia, inflammatory factors, and hyperglycemia and others, can disturb ER function and compromise the adaptive UPR, resulting in persistent ER stress in retinal cells. This leads to sustained activation of the ATF4/CHOP pathway and the IRE1/TRAF2/ASK/JNK pathway. Both JNK and CHOP attenuate the function of the pro-survival factor Bcl-2, but enhances the activity of proapoptotic Bcl-2 proteins such as Bim, Bax, and PUMA, resulting in mitochondrial dysfunction and cytochrome c release. In addition, caspase-12 is activated during ER stress, which sequentially activates caspase-7 and/or caspase-3, leading to mitochondria-independent apoptosis.

Results

ER stress induces cell death

Several study has shown that endoplasmic reticulum (ER) stress is associated with the pathogenesis of several eye diseases such as retinitis pigmentosa, glaucoma and age-related macular degeneration (AMD) [44]. However, the main question of whether ER stress actually triggers RPE cell death have not yet been studied. Tunicamycin (TM), activate the UPR by inhibiting the N-linked glycosylation, is known to synthetized proteins resulting in ER protein misfolding and is widely used to experimentally ER stress inducer in vivo and in vitro. To identify whether ER stress induced RPE cell death, I examined cell viability in H-RPE cells (the human retinal pigment epithelial primary cells) with different doses of TM (0.1 μg/mL - 2 μg/mL), ER stress inducer, treatment at 48 hours. The H-RPE cells treated low concentration TM (0.1ug/mL), had little effect on cell viability, whereas high concentration TM (0.5, 1 and 2 μg/mL) treatment induced cell death, dose dependently (Figure 1A). Additionally, cell viability was markedly decreased at 48 hours and 72 hours after TM (0.5 μg/mL) treatment in H-RPE cells (Figure 1B). These results showed that ER stress induced RPE cell death in a dose and time dependently manner (Figure 1).

ER stress activates autophagy related marker genes

Several studies have demonstrated that the ER stress and autophagy are mechanistically interconnected, in which the UPR, the key ER stress pathway, stimulates the autophagy. In recent years, ER stress is known to activate autophagy in various cell types including neuronal cell [45] and β-cells [46]. The major events in autophagy, such as the induction of phagophore and maturation, are coordinated by the LC3B and the autophagy-related protein 12-5 (ATG12-ATG5) conjugate [47]. Furthermore, robust ER stress-CHOP, is another potent transcription factor, which is involved in the induction of autophagy [48]. It has been elucidated that the expression levels of ATG5 is elevated by upregulation of the CHOP expression. Hence, to determine whether autophagy marker genes are expressed under ER stress in H-RPE cells (the human retinal pigment epithelial primary cells) and ARPE-19 cells (the human retinal pigment epithelial cell line) were treated with ER stress inducer, TM and PA (palmitic acid), respectively. Palmitic acid stimulates the synthesis of ceramides and increases reactive oxygen species, either of which may induce ER stress. I analyzed protein expression levels of LC3B, a stable marker protein associated with the biochemical detection of cellular autophagy, and CHOP, a marker for ER stress-induced apoptosis, in H-RPE and ARPE-19 cells in response to ER stress. Total protein isolated from human primary H-RPE and ARPE-19 cells after treatment with TM (0.05 μg/mL-2 μg/mL) for the different concentrations at 48 hours. Western blot revealed that TM or PA treatment increased the levels of LC3B and CHOP expression in H-RPE and ARPE-19 cells, (Figure 2A, 2C and 2E) respectively. Additionally, LC3B expression began to increase and reached maximum expression 48 hours after TM or PA treatment in H-RPE (0.5 μg/mL) and ARPE-19 cells (1 μg/mL). CHOP expression also increased after TM treatment (Figure 2B, 2D and 2F). Furthermore, I next investigated mRNA levels of autophagy related genes using quantitative real time (RT)-PCR. Total RNA isolated from human ARPE-19 cells after TM (0.1 μg/mL-2 μg/mL) treatment at 24 hours. Autophagy related genes, such as LC3B, p62, ATG5, Beclin1, mRNA expression dose dependently increased in ARPE-19 cells. GRP78 and CHOP are commonly used as markers of ER stress. As an ER

chaperone, GRP78 functions as a potent anti-apoptotic factor and confers drug resistance, whereas CHOP is a key initiating factor of ER stress-related cell death. Also, mRNA levels of CHOP and GRP78 were increased in response to TM treatment (Figure 3). Together, these results suggested that ER stress activates autophagy, which results in increased autophagy related marker genes including, LC3B, p62, ATG5, Beclin1, in human retinal pigment epithelial cells.

ER stress mediates apoptosis under autophagy

To confirm that ER stress could induce autophagy the expression of LC3B and CHOP in H-RPE cells and ARPE-19 cells, treated TM or TM with ER stress inhibitor 4-phenylbutyric acid (4-PBA), which improves the capacity of ER folding and reduces ER stress, were measured in both protein and RNA levels. Using western blot, the protein levels check form H-RPE cells and ARPE-19 cells after treatment TM (0.5 μg/mL or 1 μg/mL) or TM with 4-PBA (2.5 mM, 5 mM) at 48 and 72 hours. TM-induced CHOP and LC3B expression were decreased by 4-PBA (2.5 mM, 5 mM) in H-RPE cells (Figure 4A) and ARPE-19 cells (Figure 4B), respectively. In addition, using quantitative real time (RT)-PCR, the mRNA levels check form ARPE-19 (Figure 4C, D) cells after treatment TM (1 μg/mL) or TM with 4-PBA (2.5 mM, 5 mM) at 48 and 72 hours. Similarly, to the protein levels, the mRNA levels of CHOP and LC3B were dramatically diminished after TM with 4-PBA treatment, time and dose dependently manner. As a results showed that ER stress may be an important regulator of autophagy in human retinal pigment epithelial cells.

Relationship between ER stress and autophagy

ER stress and autophagy could function dependently, they share a number of common features, including protecting cells and inducing cell death under extreme conditions. However, the relationships between these two complicated systems are controversial and ER stress has been found to have the ability to induce, inhibit, or select autophagy [49]. To examine whether autophagy plays a role in the ER stress-induced cell death, chloroquine (CQ) was applied to inhibit the occurrence of autophagy in RPE cells. The effects of ER stress inducer, TM, with CQ combination on cell viability and protein expression were investigated. H-RPE (Figure 5A) cells and ARPE-19 (Figure 5C) cells were treated to TM (1μg/mL) or TM with CQ (50 μM) at 48 hours. CQ, autophagy inhibitor, reduced the cell viability and ER stress-induced promoted cell death. These results showed that autophagy inhibition enhanced the RPE cell death effect of ER stress. Total protein harvest from H-RPE (Figure 5B) and ARPE-19 cells (Figure 5D) were treated to TM (0.5μg/mL or 1μg/mL) for 48 hours in the presence and the absence of CQ (50μM), and then using western blotting. ER stress increased the expression protein levels of CHOP, LC3B and Cleaved caspase-3. However, the combination treatment of both TM and CQ significantly enhanced protein levels. Compared with TM treatment alone, the combination treatment of both TM and CQ upregulated the expression levels of CHOP, LC3B and Cleaved caspase-3, indicating a desert of cell death. All these results showed that autophagy protected ER stress induced cell death in human retinal pigment epithelial cells.

ER stress activates autophagy via NAD(P)H oxidase pathway

To understand which signaling pathway(s) were responsible for activating autophagy upon ER stress inducer treatment in H-RPE cells and ARPE-19 cells. I isolated protein form RPE cells after TM or TM with various inhibitors at 48 hours. I used specific inhibitor of DPI (NAD(P)H oxidase inhibitor), Mito-TEMPO (mitochondria-specific superoxide scavenger), NAC (cytosolic ROS scavenger), BAY11-7082 (NF-κB inhibitor), U0126 (MEK1/2 inhibitor), and SP600125 (JNK MAP kinase inhibitor), respectively. The H-RPE cells were treated with DPI (10μM), Mito-TEMPO (10μM), BAY11-7082 (1μM), U0126 (1μM) and SP600125 (5μM), in the presence or absence of TM, protein levels of LC3B were assessed at 48 hours after treatment (Figure 6A). ARPE-19 cells also were treated with DPI (10μM), Mito-TEMPO (10μM) and NAC (10mM), in the presence or absence of TM, protein levels of LC3B was assessed at 48 hours after treatment (Figure 6B). Mito-TEMPO, NAC, BAY11-7082, U0126 and SP600125 had no effect on LC3B protein levels, whereas DPI blocked protein levels of LC3B in response to ER stress inducer, TM. Additionally, I harvest total RNA from H-RPE cells treated TM or TM with DPI (10μM), Mito-TEMPO (10μM), BAY11-7082 (1μM), U0126 (1μM) and SP600125 (5μM) at 48hours. DPI only blocked mRNA levels of LC3B in response to TM. However, Mito-TEMPO, BAY11-7082, U0126 and SP600125 exerted no effect on LC3B expression under ER stress condition (Figure 6C). These results suggest that NAD(P)H oxidase signaling pathway may play a role in autophagy activation in response to ER stress condition in human retinal pigment epithelial cells.

ER stress-induced autophagy attenuates age-related macular degeneration marker genes

Recent studies have indicated that single-nucleotide polymorphisms (SNPs) in genes regulating innate immunity, such as complement factor-H (CFH), complement factor I (CFI), toll-like receptor-4 (TLR4), toll-like receptor-2 (TLR2) and apolipoprotein E (APOE), contribute significant susceptibility to AMD. In addition, APOE and apolipoprotein J (APOJ) [50], HDL component of HDL is apolipoprotein E and J binds to complement factor H (CFH) (via domains 5–7), thereby regulating complement activation [51]. Therefore, the effects of ER stress-induced autophagy during AMD-related genes regulation after TM treatment in the absence or presence of 4-PBA or CQ in ARPE-19 cells were assessed. mRNA levels of AMD-associated genes, including CFI, CFH, APOE, APOJ, TLR2 and TLR2 were enhanced 24 hours after TM, ER stress inducer, (1 μg/mL) treatment. However, ER stress-induced AMD marker genes diminished by 4-PBA (5 mM), but not significantly increased by CQ (50 μM) (Figure 7). These results provide that ER stress-induced autophagy could lead to cell survival and AMD-associated gene regulation in RPE cells. In other words ER stress-induced autophagy might play as a pathologic protector under ER stress-induced RPE cell death.

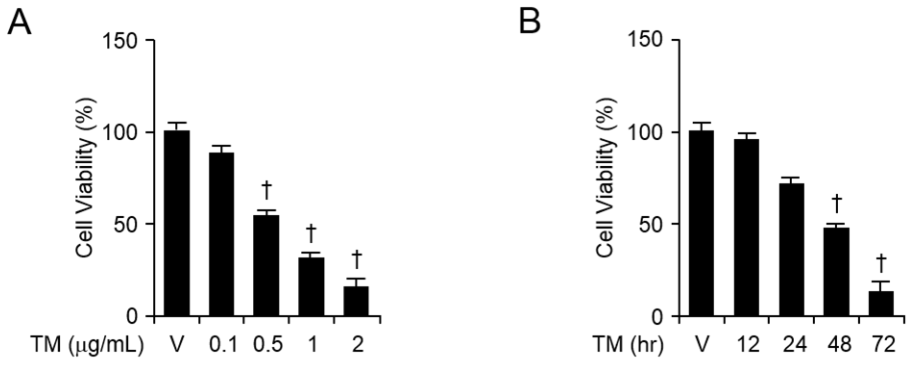

Figure 1. Cell viability was decreased in response to ER stress inducer, Tunicamycin (TM), in human RPE cells.

Primary human H-RPE cells were treated for 48 hours in various dose of TM (A). H-RPE cells were exposure to 0.5μg/mL TM, for the indicated time points (B). Values are presented as mean ± SD, n=12. †$p<0.05$, decreased the cell viability after TM treatment vs vehicle (V).

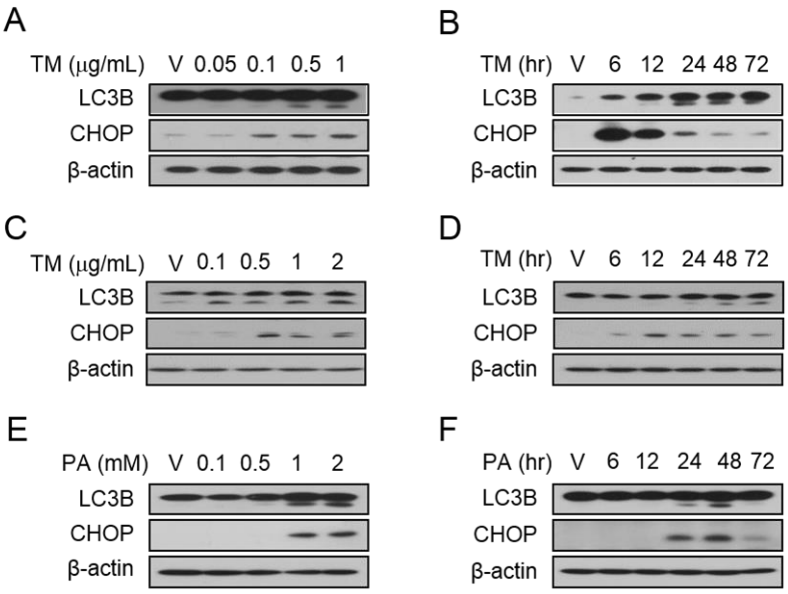

Figure 2. Autophagy related gene, LC3B, was enhanced in response to ER stress inducer, Tnincamycin (TM) and Palmitic acid (PA) in human RPE cells.

Primary human H-RPE cells (A) and ARPE-19 cells (C) were treated TM for 48 hours in dose dependently. H-RPE cells (B) and ARPE-19 cells (D) were treated TM (0.5μg/mL or 1μg/mL) for the indicated length of time points, respectively. PA was treated for 48 hours dose dependently in ARPE-19 cells (E) and PA as treated for the indicated length of time ARPE-19 cells (F). The levels of LC3B and CHOP proteins were assessed using western blotting analysis. β-actin was used as a loading control. Experiments were performed at least three independent time.

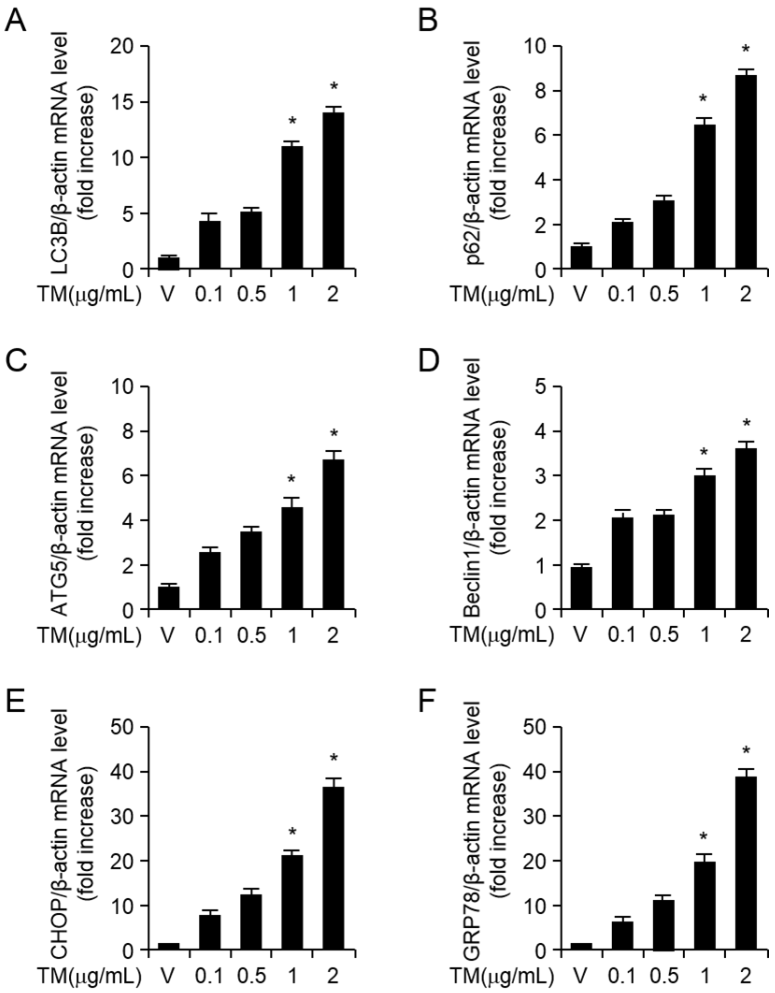

Figure 3. The mRNA levels of autophagy related genes in response to TM in human RPE cells.

Total RNA was extracted form ARPE-19 cells 24 hours in various dose of TM. mRNA expression of autophagy-related genes were analyzed. mRNA levels of LC3B (A), p62 (B), ATG5 (C), Beclin 1 (D), CHOP (E) and GRP78 (F) were analyzed by quantitative real-time RT-PCR. Human β-actin was used as a control for normalization. Expression levels of each mRNA are divided by expression of β-actin and shown as a ratio of each mRNA/β-actin. Values are presented as mean ± SD, n=3. *$p<0.05$, increased mRNA levels of autophagy-related genes after TM treatment vs vehicle (V).

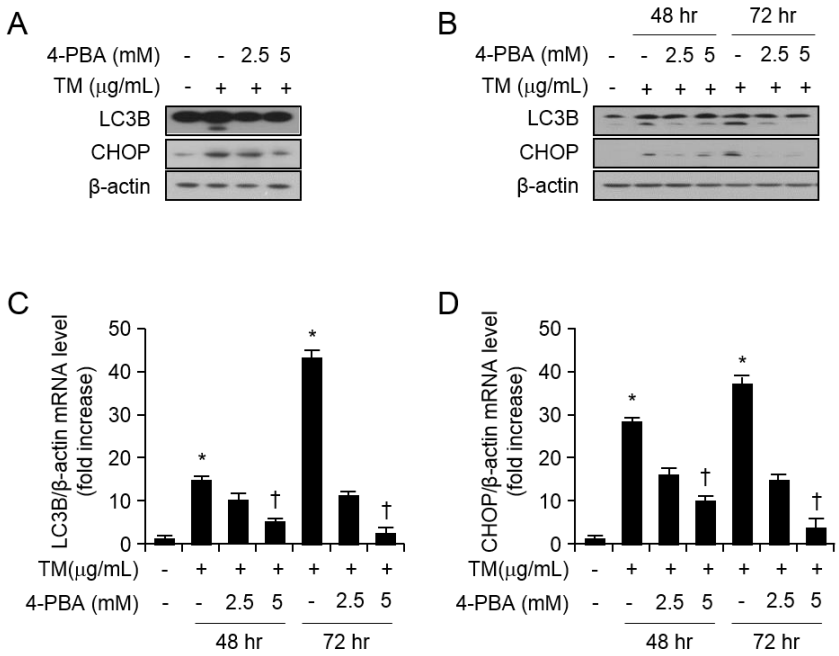

Figure 4. ER stress-induced autophagy in response to ER stress inhibitor, 4-PBA, in human RPE cells.

Primary human H-RPE cells (A) and ARPE-19 cells (B) were treated TM (0.5μg/mL or 1μg/mL) for 48 or 72 hours in the absence or presence of 4-Phenylbutyric acid (4-PBA, 2.5mM and 5mM). The levels of LC3B and CHOP proteins were assessed using western blotting analysis. β-actin was used as a loading control. Experiments were performed at least three independent times. Total RNA was extracted form ARPE-19 cells 48 or 72 hours after 1μg/mL TM in the absence or presence of 4-PBA (2.5mM and 5mM) treatment. Quantitative real-time RT-PCR was performed to assess mRNA levels of LC3B (C) and CHOP (D). For all real-time PCR analyses, β-actin was used as a control for normalization. Expression levels of each mRNA are divided by expression of β-actin and shown as a ratio of each mRNA/β-actin. Values are presented as mean ± SD, n=3. *$p<0.05$, increased mRNA levels of genes in response of TM vs Vehicle (V). †$p<0.05$, decreased mRNA levels of genes in response of TM plus 4-PBA vs TM alone.

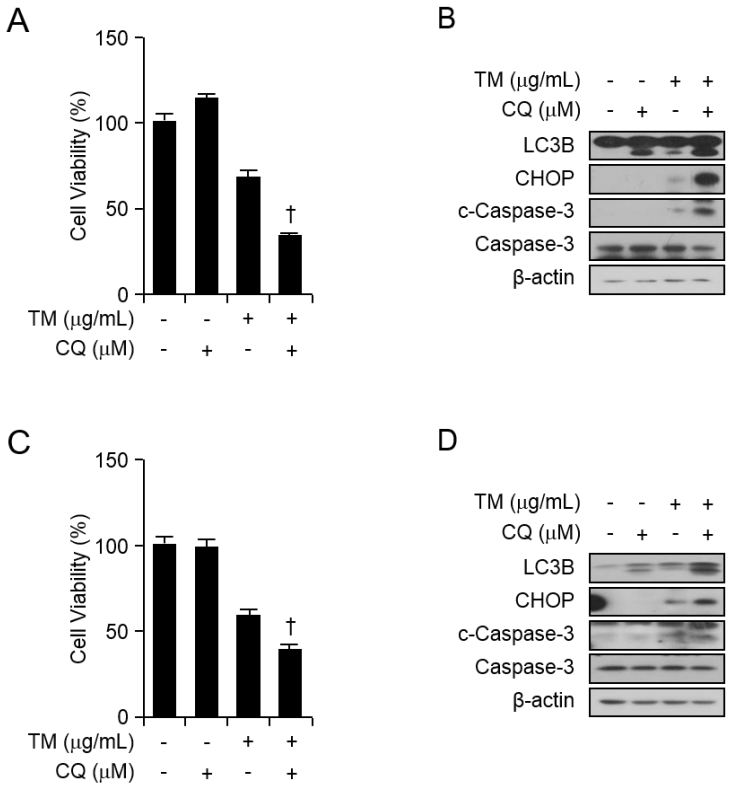

Figure 5. Cell viability was decreased in response to TM with autophagy inhibitor, Chloroquine (CQ), in human RPE cells.

The cell viability was analyzed 48 hours after TM (0.5μg/mL or 1μg/mL) treatment hours in the absence or presence of autophagy inhibitor, Chloroquine, (CQ, 50μM) in H-RPE cells (A) and ARPE-19 cells (B). Values are presented as mean ± SD, n=12. †p<0.05, decreased the cell viability in response to TM plus CQ vs TM alone. H-RPE cells (B) and ARPE-19 cells (D) were treated TM (0.5μg/mL or 1μg/mL) for 48 hours in the absence or presence of autophagy inhibitor, Chloroquine, (CQ, 50μM). The levels of LC3B, CHOP, total caspase 3 and cleaved Caspase-3proteins were assessed using western blotting analysis. β-actin was used as a loading control. Experiments were performed at least three independent times.). †p<0.05, decreased mRNA levels of genes in response of TM plus CQ vs TM al

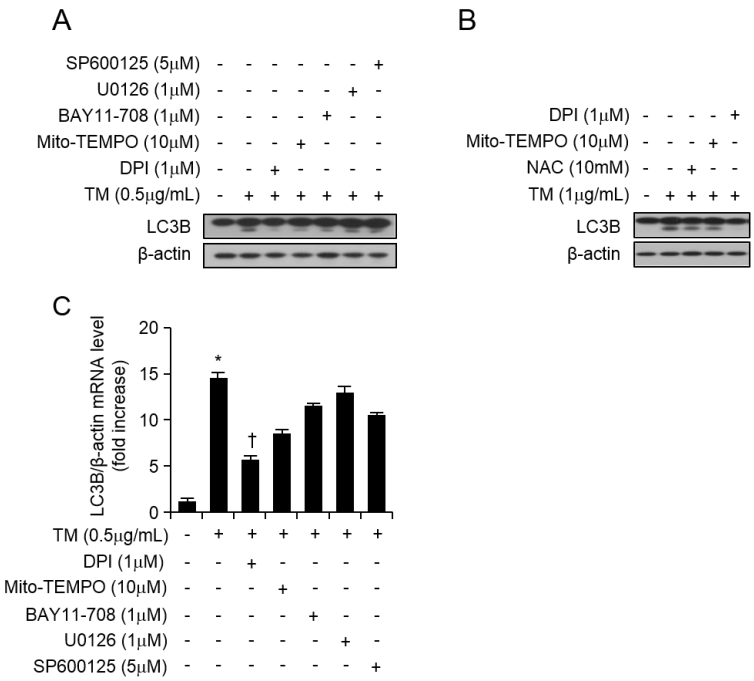

Figure 6. ER stress –induced autophagy was diminished in response to NAD(P)H oxidase inhibitor, Diphenyleneiodonium (DPI), in human RPE cells.

Primary human H-RPE cells (A) and ARPE-19 cells (B) were treated TM (0.5μg/mL or 1μg/mL) with signaling inhibitor (1 μM DPI, 10μM mito-TEMPO, 1 μM BAY11-7082, 1 μM U0126, 5 μM SP600125 or 10 mM NAC) for 48 hours. The levels of LC3B proteins were assessed using western blotting analysis. β-actin was used as a loading control. Experiments were performed at least three independent times. Total RNA was extracted form H-RPE cells (C) were treated TM (0.5μg/mL) with signaling inhibitor (1 μM DPI, 10 μM mito-TEMPO, 1 μM BAY11-7082, 1 μM U0126, 5 μM and SP600125) for 48 hours. Quantitative real-time RT-PCR was performed to assess mRNA levels of LC3B. For all real-time PCR analyses, β-actin was used as a control for normalization. Expression levels of each mRNA are divided by expression of β-actin and shown as a ratio of each mRNA/β-actin. Values are presented as mean ± SD, n=3. *$p<0.05$, increased mRNA levels of genes in response of TM vs Vehicle (V). †$p<0.05$, decreased mRNA levels of genes in response of TM plus DPI vs TM alone.

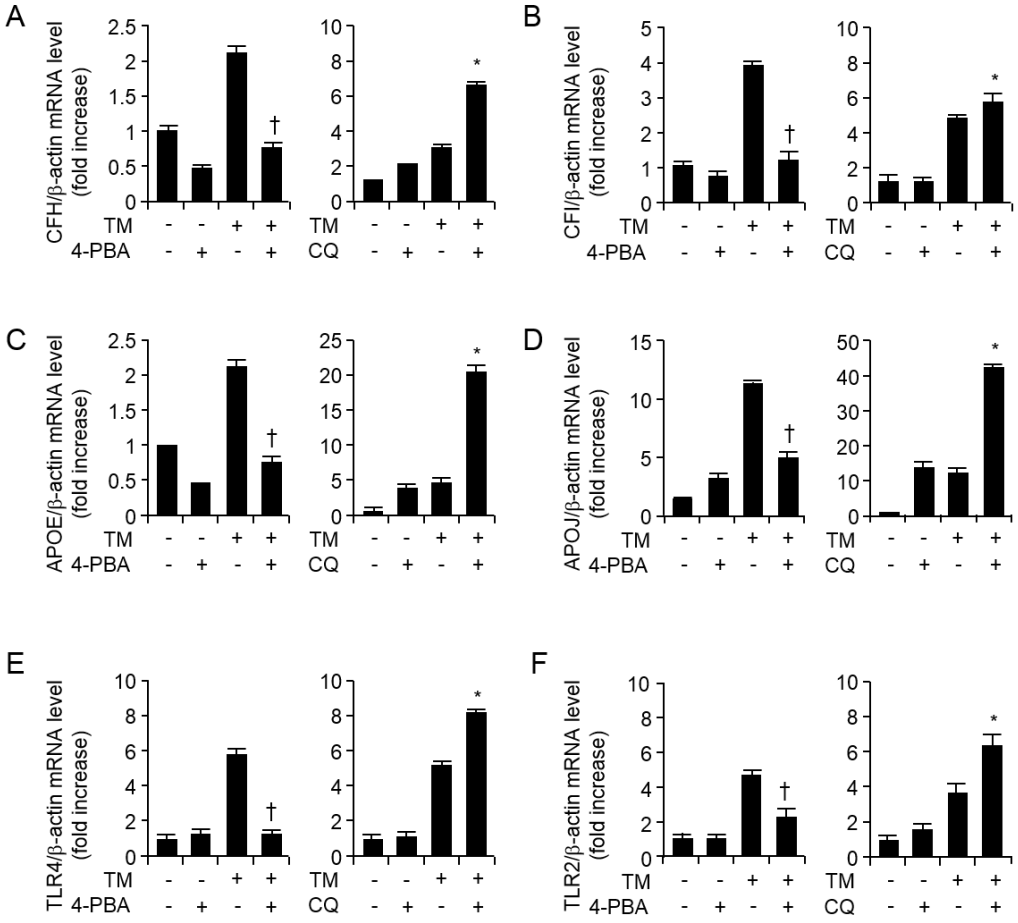

Figure 7. The mRNA levels of AMD-associated genes in response to TM with 4-PBA or CQ in human RPE cells.

Total RNA was extracted form ARPE-19 cells after TM (1ug/mL) with 4-Phenylbutyric acid (5mM) or CQ (50uM) for 48 hours. mRNA levels of CFH (A), CFI (B), APOE (C), APOEJ (D) TLR4 (E) and TLR2 (F) were analyzed by quantitative real-time RT-PCR. Human β-actin was used as a control for normalization. Expression levels of each mRNA are divided by expression of β-actin and shown as a ratio of each mRNA/β-actin. Values are presented as mean ± SD, n=3. *$p<0.05$, increased mRNA levels of genes in response of TM plus CQ vs TM alone (V). †$p<0.05$, decreased mRNA levels of genes in response of TM plus 4-PBA vs TM alone.

Discussion

The retinal pigment epithelium (RPE), a monolayer of pigmented and polarized central nervous system tissue, has important roles in retinal homeostasis. It mainly functions as absorb light energy focused on the retina by the lens, and contribute to the maintenance of photoreceptor excitability. RPE cells are constantly exposed to cellular stress that may lead to the accumulation of damaged proteins, DNA and lipids and cause tissue aggravation during the aging process [52]. AMD, a multi-factorial disease, is the leading cause of irreversible vision loss, particularly in individuals over 65 years of age [53]. AMD initially affects the RPE and over time, leads to secondary loss of photoreceptor cells. Age-related alterations in the RPE include a reduction in cell density, which can be caused by cell death resulting from accumulation of toxic substances such as drusen [54]. More recently, ER stress has been suggested as playing an important role in RPE cell injury that leads to AMD-related pathological changes [55]. Tunicamycin (TM), ER stress inducer, is characterized by an elevation of the ER stress markers GRP78 and CHOP. Additionally, TM is often used to induce a cell death in cell cultures. In line with I previous finding demonstrated that the exposure of ARPE-19 cells to TM induces a typical inflammatory response which involves PTX3 activation, reduced cell viability [56]. Emerging evidence indicate that ER stress can stimulate autophagy. Autophagy, catabolic process, is essential for cell survival in response to stress in RPE cells [57]. However, the role of autophagy in ER stress is somewhat controversial or dynamic and the signaling mechanisms linking ER stress to autophagy remain not fully delineated in RPE cells, yet. In the present work, I demonstrated that TM induces mRNA and protein levels of autophagy related marker genes, LC3B, in H-RPE cells and ARPE-19 cells. I research has indicated the expression and importance of autophagy in RPE cells. Further, to confirm that ER stress could induce autophagy using ER stress inhibitor (4-PBA) or autophagy inhibitor (CQ). ER stress

induced autophagy was diminished by ER stress inhibitor RPE cells. Also ER stress-induced cell death was increased in the presence of autophagy inhibitor in RPE cells. Furthermore, only NAD(P)H oxidase signaling pathway was involved in autophagy activation in RPE cells, however, the mitochondria-specific superoxide, cytosolic ROS, NF-κB, MEK1/2 and JNK/MAP pathways were not engaged in ER-induced autophagy activation. More importantly, AMD is a progressive retinal disease is which the early stage is characterized by relatively few small drusen within the macula. When AMD progresses, drusen size and number increase, eventually leading towards more advanced stages of AMD. AMD increased dramatically with the identification of biological complement systems. Complement can be compared between patients and control individuals in drusen. Interestingly, a significant activates the CFH and CFI genes, in addition to two other genes (APOE and APOJ), was observed in drusen within the macula of AMD patients [58]. TLR4 and TLR2 has been implicated in transmembrane signaling in response to AMD [59]. Therefore, the expression of ER stress-induced AMD-associated genes, including complement factor H (CFH), complement factor I (CFI), apolipoprotein E (APOE), apolipoprotein J (APOJ) toll-like receptor 4 (TLR4) and, toll-like receptor 2 (TLR2) were decreased by ER stress inhibitor in RPE cells. On the other hands, ER stress-induced AMD marker genes were significantly increased by autophagy inhibitor in RPE cells. These data suggest that autophagy plays a protective effects during ER stress-induced cell death in RPE cells. In this study, I provide information regarding the critical role of autophagy under ER stress conditions in the early stage of AMD development, especially the loss of RPE cells.

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Part 3.

The role of sulfasalazine under tamoxifen-induced autophagy in human retinal pigment epithelial cells.

Abbreviations

RPE	Retinal pigment epithelium
AMD	Age-related macular degeneration
POS	Photoreceptor outer segments
Tx	Tamoxifen
ATLAS	Adjuvant Tamoxifen: Longer against Shorter
SSZ	Sulfasalazine
DMARD	Disease-modifying anti-rheumatic drug
RA	Rheumatoid arthritis
SERM	Selective-estrogen-receptor-modulator
BCPT	Breast Cancer Prevention Trial
ER	Estrogen-receptor
LMP	Lysosomal membrane permeabilization
IBD	Inflammatory bowel disease
5-ASA	5-aminosalicylic acid
SPD	Sulfapyridine
MTX	Methotrexate
CFI	Complement factor I
CFH	Complement factor H
APOE	Apolipoprotein E
APOJ	Apolipoprotein J
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4

Abstract

The human retina is under constant remodeling throughout the lifetime, with various forms of cell death. Autophagic cell death has been described as early as embryonic development and organogenesis and as late as old age, in particular, in neurodegenerative diseases. The retinal pigment epithelium (RPE), a single layer of pigmented cells, is crucial physiological roles that are vital for the normal functioning of the retina. One such function is a critical role in the maintenance of photoreceptors, phagocytizing the photoreceptor outer segments (POS), which are then digested within lysosomes. As autophagy plays an important role in the homeostasis of RPE cells its impairment can lead to RPE dysfunction. The RPE dysfunction is also thought to play a role in a variety of retinal diseases including AMD, tamoxifen retinopathy, chloroquine retinopathy, as well as a variety of inherited retinal disorders [1].

Tamoxifen (Tx), a nonsteroidal estrogen receptor antagonist, is used widely as a chemotherapeutic agent for some forms of breast cancer. The current standard treatment with Tamoxifen for breast cancer is 5 consecutive years, however the global Adjuvant Tamoxifen: Longer against Shorter (ATLAS) trial has recently showed that 10 years of treatment reduced the risk of recurrence and mortality of breast cancer [2]. Thus, it is likely that the standard of care will change, resulting in a longer period of tamoxifen therapy with a likely increase in tamoxifen-induced ocular toxicity. Tx induces macula-involving retinopathy, which can compromise vision seriously, especially in women treated with high daily or cumulative doses of Tx. The RPE generally has been considered the primary target of Tx-induced retinotoxicity [3]. Because Tx is known to induce autophagy in RPE, and since autophagy can cause cell death, it seems possible that an autophagic mechanism also may be involved in Tx-induced retinopathy [4].

Sulfasalazine (SSZ), a well-established anti-inflammatory drug, is considered a disease-modifying anti-rheumatic drug (DMARD) [5]. It is used in the treatment of rheumatoid arthritis (RA),

inflammatory bowel disease, and some other autoimmune conditions. SSZ also can decrease the pain and swelling of arthritis, prevent joint damage, and reduce the risk of long-term disability (It works by reducing inflammation (swelling) inside the body). However, the effect and molecular mechanisms of SSZ in response to Tx-induced autophagy have not been investigated in RPE cells. To better understand the role of SSZ in degenerative processes underlying RPE death, I analyzed the effects of SSZ and the contribution of autophagy on the RPE death induced by Tx. In the present study, I demonstrated that SSZ, a known anti-inflammatory drug, protected the Tx-induced autophagy, with ROS signaling pathways playing a role in the molecular mechanism. Moreover, SSZ protected TAM-induced autophagy and the expression of AMD-related genes, such as complement factor I (CFI), complement factor H (CFH), apolipoprotein E (APOE), apolipoprotein J (APOJ), toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4), in human RPE cells and suppressed RPE cell death. Taken together, these results provide critical insight into the pathologic effects of SSZ during Tx-induced RPE cell death in the early development of age-related macular degeneration.

Introduction

1. Tamoxifen (Tx)

Tamoxifen (Tx) is considered a groundbreaking drug in medical oncology that has saved many lives over the past four decades and progressed to become a significant part of our healthcare [6]. Tamoxifen, sold under the brand name Nolvadex among others, is nonsteroidal triphenylethylene derivative that binds to the estrogen receptor. It has both estrogenic and antiestrogenic actions, depending on the target tissue. It is strongly antiestrogenic on mammary epithelium, hence its use in both the prevention and treatment of breast cancer; it is proestrogenic on uterine epithelium, hence the current controversy regarding its safety in cancer prevention, especially since an increased incidence of endometrial carcinoma has been found in women treated chronically with tamoxifen. It is therefore inappropriate to refer to tamoxifen simply as an antiestrogen. The term selective estrogen receptor modulator is more appropriate. In 1998, the selective-estrogen-receptor-modulator (SERM) tamoxifen achieved positive results in the Breast Cancer Prevention Trial (BCPT), leading to the Food and Drug Administration (FDA) approval of tamoxifen for risk reduction in women at high risk of breast cancer (the historic first FDA approval of a cancer preventive agent) [7]. Tamoxifen is used alone or as an adjuvant in women and treat breast cancer in women and men. It is also being studied for other types of cancer [8]. Although the current standard of care is 5 years of tamoxifen therapy, the global Adjuvant Tamoxifen: Longer Against Shorter (ATLAS) trial recently showed that 10 years of tamoxifen treatment reduced the risk of breast cancer recurrence, reduced breast cancer mortality, and reduced overall mortality [1].

1.1. The effect of tamoxifen in breast cancer cell

Breast cancer is the most common malignancy among females and affects approximately one in every ten women worldwide. There are an estimated 29 million women at increased risk for breast

cancer in this country. Tamoxifen (Nolvadex®) is a medication in pill form that has been used for more than 25 years to treat breast cancer in women and men [9, 10]. Tamoxifen is one of the most common endocrine therapy drugs. It has been shown to decrease the chance of recurrence in some early-stage breast cancers and to prevent the development of cancer in the opposite breast [11]. Tamoxifen can also slow or stop the growth of cancer cells present in the body. The primary target of tamoxifen is the estrogen-receptor (ER). The ER is a ligand-activated transcription factor that is a critical regulator of breast epithelial cell proliferation, differentiation, and apoptosis [12]. There are two ER isoforms, ERα and ERβ. The receptors differ in their cellular function, tissue distribution, as well as ligand binding properties. Both ERα and ERβ are expressed in normal and malignant tissues [13, 14]. In breast cancer, ERα is expressed in a greater proportion of cells and growth is thought to occur through both autocrine and paracrine mechanisms. About 80% of all breast cancers are "estrogen-receptor-positive (ER+)" that means the cancer cells grow in response to the hormone estrogen. This suggests that the breast cells may receive signals from estrogen that could promote their growth [15]. Tamoxifen is classified as a selective estrogen receptor modulator (SERM) and works as an anti-estrogen: While the hormone estrogen promotes the growth of breast cancer cells, Tamoxifen works by blocking estrogen from attaching to estrogen receptors on these cells [16]. By blocking the estrogen receptors, it is believed that the growth of the breast cancer cells will be halted (Figure 1) [17]. Despite its effectiveness in the adjuvant setting as well as in first-line therapy for advanced ER-positive breast cancer, approximately 20% to 30% of cases are resistant to tamoxifen. The tamoxifen-resistant cancers either have an intrinsically resistant phenotype, called innate resistance, or they acquire resistance after prolonged exposure to tamoxifen. Autophagy (macroautophagy) is a major mechanism of self-degradation in cells. Autophagy starts with the formation of double-membraned autophagosomes around misfolded proteins and damaged organelles. Autophagy starts with the formation of double-membraned autophagosomes around misfolded proteins and damaged organelles. Autophagosomes eventually fuse with lysosomes to form autolysosomes, which contain lysosomal acidic hydrolases that degrade the contents of the autophagosomes (Figure 2) [18]. Although autophagy may have evolved as a defensive response to

starvation in single-cell organisms, it has other diverse functions in multi-cellular organisms. Deficits in autophagy have also been implicated in the development of several forms of cancer, including breast cancer. Although autophagy is essential for normal cellular functions, it also has a role in cell death under certain circumstances. Autophagic cell death or cell death with autophagy have been implicated in ischemic brain injury, heart and liver disease, and myopathies [19, 20, 21]. While deficits in autophagy may induce cancer [22], several chemotherapeutic agents act by evoking autophagic cell death. For example, tamoxifen, causes autophagic vacuoles formation and autophagic cell death in breast cancer cells [23]. The mechanism underlying autophagic cell death remains unclear, but lysosomal derangements, such as accumulation of lysosomes and lysosomal membrane permeabilization (LMP), are thought to be involved.Treatment of MCF-7 cells with tamoxifen induced vacuole formation and cell death. (Figure 3) [24]. Tamoxifen is also associated with other side effects, including, blood clots, stroke, endometrial cancer and vision problems. Although longer treatment with tamoxifen increases the risk of adverse effects, the reduction in breast cancer mortality associated with longer treatment outweighs those risks [25].

1.2. The side effect of tamoxifen in retina degeneration

The oral antiestrogen tamoxifen has demonstrated efficacy in the treatment of metastatic breast cancer and as adjuvant therapy in early-stage disease. Clinical trials of tamoxifen in chemoprevention of breast cancer among high-risk women have focused attention on potential adverse effects of long-term tamoxifen use, including the possibility of ocular toxicity [26]. Toxic effects of tamoxifen on ocular were first described in 1978 and the reported incidence of ocular side effects of tamoxifen ranges from 0.9 to 11 %, induces macula-involving retinopathy, which can compromise vision seriously, especially in women treated with high daily or cumulative doses of tamoxifen. Tamoxifen can lead to corneal toxicity, progression of cataracts, retinopathy, and neuropathy. The most visually significant aspect of tamoxifen toxicity is a maculopathy [27, 28, 29]. Tamoxifen is structurally similar to other drugs with well-known retinal effects including chloroquine, chlorpromazine, thioridazine, and tilorone.

Chloroquine, specifically, has been shown by others to disrupt lysosomal function and disrupt phagocytosis in RPE cells. RPE generally has been considered the primary target of tamoxifen induced retinotoxicity. Moreover, recent reports have demonstrated that tamoxifen toxicity also affects photoreceptors as well. Tamoxifen might well induce cytotoxicity to RPE and photoreceptors. Because tamoxifen is used increasingly to treat breast cancer patients, the prevalence and clinical significance of tamoxifen induced retinopathy also are rising. However, although it is known that tamoxifen penetrates the blood-retinal barrier and induces oxidative stress, the mechanism of tamoxifen-induced retinotoxicity is not yet clear. Because tamoxifen is known to induce autophagy in human RPE cells, and since autophagy can cause cell death, it seems possible that an autophagic mechanism also may be involved in tamoxifen -induced retinopathy [1, 2, 4].

2. Sulfasalazine (SSZ)

Sulfasalazine (SSZ) is in a type of sulfa drug. You should not take it if you have a sulfa allergy. Sulfasalazine was initially developed in the 1930s to treat rheumatoid arthritis (RA) [30]. In addition, SSZ used to treat inflammatory bowel disease (IBD) and some other autoimmune conditions. It works to lower inflammation in the body [31]. Sulfasalazine is a prodrug composed of 5-aminosalicylic acid (5-ASA) linked to sulfapyridine through an azo bond. Approximately 30 percent of orally administered sulfasalazine, which therapeutically is a relatively inactive chemical, is rapidly absorbed by the small bowel and is then returned, largely unaltered, via the enterohepatic circulation into the bile. Thus, approximately 90 percent of the ingested drug reaches the large intestine as an intact molecule. In the colon, sulfasalazine is reduced by the bacterial enzyme azoreductase to its two components, sulfapyridine and 5-ASA. Thus, coliform bacteria are necessary to reduce the relatively inactive parent drug to its active moieties. The sulfapyridine is responsible for many of the side effects of sulfasalazine, while the 5-ASA is responsible for many of its beneficial effects in patients with inflammatory bowel disease. This discovery provided a rationale for the development of a drug that contains only the 5-ASA component. Unfortunately, the sulfapyridine component is necessary for the beneficial effects of

sulfasalazine in patients with rheumatoid arthritis; thus the 5-ASA drugs are not suitable for those patients. The actions of one of the major proinflammatory cytokines, IL-1, IL-2, IL-6, IL-12 and TNF-α, are also counteracted both by suppression of production of the cytokine, and by inhibition of receptor binding. Studies in animal models of autoimmune disease also support an immunomodulatory action of sulfasalazine, and proinflammatory cytokines were suppressed at the same time as measures of disease activity improved in patients with rheumatoid arthritis who received sulfasalazine (Figure 4) [32 ,33, 34]. Rheumatoid arthritis is a chronic disorder for which there is no known cure. Fortunately in the last few years, a shift in strategy toward the earlier institution of disease modifying drugs (DMARD) and the availability of new classes of medications have greatly improved the outcomes that can be expected by most patients [35]. Sulfasalazine is well established DMARD used in the treatment of patients with rheumatoid arthritis. Its effectiveness overall is somewhat less than that methotrexate, but it has been shown to reduce signs and symptoms and slow radiographic damage [36, 37]. It is also given in conjunction with methotrexate and hydroxychloroquine as part of a regimen of “triple therapy” which has been shown to provide benefits to patients who have had inadequate responses to methotrexate alone. Its mechanism of action in RA is unknown [38].

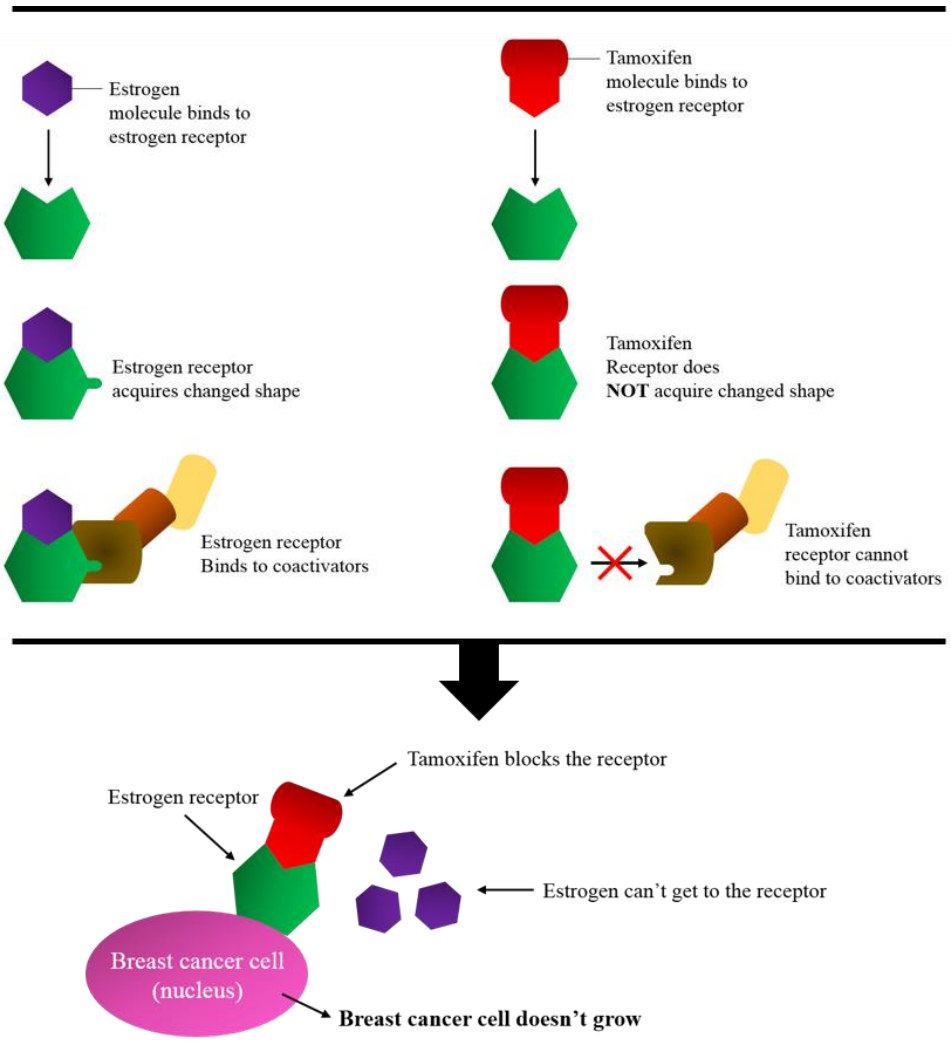


Figure 1. Schematic presentation of the mechanisms of action of endocrine treatment in breast cancer using tamoxifen [17].

Tamoxifen has been used for many years to treat breast cancer. It has been proven to be a very effective treatment. Tamoxifen can substantially reduce the chance of breast cancer coming back and of new breast cancers developing. Tamoxifen works by blocking the oestrogen receptor inside the breast cancer cell. This stops oestrogen making the breast cancer cell grow.

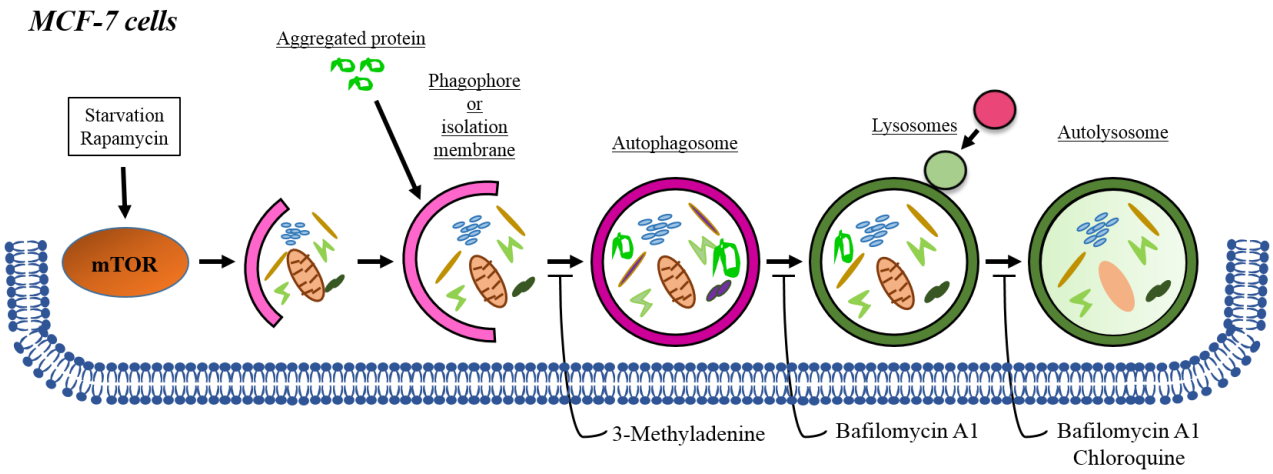


Figure 2. Autophagy: a pro-survival strategy employed by breast cancer cells [18].

One of the pro-survival strategies employed by breast cancer cells during their acquisition of dormant states is autophagy, which facilitates the recycling of damaged or unnecessary organelles and/or proteins as a means to provide energy during periods of metabolic stress.

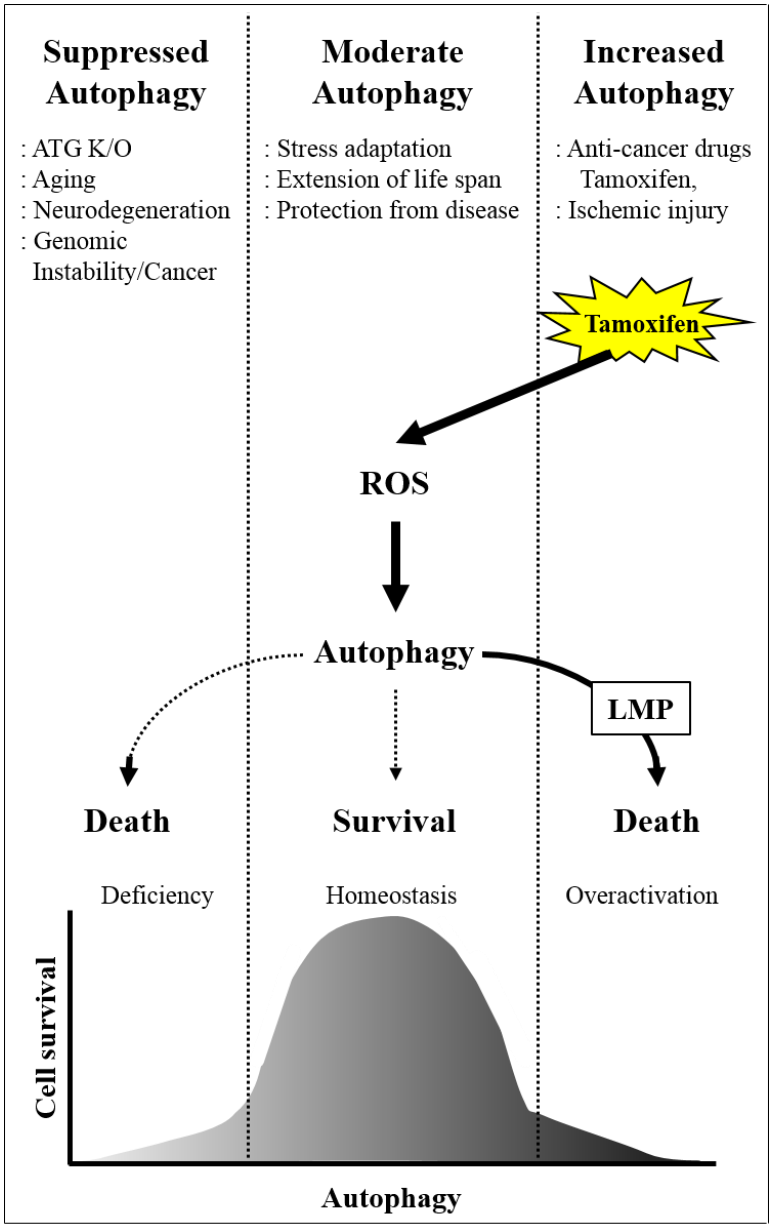


Figure 3. A diagram for toxic mechanism of action by tamoxifen [24].

Tamoxifen increases ROS production in MCF-7 cells. Increases in intracellular ROS resulted in activation of autophagy. Whereas adequate levels of autophagy may be beneficial for cell survival, excessive levels of autophagy such as induced by toxic levels of tamoxifen, may result in LMP and cell death.

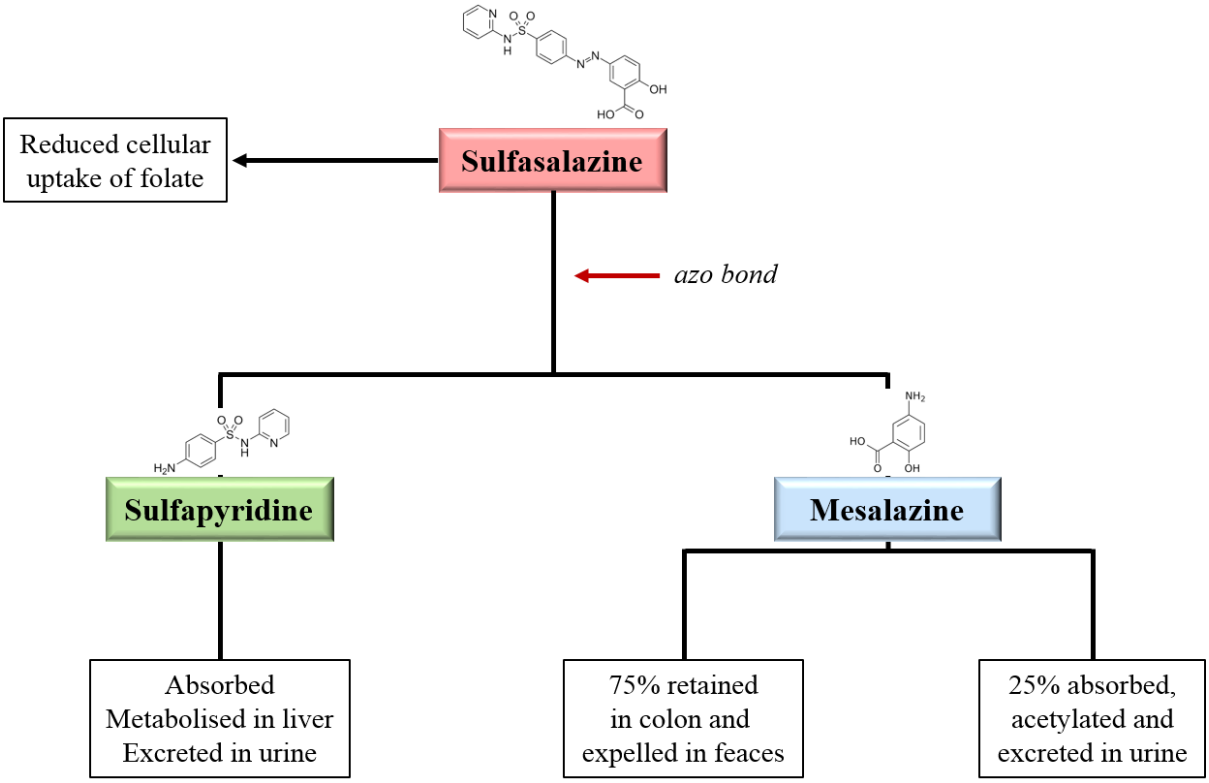


Figure 4. Metabolism and activity of sulfasalazine [32 ,33, 34].

Sulfasalazine is reduced to sulfapyridine and mesalazine in the colon by bacterial azoreductases. The two components have distinct anti-inflammatory activities. 5-ASA is retained mostly in the colon and excreted, but 30% of intact SSZ and all sulfapyridine are absorbed. Anti-inflammatory effects of mesalazine, both in the colonic epithelial cell and in peripheral blood mononuclear cells. In the treatment of inflammatory bowel disease, preparations containing 5-ASA alone have efficacy comparable to that of SSZ, but with fewer side-effects.

Results

Sulfasalazine inhibits tamoxifen-induced cell death

Several studies demonstrated that tamoxifen (Tx) induces cytotoxicity that specifically affects RPE cells [4, 39]. Tx, a nonsteroidal extrogen receptor (ER) antagonist, has been widely used as a chemotherapeutic agent against breast cancer (Figure 1A). To investigate the cytotoxicity of Tx, primary human H-RPE cells treated to different concentration (1-20 μM) of Tx for 24 hours. Cell viability was evaluated using MTT assays. The low concentration of Tx (1 μM) induced virtually no cell death, whereas high concentration of Tx (20 μM) killed almost all cells. At a concentration of 10 μM, Tx induced approximately 49.98±2.05% cell death (Figure 1B). In an effort to identify sulfasalazine (SSZ) (Figure 1A), a medication used to treat rheumatoid arthritis, did not impact cell viability when used at a broad range of concentrations (1-500 μM), suggesting the safe use of SSZ in H-RPE cells (Figure 1C). I also analyzed the effect of different concentrations of SSZ in protecting H-RPE cells and ARPE-19 cells from Tx-induced cell death. I found that SSZ at concentration of 100uM had the highest efficiency in protecting H-RPE cells and ARPE-19 cells, which gradually increased with 10uM and 50uM SSZ pretreatment, respectively (Figure 1D, E). Also, cell viability was confirmed using lactate dehydrogenase (LDH)-release assay. The protective effect of SSZ still existed when the concentration of Tx (30 μM) was caused loss of cell viability without treatment with SSZ (Figure 1F). SSZ, prodrug, is composed of 5-aminosalicylic acid (5-ASA or mesalazine; anti-inflammatory agent) linked to sulfapyridine (SPD; a sulfonamide anti-infective) through an azo bond. To further characterize the protective effects of SPD (100 μM) and 5-ASA (100 μM) on cell viability compare with SSZ in H-RPE cells after treatment with Tx (10 μM) at 24 hours. The SSZ protected up to 98% of cells in comparison with average 80% rescue when SPD and 5-ASA were used at their published concentrations [40] from

Tx-induced cell death. Interestingly, when both SPD and 5-ASA combination were significantly rescued from Tx-induced RPE cell death (Figure 1G). The methotrexate (MTX) and sulfasalazine (SSZ), a disease-modifying anti-rheumatic drugs (DMARDs), are the most commonly used effective medications to treat rheumatoid arthritis (RA). Thus, I also tested the effect of MTX in ARPE-19 cells, and observed that MTX cannot protected from Tx-induced cell death compared with SSZ (Figure 1H). Additionally, to test whether SSZ protects against Tx-induced cell death in breast cancer cell lines, I analyzed its effect on MCF-7 cells. The SSZ effects markedly abolished the Tx-induced cell death in MCF-7 cells (Figure 1I). Taken together, these results suggested that SSZ potently protects form Tx-induced cell death in human retinal pigment epithelial cells.

Sulfasalazine induces tamoxifen-Induced autophagy related genes expression

Autophagy is an umbrella term for three distinct cellular processes. Their collective purpose is the degradation of intracellular material, with each form of autophagy using different mechanisms for lysosome delivery. There are some reports that demonstrate autophagy promotes cell death [41]. Tamoxifen (Tx) is known to activate autophagy in breast cancer and colorectal cancers [20, 42, 43]. In addition, recently several studies shown that RPE cells die primarily from autophagy in response to tamoxifen. Tx toxicity of the retina is believed to be mediated by damage to the RPE through disruption of lysosomes [1, 4]. I asked whether sulfasalazine (SSZ) regulates Tx-induced autophagy in humans RPE cells. To examine that total protein harvest from H-RPE (Figure 2A) and ARPE-19 cells (Figure 2C) were treated to Tx (10 μM or 30 μM, respectively) for indicated times in the presence and the absence of SSZ (100 μM), and then using western blotting. The human RPE cells treated to Tx alone showed enhanced autophagy related protein genes, such as LC3B at 12 and 24 hours. However, the LC3B level was abolished when combination treatment of both Tx and SSZ, suggesting Tx-induced autophagy was regulated by SSZ in RPE cells. LC3B is used as a quantitative marker of autophagy since it is required for the formation of the autophagosome and its level is proportional to the amount of autophagosomes in the cell [41]. Moreover, examined the effect of SSZ on other hallmarks of cell death including, phophorylated JNK and cleaved caspase-3. Tx increased the expression of phophorylated JNK and cleaved caspase-3, indicating a desert of cell death, but SSZ decreased the cell death related protein levesls caused by Tx treatment. In addition, using quantitative real time (RT)-PCR, the mRNA levels check form H-RPE cells (Figure 2B) after treatment Tx (10 μM) or Tx with SSZ (100 μM) at 12 hours. Similarly, to the protein levels, the mRNA levels of autophagy related genes, including LC3B, Belcin1, p62 and ATG5, increased in response to Tx treatment. SSZ were dramatically diminished Tx-induced autophagy related genes in human RPE cells. Taken together, these results indicate that SSZ has the ability to regulate Tx-induced autophagy by protecting the inhibition of autophagy related genes and cell death related genes in human retinal pigment epithelial cells.

Sulfasalazine suppresses tamoxifen-induced autophagy cell death

To determine elucidate the potency of SSZ on Tx-induced autophagy in human RPE cells. I first applied an autophagy inhibitor, chloroquine (CQ) and Bafilomycin A1 (Baf-1). Bafilomycin A1 (Baf-1) inhibits autophagic flux by preventing the acidification of endosomes and lysosomes. Similar to bafilomycin A1, the chloroquine (CQ) is now widely used as an inhibitor of autophagy in both cell culture and in vivo [44]. The cell viability in H-RPE cells were treated to Tx (10 μM) or Tx with SSZ (10 μM or 50 μM) for 24 hours in the presence and the absence of autophagy inhibitor, CQ (50 μM) and Baf-1 (100 nM), and then using MTT assays. The H-RPE cells after treatment Tx with autophagy inhibitor, respectively, increased cell viability which indicated the activation and induction of autophagy. Furthermore, Tx with autophagy inhibitor in the presence SSZ, dose dependently, remarkably increased cell viability of H-RPE cells (Figure 3A, B). Having established that SSZ inhibition Tx-induced autophagy, I next used autophagy inducer, Rapamycin, whether SSZ inhibits the cell viability of H-RPE cells after Tx with Rapamycin for 24 hours. I found that the H-RPE cells after treatment Tx with autophagy inducer, Rapamycin (500 nM) decreased cell viability, however SSZ enhanced cell viability under Tx with rapamycin combination (Figure 3C). To confirm the role of SSZ in Tx-induced autophagy cell death, I verified the cell viability 24 hours after Tx (30 μM) or Tx with SSZ (100 μM) treatment in control or hLC3B shRNA expression ARPE-19 cells. The cell viability was decreased after Tx treatment to control shRNA expression ARPE-19 cells, but not in hLC3B shRNA expressing ARPE-19 cells. The cell viability were significantly increased Tx with SSZ treatment in control and hLC3B shRNA expression ARPE-19 cells (Figure 3D). Taken together, these results suggest that the SSZ function in protecting against Tx- induced autophagy in human retinal pigment epithelial cells.

Tamoxifen-mediated reactive oxygen species enhances autophagy

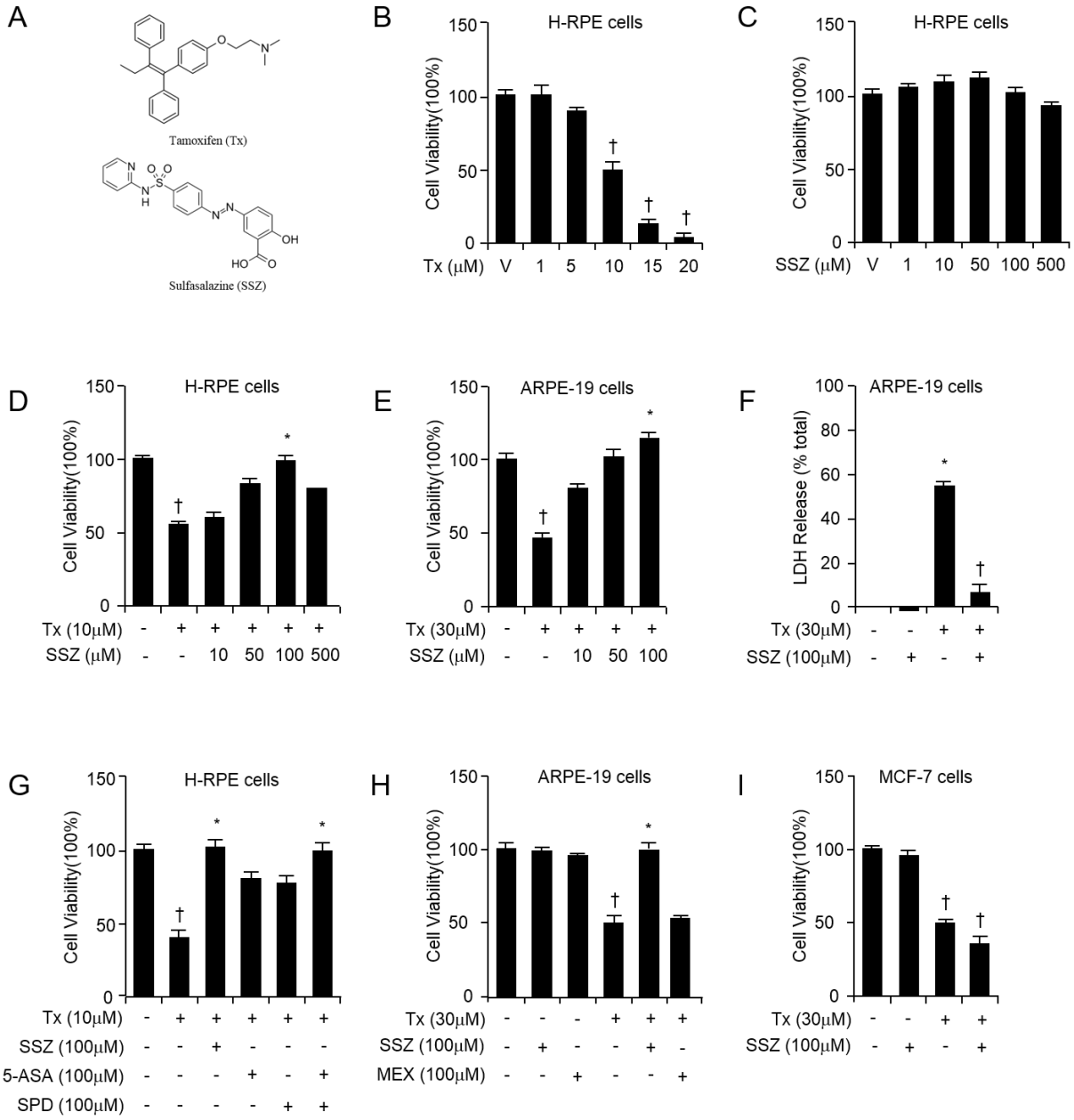
To identify the signaling molecules involved in tamoxifen (Tx)-induced cell death in human RPE cells, The cell viability in H-RPE cells were treated to Tx (10 μM) or Tx with inhibitor (10mM NAC, 1 μM DPI, 10 μM Mito-TEMPO) for 24 hours, and then using MTT assays. The cell viability of H-RPE cells decreased by 48.11±1.57% in response to Tx (10 μM). Only NAC (cytosolic ROS scavenger) rescued the cell viability of H-RPE cells up to 88.78%±3.49%, in response to Tx (10 μM) (Figure 4A). Moreover, using quantitative real time (RT)-PCR, the mRNA levels check form H-RPE cells (Figure 4B) after treatment Tx (10 μM) or Tx with inhibitor (10mM NAC, 1 μM DPI, 10 μM Mito-TEMPO) at 12 hours. The mRNA levels of autophagy related genes, including LC3B, increased in response to Tx treatment. However, only NAC was dramatically decreased Tx-induced LC3B expression in human RPE cells. In the same manner, total protein harvest from H-RPE (Figure 4C) cells were treated to Tx (10 μM) for 24 hours in the presence of inhibitor (10mM NAC, 1 μM DPI, 10 μM Mito-TEMPO), and then using western blotting. The human RPE cells treated to Tx alone showed enhanced autophagy related protein genes, LC3B, but, the LC3B level was abolished when combination treatment of NAC, suggesting Tx-induced autophagy was involved by cytosolic ROS in human retinal pigment epithelial cells.

Sulfasalazine inhibits tamoxifen–induced intracellular generation of reactive oxygen species

Previous results (Figure 4) showed that tamoxifen (Tx) increase the level of ROS in human RPE cells. Hence, I analyzed the possibility that SSZ protects to Tx-induced ROS generation and cell death in human RPE cells. The production of ROS in ARPE-19 cells were treated Tx (30 μM) or Tx with SSZ (100 μM) for 6, 12, 24 hours, and then using fluorescent probes oxidative stress detection reagent (green) for total ROS detection and superoxide detection reagent (orange), respectively. Total ROS and superoxide were increased markedly in ARPE-19 cells after Tx treatment at 12hours. However, 6 and 24 hours had no effect on ROS generation. Consistent with a role for ROS in Tx-induced autophagy, addition of the SSZ, almost completely blocked Tx-induced ROS production in ARPE-19 cells (Figure 5). These results demonstrated that SSZ protects Tx-induced ROS dependent autophagy in human retinal pigment epithelial cells.

Sulfasalazine attenuates tamoxifen-induced age-related macular degeneration marker genes

Thereafter, the effects of SSZ on AMD-associated gene expression in response to Tx (10 μM) in H-RPE cells were assessed. mRNA levels of AMD-associated genes, including complement factor I (CFI), complement factor H (CFH), apolipoprotein E (APOE), apolipoprotein J (APOJ), toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) were enhanced 12 hours after Tx treatment, but not Tx with SSZ (100 μM) in H-RPE cells (Figure 6). These results suggest that SSZ protects Tx-induced autophagy, cell death, and AMD-associated gene expression in H-RPE cells. Therefore, SSZ might play as a pathologic mediator under autopagy cell death in human retinal pigment epithelial cells**.**


A
Tamoxifen (Tx)
Sulfasalazine (SSZ)
B
H-RPE cells
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Tx (30μM) - - + +
SSZ (100μM) - + - +

Figure 1. Sulfasalazine was inhibited tamoxifen–induced cell death in human RPE cells.

The chemical structure of Tx and SSZ (A). The cell viability was analyzed 24 hours after various doses of Tx administration in H-RPE cells (B). †$p<0.05$, decreased the cell viability after Tx administration vs vehicle (V). The cell viability was analyzed 24 hours after various doses of SSZ administration in H-RPE cells (C). The cell viability was analyzed in response to Tx or Tx plus SSZ in H-RPE cells (D). †$p<0.05$, decreased the cell viability after Tx administration vs vehicle (V). *$p<0.05$, rescued the cell viability in response to Tx plus SSZ vs Tx alone. The cell viability was analyzed in response to Tx or Tx plus SSZ in ARPE-19 cells (E). †$p<0.05$, decreased the cell viability after Tx administration vs vehicle (V). *$p<0.05$, rescued the cell viability in response to Tx plus SSZ vs Tx alone. The LDH release was analyzed in response to Tx or Tx plus SSZ in ARPE-19 cells (F). *$p<0.05$, increased the LDH release in response to Tx administration vs vehicle (V). †$p<0.05$, decreased the LDH release after Tx plus SSZ vs Tx alone. The cell viability was analyzed in response to Tx, Tx plus SSZ, Tx plus 5-ASA or Tx plus SPD in H-RPE cells (G). †$p<0.05$, decreased the cell viability after Tx administration vs vehicle (V). *$p<0.05$, rescued the cell viability in response to Tx plus SSZ, Tx plus 5-ASA and SPD vs Tx alone. The cell viability was analyzed in response to Tx, Tx plus SSZ or Tx plus MEX in ARPE-19 cells (H). †$p<0.05$, decreased the cell viability after Tx administration vs vehicle (V). *$p<0.05$, rescued the cell viability in response to Tx plus SSZ vs Tx alone. The cell viability was analyzed in response to Tx or Tx plus SSZ in MCF-7 cells (I). †$p<0.05$, decreased the cell viability after Tx administration vs vehicle (V), Tx plus SSZ vs Tx alone. Values are presented as mean ± SD, n=12.

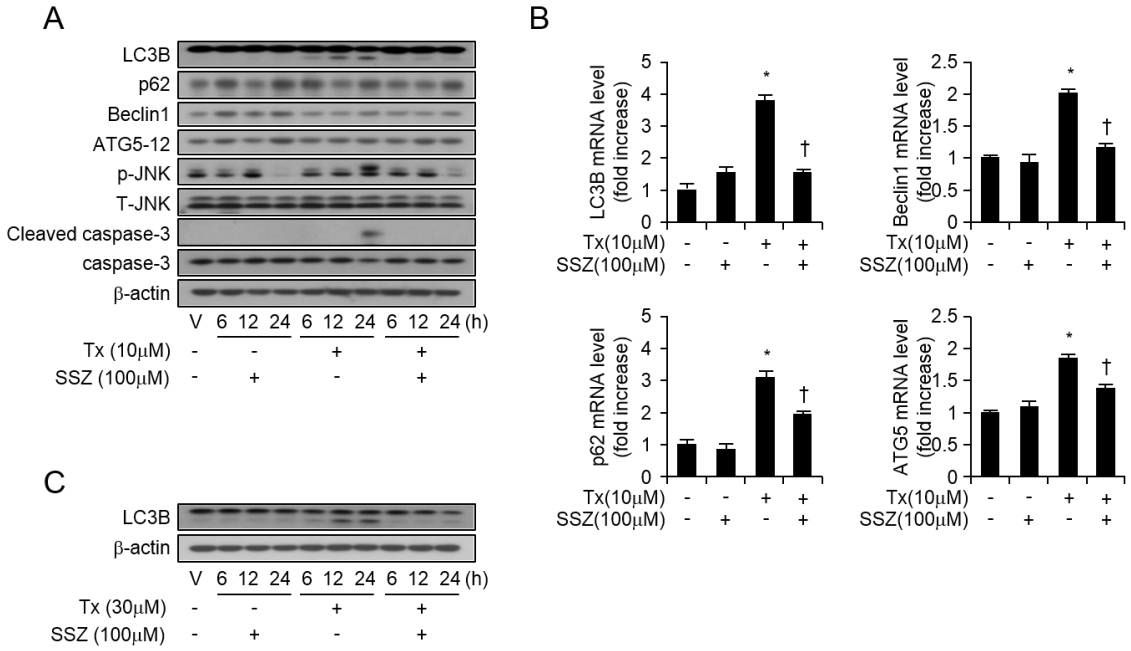

Figure 2. Sulfasalazine was enhanced mRNA and protein levels of tamoxifen-induced autophagy related genes in human RPE cells.

The levels of LC3B, p62, Beclin1, ATG5-12, total JNK, phosphorylated JNK, caspase-3 and cleaved caspase-3 proteins were assessed using western blotting analysis (A). β-actin was used as a loading control. Experiments were performed at least three independent times. . Total RNA was extracted from H-RPE cells 12 hours after Tx (10 μM) or Tx plus SSZ (100 μM) administration. Quantitative real-time RT-PCR was performed to assess mRNA levels of autophagy related genes, including, LC3B, p62, Beclin1 and ATG5 (B). For all real-time PCR analyses, mouse β-actin was used as a control for normalization. Expression levels of each mRNA are divided by expression of β-actin and shown as a ratio of each mRNA/β-actin. Values are presented as mean ± SD, n=3. *$p<0.05$, increased mRNA levels of genes in response of Tx vs vehicle (V). †$p<0.05$, decreased mRNA levels of genes in response of Tx plus SSZ vs Tx alone. The levels of LC3B proteins was assessed using western blotting analysis (C). β-actin was used as a loading control. Experiments were performed at least three independent time

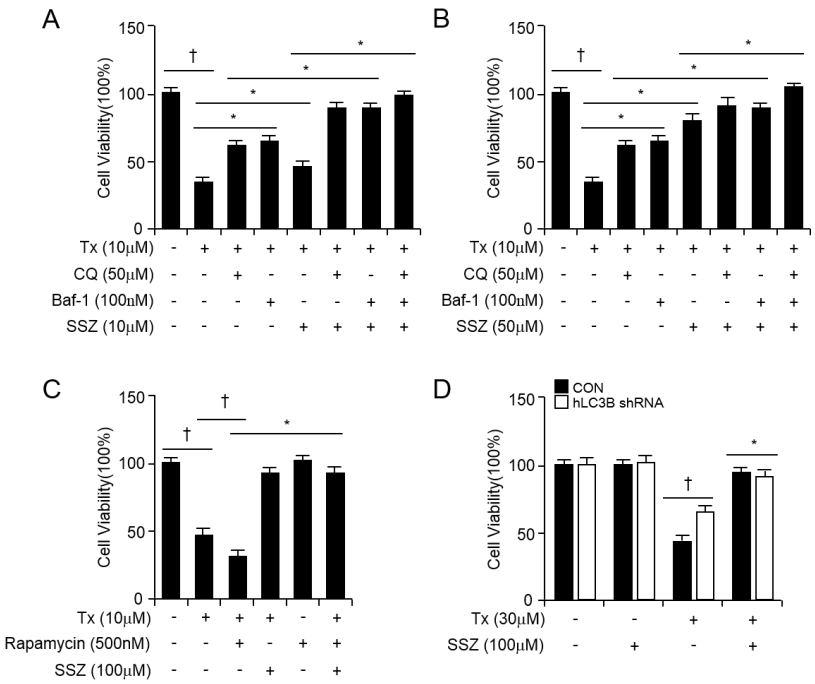

Figure 3. Sulfasalazine was decreased tamoxifen-induced autophagy cell death in human RPE cells.

The cell viability was analyzed 24 hours after Tx (10 μM) or Tx plus autophagy inhibitor (CQ and Baf-1) in the presence and the absence of SSZ 10 μM (A) or SSZ 50 μM (B) administration in H-RPE cells, respectively. †p<0.05, decreased the cell viability after Tx administration vs vehicle (V). *p<0.05, rescued the cell viability in response to Tx plus autophagy inhibitor or Tx plus SSZ. The cell viability was analyzed 24 hours after Tx (10 μM) or Tx with autophagy inducer (Rapamycin) in the presence and the absence of SSZ (100 μM) administration in H-RPE cells (C). †p<0.05, decreased the cell viability after Tx plus autophagy inducer vs Tx alone. *p<0.05, rescued the cell viability in response to Tx plus autophagy inducer in the presence SSZ vs Tx plus autophagy inducer. The cell viability was analyzed in response to Tx (30 μM) or Tx plus SSZ (100 μM) in control or hLC3B shRNA expressing ARPE-19 cells (D). †p<0.05, decreased the cell viability after Tx administration vs vehicle (V), Tx plus SSZ vs Tx alone. Values are presented as mean ± SD, n=12.

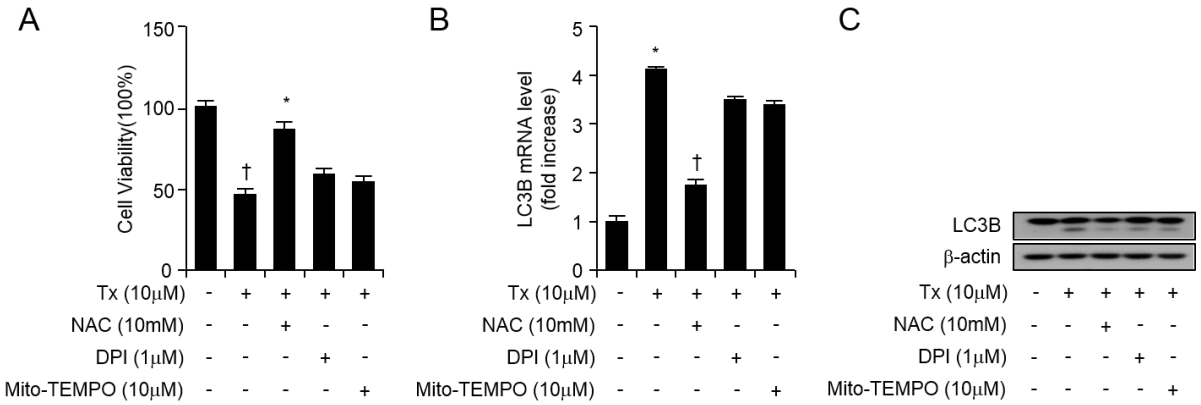

Figure 4. Tamoxifen-mediated ROS was increased autophagy in human RPE cells.

The cell viability was analyzed in response to Tx (10 μM) or Tx plus signaling inhibitors in H-RPE cells (A). †p<0.05, decreased the cell viability after Tx administration vs vehicle (V). *p<0.05, rescued the cell viability in response to Tx plus signaling inhibitors vs Tx alone. Total RNA was extracted from H-RPE cells 12 hours after Tx (10 μM) with signaling inhibitor (10 mM NAC, 1 μM DPI or 10 μM Mito-TEMPO), administration. Quantitative real-time RT-PCR was performed to assess mRNA levels of LC3B. For all real-time PCR analyses, mouse β-actin was used as a control for normalization. Expression levels of each mRNA are divided by expression of β-actin and shown as a ratio of each mRNA/β-actin. Values are presented as mean ± SD, n=3 (B). *p<0.05, increased mRNA levels of genes in response of Tx vs vehicle (V). †p<0.05, decreased mRNA levels of genes in response of Tx plus NAC vs Tx alone. Total protein was extracted from H-RPE cells 24 hours after Tx (10 μM) with signaling inhibitor (10 mM NAC, 1 μM DPI or 10 μM Mito-TEMPO), administration. The levels of LC3B proteins was assessed using western blotting analysis (C). β-actin was used as a loading control. Experiments were performed at least three independent times.

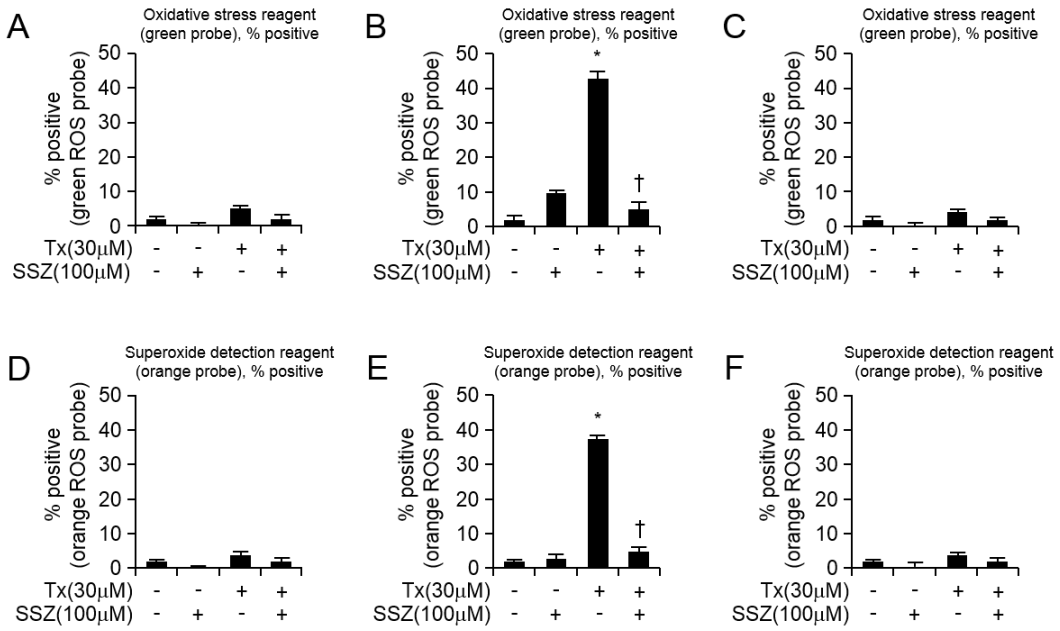

Figure 5. Sulfaslazine was disrupted tamoxifen-induced ROS in human RPE cells.

ARPE-19 cells were treated Tx (30 μM) or Tx plus SSZ (100 μM) for 6 (A, D), 12 (B, E) and 24 (C, F) hours. Oxidative stress and superoxide were assayed for by flow cytometry using the fluorescent probes oxidative stress detection reagent (green) for total ROS detection and superoxide detection reagent (orange), respectively. *$p<0.05$, increased the level of ROS in response to Tx administration vs vehicle (V). †$p<0.05$, decreased the level of ROS after Tx plus SSZ vs Tx alone.

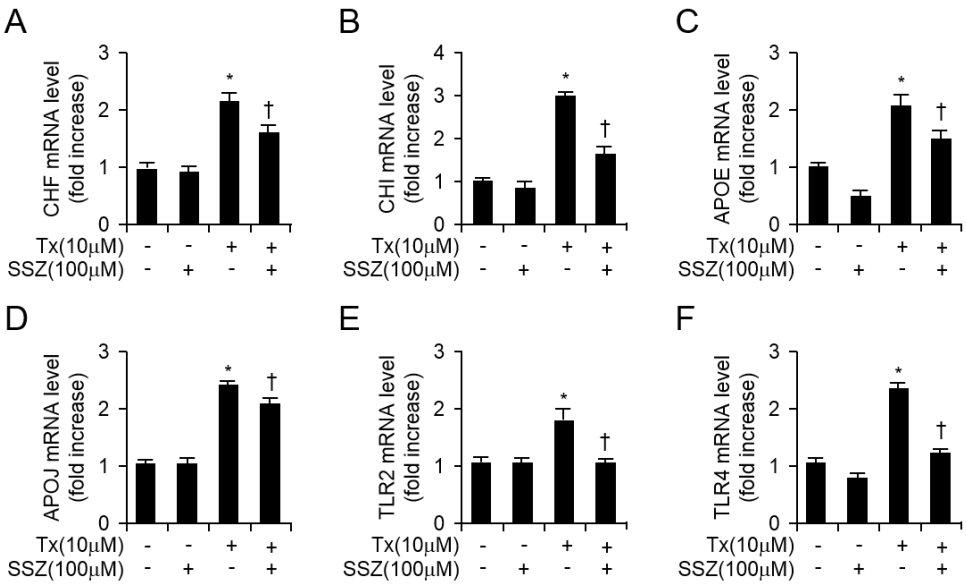

Figure 6. Sulfasalazine was abolished mRNA levels of AMD-associated genes in human RPE cells.

Total RNA was extracted form H-RPE cells after Tx (10 μM) or Tx with SSZ (100 μM) administration for 12 hours. mRNA levels of CFH (A), CFI (B) APOE (C), APOEJ (D) TLR2 (E) and TLR4 (F) were analyzed by quantitative real-time RT-PCR. Human β-actin was used as a control for normalization. Expression levels of each mRNA are divided by expression of β-actin and shown as a ratio of each mRNA/β-actin. Values are presented as mean ± SD, n=3. *$p<0.05$, increased mRNA levels of genes in response of Tx vs vehicle (V). †$p<0.05$, decreased mRNA levels of genes in response of Tx plus SSZ vs Tx alone.

Discussion

Tamoxifen is an oral estrogen antagonist drug, which is adjuvant treatment of estrogen receptor positive breast cancer when used in low doses. Although generally tolerable, tamoxifen has been relatively free of serious side effects, though ocular toxicity, including dryness, irritation, cataracts, and deposits in the retina, in the area of the macula, that result in macular edema. It is likely associated with damage to retinal pigment epithelium (RPE) cells and photoreceptors [4]. Retinal pigment epithelium cells serve a critical role in the maintenance of photoreceptors, phagocytizing the outer segment tips of photoreceptors, which are then digested within lysosomes. Retinal pigment epithelium dysfunction is thought to play a role in a variety of retinal diseases including tamoxifen retinopathy and Age related macular degeneration (AMD). Consequently, the prevention of RPE due to tamoxifen damage may play a significant role in the treatment of AMD. Sulfasalazine (SSZ), sold under the trade name Azulfidine among others, is a medication used to treat rheumatoid arthritis, ulcerative colitis, and Crohn's disease. It is considered by some to be a first line treatment in rheumatoid arthritis. Although the role of SSZ in many diseases is alternative, SSZ is an orally applicable, well-known drug, mainly used in rheumatology, with an anti-inflammatory and immunomodulatory efficacy [45]. However, the role and effects of SSZ in tamoxifen-induced signaling pathways and cell viability have not been elucidated in RPE cells. The goal of this study was to investigate the SSZ mechanism by which tamoxifen induces RPE cell death and identify targets that can be used as a potential therapy. I examined the mechanism of tamoxifen toxicity in cultured human primary H-RPE cells and cell lines ARPE-19 cells. And then, I revealed that SSZ protected human RPE cells against tamoxifen-induced cell death. Cell death can occur through several different mechanism. One event that may initiate cell death is a lysosome-dependent autophagy. Excessive autophagy is an underlying mechanism in the acute toxicity paradigm. Because tamoxifen is

known to induce autophagy in certain cancer cells [23, 46], and since autophagy can cause cell death [47], it seems possible that an autophagic mechanism also may be involved in TAM-induced cell death in RPE cells. Consequentially, SSZ has the ability to regulate Tx-induced autophagy by protecting the inhibition of autophagy related genes and cell death related genes in human RPE cells. I demonstrated that chloroquine (CQ) and bafilomycin A1 (Baf-1), which blocks the initiating event of autophagy, reduced tamoxifen toxicity. However, SSZ was effective in reducing tamoxifen toxicity in human RPE cells. Moreover, Rapamycin, which induces the initiating event of autophagy, enhanced tamoxifen toxicity. In addition, SSZ was more effective in increasing tamoxifen toxicity. Also I have observed different cell viability, in response to tamoxifen or tamoxifen with SSZ in LC3B shRNA expressing ARPE-19 cells compared with control shRNA expressing ARPE-19 cells, indicating that SSZ contribute to tamoxifen-induced cell death in human RPE cells. Previous studies have reported that tamoxifen increase the level of reactive oxygen species (ROS) and oxidative stress in breast cancer cells [48, 49]. ROS have been identified as signaling molecules in various pathways regulating both cell survival and cell death [50]. Many stimuli that induce ROS generation also induce autophagy. Hence, I analyzed that tamoxifen with ROS inhibitor, NAC, dramatically reduces LC3B expression, suggesting the involvement of ROS in the initiation of tamoxifen-induced autophagy. Moreover, addition of SSZ attenuated tamoxifen-induced ROS production, indicating that SSZ contribute to tamoxifen-induced ROS mediated autophagy in human RPE cells. This finding is consistent with other accounts of autophagic cell death. Furthermore, autophagy associated retinal degeneration, including age-related macular degeneration (AMD). Although the pathogenesis of AMD is still partly unknown, cellular autophagy plays a key role in the development of AMD. More importantly, the expression of AMD-associated genes, CFI, CFH, APOE, APOEJ, TLR2 and TLR4, were decreased in tamoxifen with SSZ treated in human RPE cells. These data suggest that autophagy and a risk for AMD were reduced in response to SSZ compare tamoxifen alone in human RPE cells. In this study, we provide information regarding the critical role of SSZ under tamoxifen-induced autophagy conditions in the tamoxifen retinopathy and early stage of AMD development, especially the loss of RPE cells.

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Material & Method

Reagents

Sodium Iodate (NaIO3; 71702), Tunicamycin (T7765), Sulfasalazine (S0883), Methotrexate (M9929), Sulfapyridine (S6252), Chloroquine (C6628), Bafilomycin A1 (B1793) and 4-phenylbutyrate (SML0309) (Sigma-Aldrich, St. Louis, MO, USA), Tamoxifen (ALX-550-095) (Enzo Life Sciences, Farmingdale, NY, USA) and 5-Aminosalicylic Acid (70265) were purchased for reagents. Retinal Pigment Epithelial Cell Growth Medium (RtEGMTM; #00195407) with supplements including 2% FBS, 2% L-glutamine, 0.5% bFGF, 0.1% GA-1000 was purchased from LONZA (Walkersville, MD, USA). Dulbecco's modified Eagle's medium (DMEM; 12800-017), fetal bovine serum (FBS; 26140-079), penicillin/streptomycin (10378-016), and 0.25% trypsin (25200-072) and other cell culture reagents were purchased from Gibco (Gaithersburg, MD, USA). Primary antibodies including phosphor-Akt (ser473; #4058, Thr308; #9275), total Akt (#9272), phospho-ERK (#4370), ERK (#4695), phosphor-JNK (#9251), JNK (#9252), phosphor-p38 (#4511), p38 (#9212), phosphor- IκBα (#9246), IκBα (#9242), cleaved caspase-3 (#9664) and caspase-3 (#9662) (Cell Signaling Technology, Inc., Danvers, MA), CHOP (sc-793) and Beclin1 (sc-11427) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), ATG5 (ab108327) (Abcam, Cambridge, MA), and LC3B (L7943), p62 (P0067), β-actin polyclonal antibody (Sigma-Aldrich Co. LLC., St Louis, MO) were used for western blotting analysis. Human pentraxin 3 (PTX3; DY1826) ELISA kit was purchased from R&D System, Inc. (Minneapolis, MN, USA). U0126 (BML-EI282), LY194002 (BML-ST420), SP600125 (BML-EI305), SB203580 (BML-EI286), BAY 11-7082 (BML-EI278) (Enzo Life Sciences, Farmingdale, NY, USA), and NAC (A7250), DPI (D2926), Mito-TEMPO (SML0737) (Sigma-Aldrich, St. Louis, MO, USA) were used for inhibitor reagents.

Human retinal pigment epithelial (RPE) cell culture

Primary human fetal RPE (H-RPE; #00195406) cells were purchased at passage one from LONZA (Walkersville, MD, USA), and all experiments were performed with cells between passage two to six. The cells were maintained in RtEGM™ medium supplemented with 2% FBS, 2% L-glutamine, 0.5% bFGF, 0.1% GA-1000. Human retinal pigmented epithelial cell lines ARPE-19 cells (CRL-2302TM) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). ARPE-19 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U⁄mL penicillin and streptomycin. Cell cultures were maintained at 37°C in a humid atmosphere incubator with 5% CO2 and 95% air. The medium was changed every 3-4 days. To passage the cells, I subcultured RPE cells at a 1:4 dilution using 0.25% trypsin and cells usually reached confluence after about four days. All experiments used for two to six passage of human RPE cells.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

For cultured cells, total RNA was isolated from cultured cells using TRIzol reagent (#15596018) (Thermo Fisher Scientific, Inc., Waltham, MA). Equal amounts of RNA were reverse transcribed with SuperScript™ III First-Strand Synthesis System (#18080-044) (Thermo Fisher Scientific, Inc., Waltham, MA) to cDNA. qRT-PCR was performed on the resulting cDNA using iQ SYBR Green Supermix (#170-8882AP) (Bio-Rad Laboratories, Inc., Hercules, CA). The comparative cycle threshold (Ct) value method, representing log transformation, was used to establish relative quantification of the fold changes in gene expression using StepOne plus system (Applied Biosystem, CA, USA). β-actin was used as an internal control (a commonly used loading control for gene degradation in PCR). Primers of human β-actin, Pentraxin 3 (PTX3), Glucose-6-phosphate dehydrogenase (G6PDH), Glutathione S-reductase (GSR), Glutathione peroxidase 1 (GPX1), Superoxide dismutase 1 (SOD1), Superoxide dismutase 2 (SOD2), Catalase (CAT), Microtubule-associated protein 1A/1B-light chain 3 (LC3B), Sequestosome-1 (p62), Autophagy related 5 (ATG5), Beclin1, CCAAT-enhancer-binding protein homologous protein (CHOP), Glucose-regulated protein 78 (GRP78), Interleukin 10 (IL-10), Transforming growth factor beta (TGFβ), Interleukin 1 beta (IL-1β), Tumor necrosis factor alpha

(TNFa), Complement factor H (CFH), Complement factor I (CFI), Apolipoprotein E (APOE), Apolipoprotein J (APOJ), Toll-like receptor 4 (TLR4) and Toll-like receptor 2 (TLR2) were purchased from Cosmo Genetech, Inc. (Seoul, Korea). The primer pairs which we used were listed in Table 1. Amplification of cDNA started with 10 minutes at 95°C, followed by 40 cycles of 15 s at 95°C and 1 minute at 60°C.

Table 1. The primers sequences were as follows.

Human gene	Forward primer sequence 5' to 3'	Reverse primer sequence 5' to 3'
PTX3	AATGCATCTCCTTGCGATTC	TGAAGTGCTTGTCCCATTCC
G6PDH	TGAGCCAGATAGGCTGGAA	TAACGCAGGCGATGTTGTC
GSR	TCACCAAGTCCCATATAGAAATC	GTGTAGGACTAGCGGTGT
GPX1	CCAAGCTCATCACCTGGTCT	TCGATGTCAATGGTCTGGAA
SOD1	GAAGGTGTGGGGAAGCATTA	ACATTGCCCAAGTCTCCAAC
SOD2	CGTGACTTTGGTTCCTTTGAC	AGTGTCCCCGTTCCTTATTGA
CAT	CGTGCTGAATGAGGAACAGA	AGTCAGGGTGGACCTCAGTG
LC3B	ACCATGCCGTCGGAGAAG	ATCGTTCTATTATCACCGGGATTTT
p62	AGGCGCACTACCGCGAT	AGGCGCACTACCGCGAT
ATG5	GCAGATGGACAGTTGCACACA	GCAGATGGACAGTTGCACACA
Beclin1	CTGGACACGAGTTTCAAGATCCT	TGTGGTAAGTAATGGAGCTGTGAGTT
CHOP	TGCTTCTCTGGCTTGGCTGAC	CCGTTTCCTGGTTCTCCCTTGG
GRP78	AGGAGGAGGACAAGAAGGAGGAC	CAGGAGTGAAGGCGACATAGGAC
IL-10	GGTTGCCAAGCCTTGTCTGA	AGGGAGTTCACATGCGCCT
TGFβ	ACCTGAACCCGTGTTGCTCT	CTAAGGCGAAAGCCCTCAAT
IL-1β	AAATACCTGTGGCCTTGGGC	TTTGGGATCTACACTCTCCAGCT
TNFα	ATGAGCACTGAAAGCATGATCC	GAGGGCTGATTAGAGAGAGGTC
CFH	TACTGGCTGGATACCTGCTC	CCTGACGGAGTCTCAAAATG
CFI	GGTGAGGTGGACTGCATTACA	CCTCCCACAATTCGTTTCCTTC
APOE	AACTGGCACTGGGTCGCTTT	GCCTTCAACTCCTTCATGGTCTCGT
APOJ	ATTTATGGAGACCGTGGCGGAGAAAG	CTGGTTACTTGGTGACGTGCAGAG
TLR2	AGTTGATGACTCTACCAGATG	GTCAATGATCCACTTGCCAG
TLR4	ACTTGGACCTTTCCAGCAAC	TTTAAATGCACCTGGTTGGA
β-actin	ATCGTGCGTGACATTAAGGAGAAG	AGGAAGGAAGGCTGGAAGAGTG

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were used to measure human PTX3 using ELISA kits from R & D Systems (Minneapolis, MN) according to the manufacturer's instructions. In brief, the ELISA plates (BD Biosciences, San Jose, CA) were coated with a monoclonal antihuman PTX3 antibody (2 μg/mL) in coating buffer (1% BSA in PBS (150 mM NaCl, 5 mM KCl, 5 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.2–7.4) for overnight at room temperature. Then the plates were blocked with coating buffer for 2 hours at

room temperature, and incubated with either recombinant human PTX3 standards or the samples collected in quadruplicate (100 μL/well) for another 2 hours. The plates were then incubated with a biotinylated human PTX3 antibody (150 ng/mL) for 2 hours, and freshly diluted streptavidin-horse radish peroxide (HRP) for 20 minutes subsequently in the dark. After each step, the plates were washed three times with the washing buffer. The chromogen substrate tetra-methylbenzidine (100 μL/well; eBioscience, Inc., San Diego, CA) was added and incubated for 5 minutes in the dark. The reaction was stopped by adding 2 N H_2SO_4 (50 μL/well), and the plates were read at 450 nm with an automatic ELISA reader (MERK SensIdent Scan, Helsinki, Finland).

Western blot analysis

The RPE cells were harvested using RIPA buffer (Tris/Cl (pH 7.6); 100 mmole/L, EDTA; 5 mmole/L, NaCl; 50 mmole/L, β-glycerophosphate; 50 mmole/L, NaF; 50 mmole/L, Na_3VO_4; 0.1 mmole/L, NP-40; 0.5%, Sodium deoxycholate; 0.5%) with 1× Complete™ protease inhibitor Cocktail (#39922700) (Roche Applied Science, Mannheim, Germany). Protein concentrations of cell lysates were determined using the Pierce BCA protein assay kit (#23225) (Thermo Scientific, Rockford, IL). The samples were resolved with 12% sodium dodecyl sulfate–PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) overnight (120 mA). Membranes were blocked for 2 hours at room temperature with a 5% nonfat milk solution in TBST buffer (20 mM Tris–HCl, pH 7.4, 500 mM NaCl, 0.1% Tween 20). The blots were then incubated with various antibodies (diluted 1:1,000) in TBST overnight at room temperature. Equal loading was confirmed with an anti-β-actin (Sigma-Aldrich Co. LLC., St Louis, MO). The blots were then washed three times in TBST and incubated with an anti-rabbit secondary antibody or an anti-mouse secondary antibody in TBST for 1 hour at room temperature. Finally, immunoblots were detected by SuperSignal® West Pico Chemiluminescent Substrate (#34580) (Thermo Fisher Scientific, Inc., Waltham, MA) and visualized after exposure to X-ray film.

Construction of shRNA expressing ARPE-19 cells

PTX3 shRNA, LC3B shRNA and nonspecific control shRNA (Sigma-Aldrich, St. Louis, MO, USA) were transfected into ARPE-19 cells using transfection reagents (#E2691) (Promega, Madison, WI) according to the protocol of the manufacturer. Briefly, for each transfection, shRNA (1 μg) was added to ARPE-19 cells for 24 hours, and stable clones expressing shRNA were further selected by puromycin (1.0 μg/mL). Cell culture medium containing puromycin was renewed every 48 hours, until resistant colonies could be identified. The expression of PTX3 and the loading control (β-actin) in stable cells was tested.

ROS detection assay

Assessment of ROS was determined by the ROS-ID® Total ROS/Superoxide Detection Kit (ENZ-51010) (Enzo Life Sciences, Farmingdale, NY, USA). Cells were seeded at 2 × 105cells per well in 96-well plates. After reagent treatment, unincorporated dye was removed by washings with 2% FBS containing PBS. Samples were then centrifuged at 1000 rpm for 3 min and the pellets were resuspended in 500 μL of 2% FBS containing PBS. And then cells were incubated with oxidative stress detection reagent (Green) for total ROS detection reagent and superoxide detection reagent (Orange) for 30 min at 37°C in the dark. After 30 min of loading measurements were performed on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer.

Cell viability assay

Cell viability was determined by the MTS assay using the Cell Titer 96 AQueous one solution cell proliferation assay kit (G358B) (Promega, Madison, WI, USA). Cells were seeded at 0.7×10^4 cells per well in 96-well plates. After reagent treatment, 20 μL of MTS solution was added to each well, and plates were incubated for an additional 2 to 4 hours at 37℃. Absorbance was then measured at 490 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA) to calculate cell survival percentages.

LDH release assay

Cells were seeded at 0.7×10^4 cells per well in 96-well plates. After reagent treatment, the positive control was reconstituted with 100 μL LDH assay buffer. At the end of incubation, the plate was gently shaken to ensure LDH was evenly distributed in the medium. In the high control wells, 10 μL cell lysis solution was added, and the plate was shaken for 1 min and incubated at 37 °C for 30 min. Quantitative analysis was performed on the cell culture supernatant (5 μL/well). For each well, 95 μL of LDH Reaction Mix (LDH substrate mix, PicoProbe, LDH Assay Buffer) was added, bringing up the total volume to 100 μL/well. Absorbance was then measured at 450 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA) to calculate LDH release percentages.

Statistical analysis

Statistical analysis: Data are represented as mean±standard deviation (SD). For comparisons between two groups, we used the Student two-tailed unpaired t test. For comparisons of timed series experiments, I performed all tests. Statistically significant differences were accepted at $p<0.05$.

망막색소상피세포 기능 장애의 분자적 기전 연구

황나래

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초록

망막색소상피(retinal pigment epithelium, RPE)는 부르크막(Bruch's membrane)의 위층에 놓여있는 육각형의 단세포층으로 색소를 띄는 특징을 가지며 주위조직인 광수용체 및 맥락막 모세혈관층과의 상호작용을 통해 시기능을 수행하는데 중요한 역할을 한다. 또한, 망막색소상피세포는 신체 내에서 광수용(light absorption), 물질 수송(epithelial transport), 시각회로 조절(visual cycle), 식세포작용(phagocytosiss), 호르몬 분비 및 면역조절 작용 등의 기능을 수행한다. 노인성 황반변성 (AMD; Age-related macular degeneration)은 망막의 중심부에 위치한 황반에 이상이 생겨 시력이 급격히 떨어지는 병으로 주로 50세 이상에서 노화로 인해 발생하며 노년기 시력 상실의 주요 원인으로 손꼽힌다. 노인성 황반변증의 정확한 발병 기전은 거의 알려지지 않았지만, 최근 들어 병리생리학 원인으로 망막색소상피세포의 손상과 세포사멸이 관여한다고 알려져 있다. 본 연구에서는 산화 스트레스, 소포체 스트레스 그리고 타목시펜에 노출되었을 때 망막색소상피세포의 기능 장애와 사멸을 유도하는지와 그 과정에 관여하는 신호전달 분자들에 대한 메커니즘에 대해 알아보고자 하였다.

첫째, 요오드산나트륨(sodium iodate, $NaIO_3$)에 의한 산화 스트레스가 발생하면 망막색소상피세포에서 펜트락신3(PTX3)의 발현이 RT-PCR과 ELISA을 통해, mRNA와 단백질과 수준에서 증가함을 확인 하였다. 또한 산화 스트레스로 인해 증가된 펜트락신3는 활성 산소 종(ROS), AKT와 ERK의 활성화되었고, 이는 각 저해제에 의해 펜트락신3의 발현이 감소됨을 확인 하였다. 뿐만 아니라, 산화 스트레스 상황에서 항산화 효소인 G6PDH, CAT, GSR의 발현은 증가 하였지만, 펜트락신3를 shRNA를 이용해 형질주입시킨 망막색소상피세포에서는 항상화 효소의 변화가 없었다. 펜트락신3의 가능을 확인하기 위해 요오드산나트륨의 농도를 높여서 망막색소상피세포의 세포 사멸을 유도한 후, 활성 산소 종, AKT 저해제로부터 세포 생장이 다시 회복 되었다. 추가로, 펜트락신3

shRNA 형질주입 된 망막색소상피세포에서는 높은 농도의 요오드산나트륨을 노출시키자 컨트롤 벡터가 형줄주입 된 세포에 비해 세포 생존률이 현저히 증가 하였다. 마지막으로, 컨트롤벡터가 형질 주입된 세포는 산화적 스트레스 상황에서 노인성 황반변증 마커 유전자인 CFI, CFH, APOE 그리고 TLR4의 발현이 증가되었지만, 펜트락신3 shRNA 가 형질주입 된 세포에서는 유전자들의 발현 변화가 없었다. 위의 결과들은 산화 스트레스 상황에서 발현되는 펜트락신3가 망막색소상피세포의 사멸을 가속화시키는 것을 보여준다.

둘째, 소포체 (Endoplasmic Reticulum, ER)는 칼슘의 저장, 신호전달 그리고 단백질의 폴딩과 운송에 대해 전문화된 세포 내 소 기관으로 세포 내 항상성 및 세포 외 자극의 변화에 매우 민감하다. 소포체 내에 잘못된 폴딩 단백질이 증가하는 소포체 스트레스는 자가포식과 세포 사멸에 밀접하게 연관되어 있다. 망막색소상피세포에서 소포체 스트레스를 유도하게 하는 튜니카마이신(tunicamycin, TM)과 팔미트에시드(palmitic acid, PA)을 이용하여 소포체 스트레스를 유도하게 되자 세포사멸이 일어나게 되고, 자가포식의 마커로 알려진 LC3B와 소포체 스트레스 마커로 세포사멸과 관련된 CHOP 발현이 증가되는 것을 단백질 수준에서 확인 하였다. 또한 RT-PCR을 통해 튜니카마이신 농도에 따른 자가포식 마커 LC3B, p62, ATG5, Beclin1 과 소포체 스트레스 마커 CHOP, GRP78의 발현을 mRNA 수준에서 증가됨을 확인 하였다. 더 나아가 소포체 스트레스로부터 증가된 자가포식의 활성이 소포체 스트레스 저해제(4-PBA)로부터 감소된다는 것을 확인 할 수 있었다. 또한 소포체 스트레스로부터 활성화된 자가포식의 기능을 확인하기 위해 자가포식 저해제(chloroquine, CQ)를 이용하여 망막색소상피세포의 세포사멸을 확인하자, 저해제로 인해 세포 사멸이 더 촉진 된다는 것을 통해 자가포식은 세포 사멸의 유도를 막는다는 것을 확인 할 수 있었다. 소포체 스트레스로부터 유도되는 자가포식는 니코틴(산)아미드 아데닌 디뉴클레오티드 인산(燐酸)(NADP)의 환원형, (NADPH) 산화효소의 신호가 관여 한다는 것을 저해제를 이용하여 확인 할 수 있었으며, 마지막으로 노인성 황반변성 마커들은 소포체 스트레스 유도제로부터 mRNA의 발현이 증가하게 되고 이 때 소포체 스트레스 저해제에 의해 감소한다. 그러나 자가포식 저해제로 인해 다시 마커들의 발현이 회복된다는 것을 확인하였다. 위의 결과들은 소포체 스트레스로부터 유도되는 망막색소상피세포의 사멸을 자가포식의 활성을 통해 이는 세포 사멸을 방어하는 것을 보여준다.

셋째, 타목시펜(tamoxifen)은 유방암에 사용되는 항에스트로겐 치료제로 안과 질환들의 부작용 등을 발생 시킬 수 있다. 설파살라진(sulfasalazine)은 류마티스관절염, 크론병과

같은 염증성 질환에 사용되는 항 염증제이다. 타목시펜으로부터 망막색소상피세포의 사멸을 유도하는 과정에서 설파살라진이 세포사멸을 억제하는 것을 MTS와 LDH assay를 통해 확인 할 수 있었다. 또한 타목시펜으로 인한 세포 사멸에서 다양한 세포 과정 중 어떠한 신호분자들이 관여하는지를 알아보기 위해 mRNA와 단백질 수준에서 확인하였다. 결과적으로, 자가포식 대표 마커인 LC3B와 세포 사멸의 마커인 인산화된 JNK, caspase3의 발현이 타목시펜에 의해 증가하였고, 이 때 설파살라진이 증가된 LC3B, 인산화된 JNK, caspase3의 발현을 감소시켰다. 타목시펜으로 활성화된 자가포식 과정에서 자가포식 저해제(chloroquine, CQ)로 인해 세포 사멸이 억제 되고, 자가포식 유도제(rapamycin)로부터 세포사멸이 촉진 된다는 것을 확인 할 수 있었으며, 동시에 설파살라진이 활성화된 자가포식을 억제하는 결과를 얻었다. 뿐만 아니라, LC3B shRNA 형질주입 된 망막색소상피세포에서는 타목시펜을 노출시키자 컨트롤 벡터가 형질주입 된 세포에 비해 세포 생존률이 현저히 증가하였으며, 설파살라진에 의해 세포 생존률이 더 회복됨을 확인 하였다. 타목시펜에 의한 자가포식 활성에서 활성 산소 종(ROS)가 증가하게 되고, 설파살라진이 증가된 활성 산소 종을 감소 시킴으로써 세포 사멸을 억제하였다. 마지막으로 망막색소상피세포에서 타목시펜에 의한 노인성 황반변성 마커들은 모두 증가하였고, 설파살라진이 증가된 마커들의 발현을 감소시킨 결과를 얻을 수 있었다. 위의 결과들은 타목시펜으로부터 유도되는 망막색소상피세포의 사멸을 설파살라진이 억제 한다는 것을 보여준다.

따라서, 본 연구에서 산화 스트레스, 소포체 스트레스, 타목시펜을 통해 망막색소상피세포의 사멸을 유도하고, 이 과정 중에 관여하는 신호 분자 메커니즘에 대해 처음으로 규명하였다. 위의 연구는 망막색소상피세포의 기능 장애와 사멸의 원인이 되는 노인성 황반변성을 포함하는 망막 질환의 예방과 치료목적의 약물 개발에 도움이 될 것으로 생각된다.