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Oxidative stress induced cellular signaling in RPE cells

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1. ABSTRACT

Oxidative stress is an important factor in the etiology of age-related macular degeneration. In the retinal pigment epithelium, oxidative stress induces protective pathways, notably the phosphatidylinositide 3-kinase (PI3K)/Akt and the nuclear factor erythroid-2 related factor 2 (Nrf2) pathways, but also vascular endothelial growth factor (VEGF) and neuroprotectin D1 (NPD-1) signaling conduct cell protection. Strong oxidative insults result in cell death, mainly mediated via a mitochondrial apoptotic pathway, including cytochrome c release and caspase activation. The role of mitogen activated protein kinases (MAPK) in oxidative stress signaling is diverse and conflicting, conducting protective as well as apoptotic pathways, in addition to involvement in a variety of other cell responses, such as VEGF or matrix metalloproteinases (MMP) upregulation. In addition to signaling deciding cell fate, first insights in inflammatory and extracellular matrix-altering signaling are emerging.

2. INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of legal blindness in the industrialized world (1). The etiology of AMD is complex and includes demographic, genetic and environmental aspects (2). Oxidative stress has been widely acknowledged as an important factor contributing to its pathology. A cell-biological model of AMD development presents a concept in which AMD consists of a combination of age-related and AMD-specific changes. In both normal aging and AMD, oxidative stress causes injury of the retinal pigment epithelium (RPE) and the choriocapillaris, which may result in chronic inflammation and altering of the extracellular matrix. In turn, these alterations impair the supply of oxygen, further damaging the tissue and finally resulting in either atrophy or neovascularization (3). Retinas of patients with AMD show increased oxidative damage and drusen contain high amounts of oxidized proteins (4,5). Also, eyes of AMD patients have a higher

expression of antioxidant enzymes (6,7). The age-related eye disease (ARED) study has shown that antioxidants combined with zinc reduce the progression to advanced stages of AMD, both of the dry and the wet form (8).

The retinal pigment epithelium (RPE), a highly pigmented monolayered epithelium located on the Bruch's membrane between the neuroretina and the choriocapillaris, performs many functions that are indispensable for sustaining vision and is exposed to high amounts of oxidative stress (9,10). Additional to intracellular exposure to reactive oxygen species, the environment and the function of the RPE add profoundly to its oxidative burden. Due to the high oxygen demand of the retina and especially of the macula, oxygen tension surrounding the RPE is very high (11). Light exposure has been linked to the development of AMD, possibly through oxidative stress related pathology (12,13). Blue light illumination induces a wide variety of reactive oxygen species (ROS) in the RPE and increases ROS production by the mitochondria while light-induced oxidation is positively associated with age (14-16). Additionally, highly toxic photosensitizers, including the phototoxic age-pigment lipofuscin, add to the oxidative burden (17). Intracellularly, in addition to the mitochondrial electron transport chain, lipid peroxidation from phagocytosed rod outer segments is the main source of intracellular oxidative stress in the RPE (18). The RPE is specifically adapted to a high oxygenated environment, which is essential for its durability (19). The ability of the RPE to cope with oxidative stress, however, decreases with age and the lack of oxidative defense can, at least in a mouse model, directly result in an enhancement of choroidal neovascularization (20).

The topic of this review is to create an overview of the pathways induced by oxidative stress in RPE cells. Oxidative stress generally induces a variety of responses in the cells which are strongly dependent on the type of the cell, as well as the type and concentration of the stressor, and of the time course of stimulation. Depending on the severity of the insult, these responses can either be adaptive or lead to cell death. While oxidative stress induced pathways are rather well described in some cell types, the information on signaling cascades in the RPE is sparse. Caution is to be taken when translating signaling pathways from one cell type to another as the reaction of a given cell type to a given stimulus may differ from the reaction of RPE cells. In this review, pathways that have been shown to be activated by oxidative stress in the RPE are presented, mainly concerning cell fate but also presenting inflammatory and extracellular matrix altering pathways.

3. OXIDATIVE STRESS PATHWAYS

3.1. Oxidative stress stimuli

Oxidative stress can be induced by a variety of molecules and chemicals. Free radicals that contain one or more unpaired electrons and are capable of independent existence, oxygen species, which have been elevated to a higher energy level (e.g. singlet oxygen) or strong oxidizing agents (e.g. hydrogen peroxide (H_2O_2)), are summarized as reactive oxygen species of which hydrogen peroxide, superoxide anion, hydroxyl radical, nitric oxide, singlet oxygen, lipid peroxyl radicals and peroxynitrites are pathophysiologically most relevant. Hydrogen peroxide is generated as a byproduct of the ß-oxidation of long-chain polyunsaturated fatty acids, which make up a great portion of the lipids in ingested photoreceptor outer segments and, if applied extracellularly, readily crosses the plasma membrane. Superoxide anion (O_2^-) is a byproduct of mitochondrial respiration and is converted into H_2O_2 by superoxide dismutase (SOD) 2 (21). In the presence of redox active metal ions (e.g. Fe^{2+}), H_2O_2 may generate highly reactive hydroxyl radical (OH*) via Fenton chemistry, which is considered to be responsible for the majority of mtDNA damage. O_2^- may additionally react with nitric oxide (NO) to generate NO-derived nitrogen oxides ($ONOO^-$), which causes lipid peroxidation (22,23). 4-hydroxynonenal (4-HNE) is a product of the oxidation of polyunsaturated fatty acids and one of the major reactive aldehydes. It reacts with nucleophilic sites of proteins and nucleic acid (24). Both H_2O_2 and 4-HNE are commonly used to experimentally induce oxidative stress in cell cultures. Another chemical oxidant applied is t-butylhydroperoxide (t-BH). It is relatively stable and readily permeates the cell membrane. Once inside the cell, it induces oxidative stress by 2-electron oxidation and by metal ion and metalloprotein-catalyzed radical processes (25,26). Other sources of oxidative stress injuries are UV-light, hypoxia and lipofuscin (17,27,28). One of the main environmental factors inducing oxidative stress that is strongly connected to AMD development is smoking (13,29). Cigarette smoke contains a vast amount of radicals including ROS, NO, nitrogen dioxide, peroxynitrite and peroxynitrate (30,31) and can directly induce oxidative damage in murine RPE (32). Recently, mononuclear phagocytes have been shown to induce oxidative changes in RPE cells that were accompanied by apoptosis (33).

3.2. Signaling of life and death

3.2.1. Protective signaling

The RPE is adjusted to exist in an environment of high oxygen tension and has a variety of mechanisms to protect itself from oxidative stress induced injuries (19). Most important factors of protective signaling in the RPE are phosphoinositide 3-kinase (PI3K) and nuclear factor erythroid-2 related factor 2 (Nrf2); additional factors discussed below are vascular endothelial growth factor A (VEGF-A, VEGF) and neuroprotectin D1 (NPD1). The signal transduction pathways of these factors overlap and interact, which is depicted in an integrative schematic of protective pathways of the RPE in figure 1.

3.2.1.1. Phosphoinositide 3-kinase

The PI3K/Akt pathway plays a major role in cell survival signaling and is implicated in many biological responses to extracellular signals. It mediates cell survival by phosphorylating and thereby inactivating several proapoptotic factors. PI3K is a heterodimer consisting of a 110 kDa subunit and a regulatory an 85 kDa subunit. When activated, PI3K catalyses the conversion of phosphatidylinositol-3,4-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate ($PtInP_3$), phosphorylating the phosphatidylinositol at the D3 position

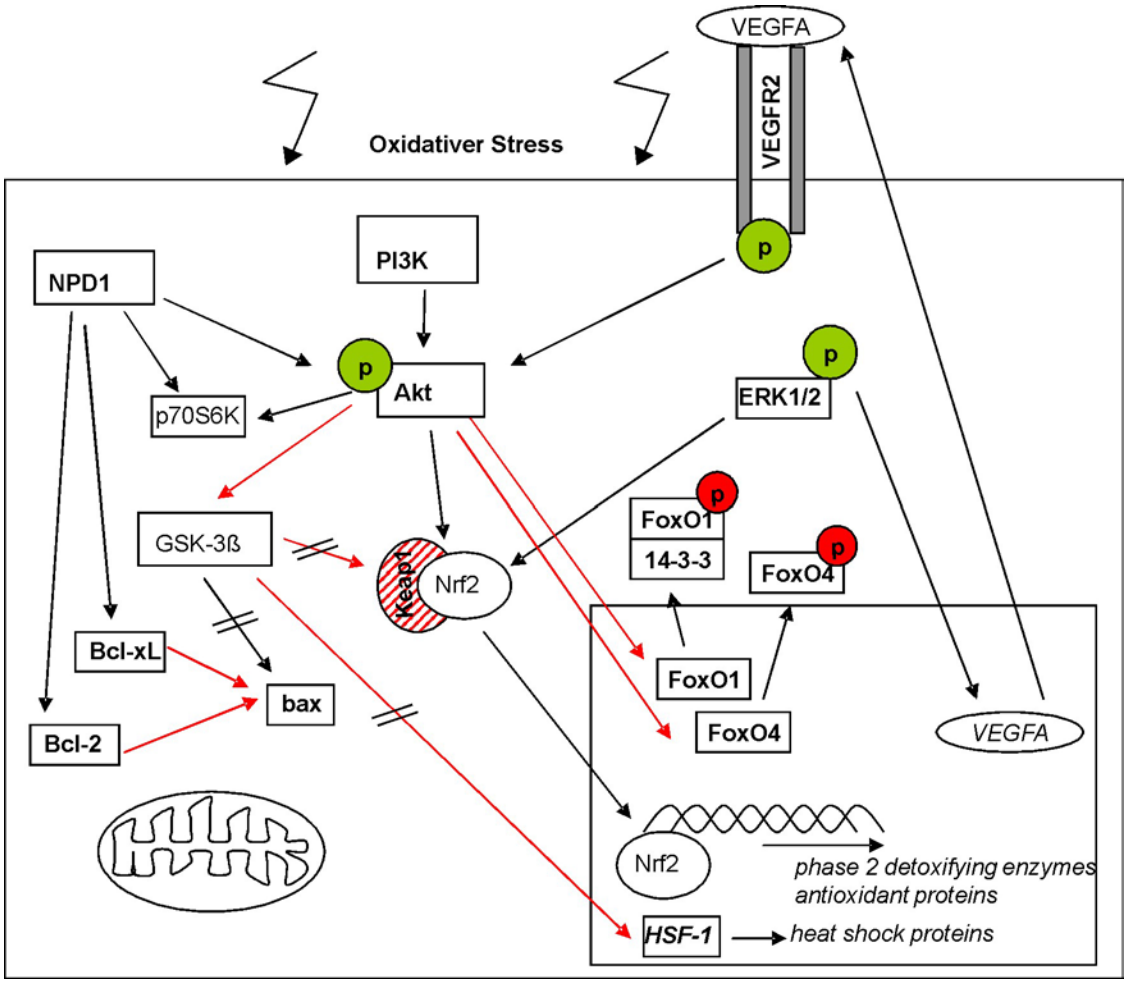

Figure 1. An integrated summary of protective pathways is given. Only interactions that have explicitly been shown for the RPE are considered. Akt is activated by oxidative stress via the activation of PI3K, by VEGF and NPD1. Akt activates Nrf2 and inactivates the transcription factors FoxO1 and FoxO4. Additionally, GSK-3ß is inhibited, preventing its activation of Bax and its inactivation of Nrf2 and HSF-1. p70S6K is activated by Akt and NPD1. NPD1 also upregulates Bcl-2 and Bcl-xL, which in turn inhibit Bax.

of the inositol ring. PtInP3 recruits pleckstrin homology domain-containing proteins to the plasma membrane, as the protein kinase Akt, also called protein kinase B, which is in turn phosphorylated and activated (34). Akt phosphorylates a wide variety of proteins on serine and threonine residues. Most downstream targets of Akt become inactivated by the phosphorylation event. These targets include, among others, glycogen synthase kinase (GSK) 3ß, Bad, caspase 9, the transcription factors forkhead and nuclear factor '*kappa*-light-chain-enhancer' of activated *B*-cells (NFkB), mammalian target of rapamycin (mTOR) and Raf protein kinase (35). Akt is active in the cytoplasm as well as in the nucleus.

Akt is activated in the RPE by oxidative stress in a dose dependent manner and a transient activation is important for RPE cell survival, as the inhibition of Akt enhances H_2O_2 mediated cell death. Phosphorylation of the proapoptotic molecules FoxO1 (forkhead in rhadomysarcoma), GSK3ß and FoxO4 (acute lymphocyte leukemia-1 fused gene from chromosome X) has been observed in the RPE (36). FoxO1 and FoxO4 are transcription factors that belong to subfamily forkhead box class O (FoxO) of the forkhead transcription factor family. Member of these families have been shown to be involved in a variety of oxidative stress responses (37). FoxO1 is a proapoptotic transcription factor, which is released from the DNA when phosphorylated. Free FoxO1 forms a complex with 14-3-3 protein, is shuttled out of the nucleus and retained in the cytoplasm, functionally inactivated (38,39). Phosphorylation of FoxO4 also translocates FoxO4 from the nucleus to the cytoplasm, inactivating the factor (40). Another member of the FoxO family, FoxO3, is involved in the regulation of complement factor H (CFH) in response to oxidative stress in the RPE and will be discussed below (41). GSK3ß is constitutively active in unstimulated cells and phosphorylates, thereby down-regulates, the activity of transcription factors such as activator protein (AP) 1, CREB and c-Myk. It phosphorylates proteins to promote their degeneration, is involved in the translocation of Bax

to the mitochondria and might impair the expression of heat shock proteins by inhibiting heat shock factor protein (HSF-)1 activation. GSK3ß is also involved in the regulation of Nrf2 (discussed below). Phosphorylation of GSK3ß by Akt turns off its catalytic activity, resulting in the activation of otherwise repressed pathways (34,42,43) (Figure 1).

Akt is also the main activator for mammalian target of rapamycin (mTOR), a serine/threonine kinase, which in turn is required for the activation of p70 S6 kinase (p70S6K) and the eukaryotic initiation factor 4E binding protein1 (4E-BP1), which is then released from the translation initiation factor eIF4E, increasing protein synthesis at the ribosome (44-46). Activation of mTOR is strongly associated with cell growth and proliferation. In the RPE, phosphorylation of mTOR and p70S6K is time- and dose-dependently initiated after oxidative stress, and the inhibition of this phosphorylation increases H_2O_2-induced apoptosis (47). The activation of mTOR in the RPE is involved in cell protection, not cell proliferation.

3.2.1.2. Nrf2

Nrf2 is a basic leucine zipper transcription factor that keeps up cellular redox homeostasis and mediates protection of cells from oxidative stress and electrophilic agents. It controls the expression and induction of a wide variety of defensive genes encoding antioxidant proteins and detoxifying enzymes, and is regarded as the most important mechanism to protect cells against acute oxidative damage. Nrf2 is mainly controlled by Kelch-like ECH-associated protein (Keap) 1. It is thought to be retained in the cytoplasm by Keap1 in an Nrf2/Cul3/Rbx1 complex, which facilitates the ubiquitylation and degradation of Nrf2 through the 26S proteasome. When the cell is exposed to oxidative stress, modifications of reactive cysteine in Keap1 lead to a physical release of Nrf2, which consequently translocates to the nucleus (48). This model has been challenged by Nguyen *et al.*, who consider Nrf2 to be primarily located in the nucleus (49). The interaction between Nrf2 and Keap1 is believed to represent a transient event taking place in the nucleus, where Nrf2 is targeted for ubiquitylation or shuttled out of the nucleus (50,51). In the nucleus, Nrf2 forms heterodimers with other basic leucine zipper genes including Jun (c-Jun, Jun-D, Jun-B) and small Maf proteins (48). As a heterodimer, Nrf2 binds to an enhancer sequence designated as the antioxidant response element (ARE) which controls the expression of phase 2 detoxifying enzymes and antioxidant proteins in response to a wide variety of stimuli, e.g. xenobiotics, heavy metals or UV light. Many genes that convey RPE resistance to oxidative stress are controlled by Nrf2, such as catalase, superoxide dismutase, heme oxygenase-1 or glutathione generating enzymes (52). Other pathways have been described to be involved in the regulation of Nrf2 transcription, e.g. PI3K (see above), or MAKP pathways (see below).

In the RPE, Nrf2 has been shown to be induced by a variety of stressors, such as cigarette smoke extract, hydroquinone, 4-HNE or acrolein (53-56). The PI3K pathway is important for Nrf2 protection against oxidative stress in the RPE (55). PI3K/Akt activity is required for Nrf2 activation and Nrf2-dependent GSH synthesis, while active Akt is sufficient to enhance Nrf2 activation. Furthermore, translocation of Nrf2 into the nucleus was prevented by PI3K inhibitors (55). This involvement of PI3K is not universal, as in cell types other then RPE, Nrf2 activity induced by the same agent (4-HNE) has been shown to be independent from the PI3K pathway (57). Another connection to the PI3K pathway is the regulation of Nrf2 activity by GSK3ß, which in turn is deactivated by PI3K. GSK3ß phosphorylates Fyn, which then translocates to the nucleus, where it binds to Nrf2. Bound Nrf2 is transported out of the nucleus, binds to Keap1 and is degraded (58). GSK3ß has also been shown to directly phosphorylate Nrf2, which excludes Nrf2 from the nucleus (59) (Figure 1).

In RPE cells, the mitogen activated protein kinase (MAPK) ERK1/2 and p38 were shown to be important for the activation of Nrf2, but not for basal activity and our own data indicates a species specific involvement of ERK1/2 in Nrf2 activation (Koinzer, unpublished data) (54). Activation of MAPK does not facilitate the release of Nrf2 from Keap, but is required for the translocation to the nucleus (60). While Nrf2 has MAPK phosphorylation sites, direct phosphorylation is not considered to be an important factor for Nrf2 activation. Moderate effects on activation and translocation could be seen after direct phosphorylation, but the effect of MAPK is considered to be indirectly mediated by additional proteins (61).

3.2.1.3. VEGF

VEGF is the major angiogenic factor in embryonic development, a major factor in the pathology of exudative AMD and involved in a variety of other retinal disease, such as diabetic retinopathy or retinopathy of prematurity (62). Physiologically, it has important functions for the maintenance of the choroid and the retina (63,64). It is secreted by the RPE and has recently been described to be an autocrine survival factor for RPE cells following oxidative stress stimulation (65). In order to exert its function, VEGF has to bind to its receptors, VEGF receptor 1 (VEGFR-1) or VEGF receptor 2 (VEGFR-2), of which VEGFR-2 is considered to be the main receptor mediating functional signaling. VEGFR-2 is a tyrosine receptor kinase which phosphorylates itself upon activation and activates, among others, the PI3K/Akt (described above) and MAPK (described below) pathway. Also involved in VEGF binding are the co-receptors neuropilin 1 and 2 (62).

VEGF is upregulated after oxidative stress in the RPE, secretion being more elevated at the apical side than the basolateral side, indicating a protective role for the neuroretina under oxidative stress (65-67). A specific upregulation of $VEGF_{189}$ has been reported, which is generally not expressed in the adult RPE (68,69). The underlying mechanisms of the upregulation in the RPE are not known in detail but have been shown to be dependent on ERK1/2 activation, while both pathological and physiological VEGF expression are partly regulated by p38 (67). Oxidative stress also induces the upregulation of VEGF-R1 and VEGF-R2 and the phosphorylation of

VEGF-R2 (70). VEGF autocrine signaling protects RPE cells via VEGFR-2 and the PI3K/Akt pathway (described above), as inhibition of VEGF-R2 phosphorylation abrogates Akt phosphorylation and elevates cell death (65) (Figure 1). VEGF has been shown to induce its own expression in the RPE as well as in endothelial cells (71,72), so induction of (protective) VEGF by oxidative stress might result in a positive feedback loop, possibly linking oxidative stress-induced protective signaling to AMD progression.

3.2.1.4. Neuroprotectin D1

NPD1 is a dihydroxy-containing derivate of docosahexaenoic acid, an omega 3 fatty acid highly concentrated in photoreceptors, brain and retinal synapses (73). It generally exerts antiapoptotic and anti-inflammatory effects. RPE cells synthesize NPD1 in culture. NPD1 synthesis is enhanced by oxidative stress stimulation, which protects against the both single-dose and repetitive oxidative stress (74,75). The production of NPD1 is dependent on 15-lipoxygenase-1 (75). NPD1 induces an elevated phosphorylation of Akt, mTOR and p70S6K during oxidative stress, activating both the PI3K/Akt and mTOR/p70S6K protective pathways (see above) (47). Additionally, NPD1 prevents RPE apoptosis by upregulating Bcl-2 and attenuating the expression of proapoptotic Bax and Bad (Figure 1). It enhances the heterodimerization of Bax with Bcl-xL, thereby inhibiting the formation of Bax homodimers, which in turn prevents the release of cytochrome c from the mitochondria (discussed in detail below). Additionally, NPD1 diminishes caspase 3 activation (73,74,76). NPD1 enhances the dephosphorylization of Bcl-xL by the protein phosphatase 2A, thereby downregulating oxidative stress induced phosphorylation of Bcl-xL and rescuing its antiapoptotic function (76).

3.2.2. Cell death pathways

Retinal pigment epithelial cells are to a high degree resistant to oxidative stress (19). High concentrations of or prolonged stimulation with oxidative stress, however, induce cell death. In the following chapters, degenerative signaling in the RPE after oxidative stress is discussed. A main executioner of cell death in the RPE is the mitochondrial pathway. Both pro- and antiapoptotic players of this pathway, including the heat shock proteins (Hsp), are presented. Additionally, p53, the oxidative stress sensor 66kDa proto-oncogene Src homologous-collagen homologue protein (p66Shc) and bone morphogenic protein 4 (BMP4), which all can also induce a senescent phenotype, as well as lipid oxidation product 4-HNE, are discussed. An integrated picture of the apoptotic pathways of the RPE is given in figure 2.

3.2.2.1. Mitochondrial pathway

In addition to their role as the main source of energy of the cell, mitochondria are important mediators of cells death. The main event in mitochondrial signaling and control of apoptosis is the permeabilization of the mitochondrial outer membrane and the release of proapoptotic proteins into the cytosol, such as cytochrome c, AIF or HtrA2/Omi, which can mediate caspase-dependent and caspase-independent cell death pathways. Mitochondrial membrane permeabilization is a complex process, which can be induced by mitochondrial permeability transition (MPT) pore-dependent and Bcl-2 family-dependent mechanisms (77).

3.2.2.1.2. Mitochondrial Permeability Transition

Mitochondrial permeability transition is caused by opening of non-specific pores in the mitochondrial inner membrane, allowing ions and small molecules to pass freely across the inner membrane. This leads to a loss of mitochondrial membrane potential and to an uncoupling of oxidative phosphorylation, increased generation of ROS and depletion of ATP. It also leads to osmotic swelling and eventually to the rupture of the outer membrane, releasing molecules of the intermembrane proteins into the cytosol, such as cytochrome c, apoptosis-inducing factor (AIF), or high temperature requirement protein 2 (HtrA2/Omi), which will be discussed in detail below (78,79). In t-BH-treated RPE cells, an early decrease of mitochondrial membrane potential is observed, followed by caspase activation and DNA fragmentation (80).

3.2.2.1.3. Bcl-2 family

The Bcl-2 family includes a variety of pro- and antiapoptotic proteins, which all share Bcl-2 homology (BH) domains. They are divided in three groups: antiapoptotic molecules that contain BH domains 1-4 such as Bcl-2, Bcl-xL and Mcl-1; proapoptotic molecules that contain BH domains 1-3, such as Bax and Bak, and a third class of proapoptotic molecules that only contain BH domain 3, such as Bad, Bik, Bim, Noxa and Puma, of which some are under transcription regulation of p53 (discussed below) or FoxO3 (81-83). Bcl-xL is a major antiapoptotic Bcl-2 protein which protects against apoptotic and necrotic cell death (84). It generally promotes cell survival by regulating electric and osmotic homeostasis of mitochondria and forms small calcium-dependent, cation selective ion channels that protect mitochondria from osmotic damage (85,86). Additionally, Bcl-xL prevents the release of cytochrome c by binding and inactivating proapoptotic family members, such as Bax or BH3-only molecules (87,88). Phosphorylation of Bcl-xL at Ser62 changes it to a proapoptotic protein (76) (Figure 2). In the RPE, Bcl-xL is highly expressed and required for RPE cell survival, especially under oxidative stress (89,90). Bcl-xL phosphorylation is enhanced in RPE cells after H_2O_2 induced oxidative stress which is intensified by PP2A inhibitors. NPD1 downregulates oxidative-stress induced phosphorylation by increasing PP2A activity and enhances the heterodimerization of Bcl-xL with Bax in the RPE (76). Bcl-2 sequesters BH3-only proteins and neutralizes activated Bax/Bak in the mitochondrial membranes (88). Additionally, Bcl-2 can regulate the Ca^{2+} homeostasis in the endoplasmatic reticulum. Bcl-2 expression is reduced in RPE cells treated with H_2O_2 (91).

Bax is an important factor in mitochondrial outer membrane permeabilization. Bax resides in the cytosol of healthy cells (92,83). Apoptotic insults result in Bax activation and its translocation to the mitochondria where it assembles into high-molecular weight complexes (93,94).

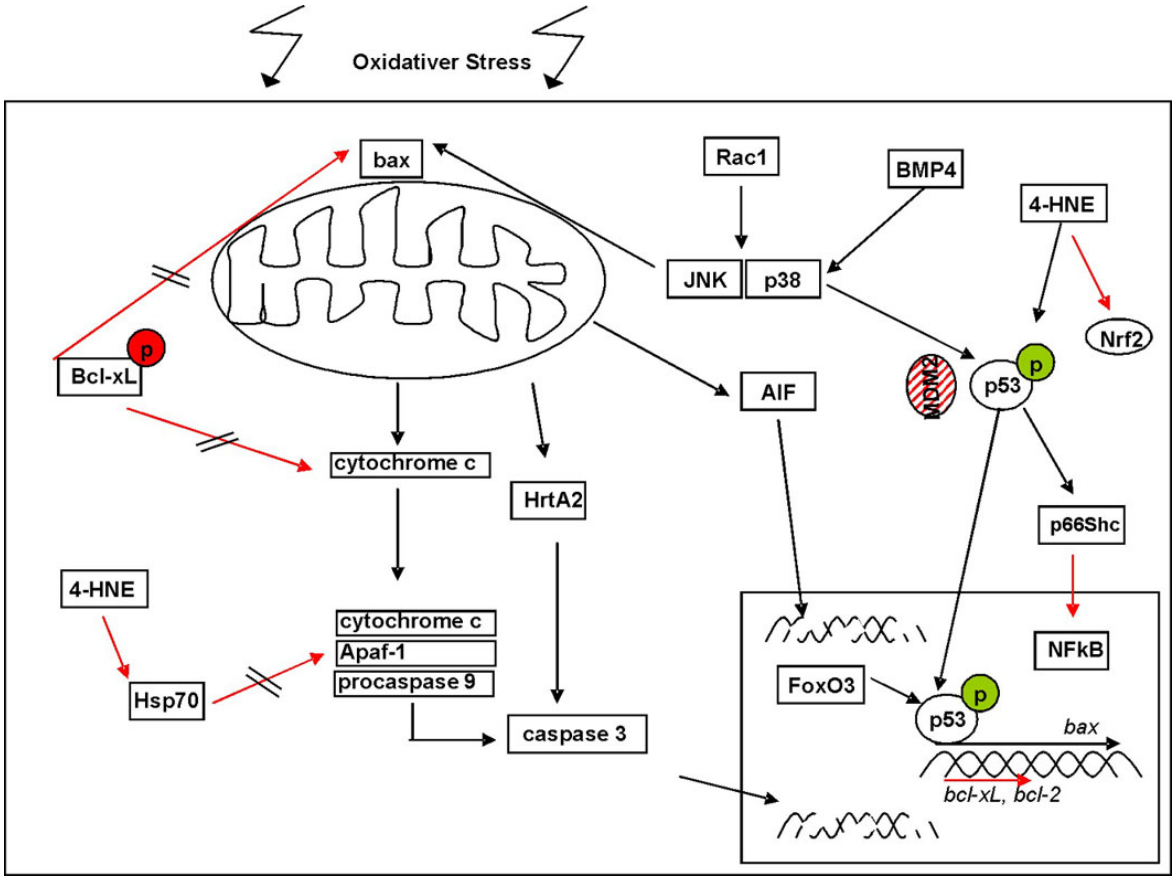

Figure 2. An integrated summary of cell death inducing pathways is given. Only interactions that have explicitly been shown for the RPE are considered. The cellular death pathway following oxidative stress stimulus follows the classical pattern of the mitochondrial death pathway. Oxidative stress induces a release of proapoptotic factors such as HtrA2/Omi, AIF and cytochrome c. Cytochrome c release leads via the apoptosome to a caspase dependent DNA degradation and cell death, while AIF is involved in caspase independent DNA degradation and cell death. Bax is regulated by MAPK, which are activated via Rac1 and BMP4. Bcl-xL is inactivated via phosphorylation and cannot exert its antiapoptotic function. The transcription factor p53 is activated via MAPK pathways and by 4-HNE. 4-HNE alters Hsp70, which in turn can no longer inhibit the formation of the apoptosome.

Bax heterodimerizes with Bcl-2 (95), interacts with BH3-only proteins, is inserted in the outer membrane and undergoes a conformational change. The exact mechanism as to how Bax permeabilizes the outer membrane is under debate (96,97).

In RPE cells, H_2O_2 treated cells display a enhanced transcription of Bax which is accompanied by an increase of caspase 9 and 3 (discussed below) and oxidative stress induces Bax translocation to the mitochondria, which is dependent on the activation of Rac1, c-Jun N-terminal Kinase (JNK) and p38 (discussed below) and induces the release of AIF from the mitochondria (discussed below) (98,99). Also, UV light induces Bcl-2 and Bax expression in RPE cells (100) (Figure 2).

3.2.2.1.4. Released factors and downstream pathways

For the induction of caspase mediated cell death, cytochrome c is a most important factor. When in the cytosol, cytochrome c binds to the apoptosis activating factor (Apaf-1) which recruits and thereby activates procaspase-9 in a complex, called apoptosome, which is tightly regulated by heat shock proteins (hsp70, hsp90, discussed below) (101,102). Activated caspase 9 cleaves and thereby activates the "executioner" caspase 3 (101,103). Release of cytochrome c, activation of caspase 9 and activation of caspase 3 after oxidative stress in the RPE have been shown in studies using t-BH and hydrogen peroxide (80,91).

Caspase mediated cell death is supported by the released protein HtrA2/Omi, a serine protease, which promotes caspase activation by neutralizing the endogenous inhibitor of caspases (IAP) (104). HtrA2/Omi is localized at the mitochondria intermembrane space where it remains until it translocates to the cytosol after apoptotic stimuli (105). Additional to its caspase promoting activity, it can mediate caspase-independent death through its own protease activity, e.g. by cleaving and degrading X-linked inhibitor of apoptosis (XIAP) (106).

H_2O_2-induced oxidative damage in the RPE

results in an HtrA2/Omi translocation from the mitochondria to the cytosol, leading to RPE cell apoptosis via a caspase-mediated pathway (107).

Additional to caspase-executed cell death, mitochondria can mediate caspase-independent cell death as well, which nevertheless may display apoptotic features (78). Apoptosis inducing factor (AIF), in healthy cells important for the maintenance of the mitochondrial respiratory chain, can mediate caspase-independent cell death when translocated to the nucleus where it induces DNA fragmentation (108). The release of AIF from the mitochondria has been shown after oxidative insults in the RPE (99).

3.2.2.2. p53

A factor closely connected to mitochondria mediated cell death is p53. The p53 pathway is composed of a network of genes responding to intrinsic and extrinsic stress signal which lead to cell cycle arrest, cell senescence or cellular apoptosis. In response to such a stress signal, p53 is activated by post-translational modification and can induce cell death via transcriptional and transcription-independent functions, while these two modes of apoptosis induction cooperate and complement each other (109). A negative regulator of p53 is mouse double minute 2 (MDM2), an ubiquitin ligase that blocks p53's transcriptional activity and mediates its degradation (110). As a transcription factor, p53 induces several proapoptotic proteins such as Bax, Puma, Noxa and Bid and represses the transcription of Bcl-2 and Bcl-xL. It also effects the expression of numerous other factors (110). For the transcription-independent pathways, p53 is accumulated in the cytosol and the mitochondria, where it interacts with members of the Bcl-2 family. p53 activates Bak, Bax and Bad while inhibiting the antiapoptotic effect of Bcl-2 and Bcl-xL (109). The forkhead transcription factor FoxO3 promotes cytosolic accumulation of p53 and apoptosis (111) (Figure 2). A downstream factor of p53 is the adaptor protein p66Shc (112) (discussed below). In the RPE, hydrogen peroxide induces an elevation of p53, and 4-HNE increases the level and phosphorylation of p53, induces its accumulation in the nucleus and causes the degradation of MDM2 (91,113). Hypoxia as well as blue light exposure of A2E-laden RPE also induce an elevation of p53 (114,115).

3.2.2.3. p66Shc

p66Shc is an adaptor protein (116), which interacts with other proteins mediating cell signaling, but lacking genuine enzyme activity. p66Shc mediates tyrosine kinase signaling, where it serves as a negative regulator, inhibiting Ras/MAPK pathways (117). It was recently identified as an intracellular oxidative stress sensor which plays a prominent role in oxidative stress induced apoptosis and in the life span of animals (118). Its apoptotic properties are dependent on the JNK/ERK dependent phosphorylation of Ser36 which is induced by oxidative stress (119) (Figure 2). p66Shc increases intracellular ROS, probably by inner membrane oxidation of cytochrome c (120). Additionally, it acts as a downstream effector of p53, which in turn increases the stability of p66Shc (112).

A knock down of p66Shc in the RPE reduces intracellular ROS and increases the expression of several antioxidant enzymes. RPE cells lacking p66Shc display an increased NFkB activity and are less susceptive to oxidative stress induced apoptosis (121). These results indicate an important function of p66Shc in oxidative stress induced cell death in the RPE.

3.2.2.4. Bone morphogenetic protein 4

BMP4 is an important regulator of differentiation, senescence and apoptosis in many different cells and tissues, including the eye (122). BMP4 is highly expressed in RPE and Bruch's membrane of atrophic AMD and mediates oxidative stress induced senescence *in vitro* via Smad and p38 pathways. In contrast, in neovascular AMD lesions, BMP4 expression in RPE is low. It might be involved in the molecular switch determining which phenotype pathway is taken in the progression of AMD (123). Chronic exposure to sublethal doses of oxidative stress can increase the BMP4 expression in the RPE and induce a senescence phenotype (124). BMP4 mediates RPE senescence via the activation of Smad and p38 pathways to activate p53 and increase the expression of $p21^{WAF1/cip1}$, and to decrease phospho-Rp. The BMP4 mediated RPE senescence can be inhibited by a BMP4 antagonist and a phospho-p38 inhibitor. The interaction of p53 with the BMP-Smad pathway is not elucidated yet, but the possibility of post-translational modifications of p53 by Smad1/5 which activate p53 dependent transcription has been discussed (123).

3.2.2.5. Heat shock proteins

Heat shock proteins are molecular chaperones which assist in the folding of polypeptides and assist misfolded proteins to regain their native state. Furthermore, they play a critical role in modulating apoptotic pathways (102) and play important roles in saving damaged cells, which might also be of clinical and pharmacological importance (125).

Heat shock proteins are classified into distinct families, of which Hsp90, Hsp70 and small heat shock proteins will be discussed. Hsp90 can prevent the formation of the apoptosome complex by inhibiting the oligomerization of Apaf-1 (126). It maintains the activity of Akt by inhibiting its dephosphorylation (127). The Akt/Hsp90 complex also inactivates JNK-mediated cell death pathway (discussed below) by inactivating ASK-1, one of the activators of JNK (128). Additionally, Hsp90 is involved in the activation of NFkB, as Hsp90 can cause the dissociation of NFkB from its inhibitor IkappaB (129). Hsp90 is upregulated in the RPE of AMD patients and was shown to be involved in the protection against 4-HNE induced cell death in the RPE, as its inhibition with Geldanamycin increases 4-HNE induced cell death (6,130).

Hsp70 inhibits the formation of a functional apoptosome by direct interaction with Apaf-1, protects against forced expression of caspase 3 and prevents the translocation of Bax from the cytoplasm to the mitochondria (131-133). Also, it binds to AIF, restricting its translocation to the mitochondria and prevents the

activation of JNK (134,135). In the RPE, increased resistance to chronic oxidative stress is correlated to a higher expression of Hsp70 and 4-HNE modifies Hsp70, impairing its function (54,136) (Figure 2).

Hsp27 belongs to the family of small heat shock proteins. Hsp27 can maintain mitochondrial stability and redox homeostasis in cells and interacts with the apoptotic signaling pathways at many steps (137). It prevents the release of cytochrome c from the mitochondria, prevents the assembly of the apoptosome by sequestering cytochrome c away from Apaf-1 and mediates the inhibition of procaspase-3 (138-140). Hsp27 is also involved in the stabilization of Akt (141). High levels of Hsp27 are expressed in the RPE of the rat retina and in Arpe19 cells. Oxidative injury activates the translational and transcriptional activation of Hsp27 (142). Its activity is regulated by phosphorylation, which seems to be important for its antiapoptotic properties (143). Phosphorylated Hsp27 is upregulated in the RPE of AMD patients (136,144). Non-lethal oxidative injury induced with HQ leads to an mRNA upregulation, dimer formation and Hsp27 phosphorylation in the RPE *in vitro* and *in vivo*, which is mediated by p38 and ERK1/2 (144). AlphaB-cystallin is closely related to Hsp27 and interferes with the processing of the precursor of caspase-3 (145,146). Additionally, it can inhibit apoptosis through sequestration of Bax and Bcl-xS in the cytoplasm (147). AlphaB-crystallin is expressed in the RPE and can protect against hydrogen peroxide induced caspase 3 activation (148).

Assessing the role of Hsp in RPE protection, one has to consider that the Hsp-inhibitor quercitin protects RPE cells from oxidative damage (149). However, this effect is attributable to the antioxidative rather than to the Hsp-inhibiting properties of quercitin.

3.2.2.6. 4-HNE

Under conditions of oxidative stress, light exposure, hyperglycemia, smoking or vitamin E deficiency, polyunsaturated fatty acids (PUFA) react with free radicals to form lipid peroxidation products (53,150-152). 4-hydroxynonenal is produced from oxidation of n-6 PUFA and is one of the major reactive aldehydes. It is considered an important second messenger for cell cycle arrest, differentiation and apoptosis and reacts with nucleophilic sites in proteins and nucleic acids (153-155). In astrocytes, 4-HNE activates Nrf2, NFkB and cFos (156). In RPE cells, however, an activation of NFkB or AP-1 is not seen (130). Additionally, it modifies proteins, especially Hsp70, which is also shown for RPE cells, or the Nrf2 inhibiting protein Keap1 (54,154,157). It is mainly detoxified by gluthatione-S-transferases (158). In the RPE, 4-HNE has been shown to activate Nrf2 and to increase GSH synthesis (54). Inhibition of PI3K enhances oxidative protein modification by 4-HNE (54). It directly induces p53-mediated apoptosis in the RPE (113) (Figure 2). 4-HNE induced cell death, however, differs from hydrogen peroxide induced cell death. In hydrogen peroxide induced cell death, we see an activation of NFkB and AP-1, which is not seen in 4-HNE induced cell death (18,130).

3.2.3. MAPK

A common mechanism of signal transduction after a plethora of signals is the sequential activation of protein kinase within the mitogen-activated protein kinase pathways. MAPK are a family of serine/threonine kinases which are strongly involved in oxidative stress mediated signaling and of which JNK, p38 and ERK1/2 are the best characterized. Each of these kinases mediates a vast variety of different reaction, both adaptive and apoptotic, and these kinases are important factors in integrating various external signals in order to coordinate the appropriate response of the cell. Generally, all MAPK are activated by MAPK kinases (MAP2K), which in turn are activated by a family of MAP3K (159). The cascade is usually started either by a small GTP-binding or an adaptor protein.

3.2.3.1. p38

The activation of p38 influences cellular processes such as inflammation, cell growth, survival and apoptosis by interacting with signaling pathways and regulating gene expression. The MAPK p38 is activated by MKK3 and MKK6, which in turn are phosphorylated by MLK3. MLK3 is activated by small G-proteins Rac1 and cdc42 (160).

In the RPE, data on p38 is not consistent. It is activated after H_2O_2 and t-BH, while in other studies, no activation of p38 after t-BH was found (99, 161-164). Also, the effect of p38 on RPE cell survival is controversial. In some studies, p38 displayed proapoptotic properties, other studies found protective effects of p38 activation, with p38 (and JNK) involved in GSH upregulation (99,161-163). Our own laboratory found a proapoptotic effect of p38 after H_2O_2 stimulation but not after t-BH stimulation (Koinzer, unpublished), so the effect of p38 might strongly depend on the reactive oxygen species, duration and concentration of stimulation, species and density of cell culture used (162). A simple dependency on the oxidative stimulus cannot be seen. MAPK p38 induces the transcription and activation of different members of AP-1 transcription factors (165,166). Depending on concentration and duration of the oxidative stimulus, different factors are activated and may mediate different cellular responses (discussed in more detail below) (167). Additionally, p38 is involved in the upregulation of IL-8 and IL-6 after oxidative stress and of matrix metalloproteinase (MMP)-3 (168,169) (discussed below).

3.2.3.2. JNK

The c-Jun N-terminal Kinase (JNK) is also referred to as the stress activated protein kinase (SAPK). JNK is activated by dual specificity kinases MKK4 and MKK7 which phosphorylate JNK on critical threonine and tyrosine residues in order to activate it (170). MKKs can be activated by a number of pathways, e.g. MEKK1-4, MLK and ASK1, of which MEKK4 is specific for JNK and of which MEKK1 and ASK1 can be directly activated by oxidative stress (159,171-174). Upstream, cdc42 or Ras/Rac can induce the activation of the JNK pathway (175). JNK has a wide variety of targets. One important function of JNK is the activation of AP-1 transcription factors (176) (see below). The activation pathway of JNK

in the RPE has hardly been investigated so far, but an involvement of the Rac1 in oxidative stress JNK activation has been shown (99). Additionally to oxidative stress, the JNK pathway can be activated by a wide variety of stressors, e.g. chemotherapeutic drugs, UV light, irritation or heavy metals (159). While activation of JNK is generally proapoptotic, the JNK pathway exhibits antiapoptotic features in some cell types and can phosphorylate and activate Nrf2 (177,178). The duration of JNK activation might be a factor in determining the induction of pro- or antiapoptotic pathways (179), but also the cellular localization or isoform may play a part in that decision (180-182). In the RPE, the involvement of the JNK pathway in oxidative stress response is not fully elucidated and studies display conflicting results. While JNK is activated after stimulation with hydrogen peroxide, the role of JNK activation has been described as proapoptotic or, at least in combination with 5-deoxy-Delta (12,14)-prostaglandin J82, as protective, where it is involved in GSH upregulation (99,161,162). After stimulation with t-BH, on the other hand, our lab did not find any proapoptotic properties of JNK (Koinzer, unpublished). In blue light induced cell death, JNK exhibited a protective effect, while UV-light and 4-HNE induced prolonged JNK activation was proapoptotic (113,115,183). Further research is needed to better elucidate the involvement of different JNK isoforms and their cellular localization in oxidative stress signaling in the RPE.

3.2.3.3. ERK1/2

ERK1/2 (p42/p44) is generally considered to be involved in proliferation and differentiation, but may also participate in apoptotic responses. Most important regulators of ERK1/2 are the MAP2K MEK1 and MEK2. ERK1/2 is a ubiquitous serine/threoine kinase that phosphorylates a high number and wide variety of substrates, most of them regulatory proteins, either in the cytosol or after translocation into the nucleus. ERK1/2 is also involved in the regulation of AP-1 transcription factors (184). The specificity of ERK1/2 activation is regulated by its pathway of activation, by subcellular localization of the kinase, and also by strength and duration of the inducing signal (185). ERK1/2 is often antiapoptotic, e.g. by phosphorylation of Bad or by translocating Nrf2 to the nucleus (60,186). In the RPE, ERK1/2 is readily activated after stimulation with hydrogen peroxide and weakly after t-BH (161,162,164,187,188). Our own data suggests an ERK1/2 involvement in cell death pathways after t-BH stimulation, which displays a species dependent pattern (Koinzer, unpublished data). ERK1/2 is also phosphorylated in response to UV light, where it is probably has proapoptotic functions (189). After hydrogen peroxide stimulation, ERK1/2 does not seem to be involved in cell death but to be involved in oxidative stress induced proliferation and can be protective if induced by PEDF (161,162,187,190). Additionally, ERK1/2 seems to be involved in the activation of Nrf2 in RPE after stimulation with 4-HNE (54). ERK1/2 is involved in oxidative stress induced upregulation of VEGF after t-BH stimulation and in MMP-1 and MMP-3 secretion (67,169).

3.2.3.4. AP-1 transcription factors

AP-1 transcription factors are a family of the basic region leucine zipper proteins Jun, Fos, Maf, Far and ATF gene subfamily, which form homo- and heterodimers in order to bind to the DNA to induce transcription. Their expression and activation are induced by many factors, including oxidative stress and can occur via a variety of pathways, especially MAPK pathways (165,184,191). These dimers bind to regulatory elements present in promoter and enhancer regions of many genes, regulating a wide range of cellular processes, including proliferation and apoptosis, depending on the cellular context (176). In the RPE, a general activation of AP-1 in response to H_2O_2 but not after 4-HNE stimulation has been shown (18,130). In response to hydrogen peroxide, FosB and c-Fos, and to a lesser extent Fra-1 and ATF3, are upregulated in a dose-dependent manner, while JunB and c-Jun are induced in a threshold response. The investigated transcription factors are induced in a time-dependent manner, specific for each individual protein (167). A model of transcriptional regulation by AP-1 transcription factors is the dimer ratio control. AP-1 proteins homo- and heterodimerize with each other. Relative levels of different AP-1 dimers in the nuclear and cytosolic pool differentially modulate AP-1 gene expression at each transcription factor locus through autoregulatory mechanisms, resulting in differing cellular responses according to the presence of different dimers, allowing a highly flexible response of the cell (167). As AP-1 proteins are differentially regulated by the concentration of the stressor and differentially regulated in a time dependent manner, this model might well explain the inconsistent effects on and of MAPK activation obtained in studies using different approaches to induce oxidative stress.

3.3. Inflammatory pathways

3.3.1. Complement system

The complement system is component of the innate immune system and a number of studies have associated the complement system with the development of AMD (192). It is divided into three different pathways, classical, alternative and lectin, of which the alternative pathway is most strongly associated with AMD. Complement factor H is a negative regulator of the alternative pathway, inhibiting several steps of this pathway and promoting degradation of activated components. The polymorphism Y402H, which displays a reduced binding to C-reactive proteins and heparin and thus is less inhibitory, has been shown to display a higher risk for developing AMD (193). In the eye, the RPE is a local source of CFH (194). Oxidative stress reduces the ability of interferon-gamma to increase CFH expression in the RPE (41). Interferon-gamma- induced increase in CFH is mediated by transcriptional activation of Stat1 and its suppression by oxidative stress is mediated by acetylation of a member of the forkhead family, FoxO3. Acetylation of FoxO3 enhances its binding to the CFH promoter and inhibits the interaction of Stat1 with the CFH promoter (41). Additionally, the phagocytosis of oxidized photoreceptor outer fragments reduces CFH mRNA level (195).

Oxidative stress also alters the expression of regulators of complement activation, which protect cells from the complement system. In the RPE, oxidative stress

decreases the expression of DAF and CD59. This reaction is specific for the RPE and is not seen after hypoxia (196).

3.3.2. Interleukin secretion

Interleukin-6, a proinflammatory cytokine which is involved in autoimmune responses and associated with dry AMD, is expressed at low level in cultured RPE and is induced by a variety of insults, such as endotoxin or cytokines (197-199). Sublethal doses of hydrogen peroxide increase IL-6 expression in the RPE in a dose dependent manner. The upregulation of IL-6 is mediated by a nuclear translocation of NFkB, which is dependent on p38 phosphorylation (200).

Interleukin-8 (CXCL8) is a proinflammatory chemokine that has strong leukocyte chemotactic and proangiogenic properties (201). It is induced in the RPE by a variety of oxidative insults, such as phagocytosis of oxidized photoreceptor outer segments (POS), oxidized cholesterols, A2E, hydrogen peroxide and paraquart, but not by t-BH (168,202,203). The underlying pathway seems to be dependent on proteasome inhibition. A short term protease inhibition results in an NFkB dependent downregulation of IL-8 secretion, while a long-term inhibition upregulates IL-8 via a MKK3/6 activated p38 signaling (168). The phagocytosis of oxidized POS also induces the upregulation of MCP-1, a monocyte chemoattractant, which is dependent on NFkB (202).

3.4. Extra cellular matrix

The turnover of the extracellular matrix, especially Bruch's membrane, is regulated by a balanced system of matrix metalloproteinases and their inhibitors (tissue inhibitors of metallo proteinases, TIMP). The RPE secretes several MMPs and TIMP (204). MMP-2 is the major RPE enzyme for the degradation of the Bruch's membrane components collagen I, collagen IV and laminine. Non-lethal oxidative stress increases pro-MMP-2 protein in the RPE, but downregulates MMP-2 activity (205). To be activated, pro-MMP-2 forms a complex with MMP-14 and TIMP-2. Oxidative injury decreases the expression of MMP-14 and TIMP-2 in the RPE, indicating that oxidative stress disrupts enzymatic cleavage of pro-MMP-2 (206,207). MMP-9, on the other hand, is increased after oxidative stress (208). Also, oxidative stress increases MMP-1 and MMP-3 release in a MAPK dependent manner, with MMP-1 dependent on ERK1/2 and MMP-3 dependent on ERK1/2 and p38 activity. The activation of MMPs results in a degradation of collagen 1 (166). Prolonged, but not transient, non-lethal oxidative stress also induces an increase of collagen IV (205).

4. SUMMARY AND PERSPECTIVE

RPE cells answer to oxidative stress in a complex and interacting manner. This is even further complicated by the fact that different stressors, all designated as models for oxidative stress, induce different responses (209). As for models of oxidative stress, in addition to "pure" chemical oxidative stress, such as hydrogen peroxide or t-BH, 4-HNE, UV-light and even hypoxia have been used, which all induce different pathways. And even in chemically induced oxidative stress, the reaction of the RPE to hydrogen peroxide might differ from the reactions to t-BH.

The RPE cell is rather resistant to oxidative stress and the published data indicates the Akt pathway to be a main factor in the pathway of protection. Akt is activated by oxidative stress via the activation of PI3K. Additionally, Akt is activated by VEGF and NPD1, factors that are both induced by oxidative stress and that both mediate RPE protection. Besides the initiation of protein synthesis and inhibition of proapoptotic proteins, Akt is strongly involved in Nrf2 protection, which in turn is vital for RPE protection against oxidative stress (Figure 1).

The cellular death pathway following oxidative stress stimulus seems to follow the classical pattern of the mitochondrial death pathway. Oxidative stress induces a change in mitochondrial membrane potential and a subsequent release of proapoptotic factors such as HtrA2/Omi, AIF and cytochrome c. Cytochrome c release leads to a caspase dependent DNA degradation and cell death, while AIF is involved in caspase independent cell death. Bcl-2 family members are strongly involved in the regulation of cell death, in particular Bcl-xL and Bax. Also, heat shock proteins have been shown to be involved in RPE cell death regulation (Figure 2).

The involvement of MAPK in oxidative stress signal transduction pathways is controversial and seems to be dependent on the stimulus used to inflict oxidative stress. The MAPK kinases p38 and JNK have been shown to be proapoptotic, protective or not involved at all. ERK1/2 seems to be generally activated, while the cell's reaction seems to be dependent on the activation pattern. Besides cell death, ERK1/2 is involved in oxidative-stress-induced VEGF upregulation. The conflicting results of the MAPK may be explained by their substrates of the AP-1 transcription factor family that form different heterodimers depending on length and severity of the oxidative insult. These levels of various AP-1 dimers may differentially modulate the cellular response.

Inflammatory responses can be induced by oxidative stress, as has been shown for the FoxO3 dependent downregulation of CFH and the p38 dependent secretion of IL-8 and IL-6, but more data is needed to elucidate the pathways that modulate the inflammatory response in the RPE. The investigation of oxidative stress induced modulation of the extracellular matrix and its underlying pathways is just at the beginning.

The present data indicates a strong involvement of oxidative stress induced pathways in AMD pathology. Further research is needed to elucidate the response of RPE cells to oxidative stress.

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Abbreviations: 4E-BP1: eukaryotic initiation factor 4E binding protein1, 4-HNE: 4-hydroxynonenal, AIF: apoptosis-inducing factor, AMD: age-related macular degeneration, AP-1: activator protein 1, Apaf-1: apoptosis activating factor, ARED: age-related eye disease, BH: Bcl-2 homology, BMP4: bone morphogenetic protein 4, CFH: complement factor H, ERK1/2: extracellular signal-regulated kinase 1/2, FoxO1: forkhead in rhadomysarcoma (FKHR), FoxO4: acute lymphocyte leukemia-1 fused gene from chromosome X (AFX), GSK3ß: glycogen synthase kinase 3ß, HtrA2/Omi: high temperature requirement protein 2, IAP: inhibitor of caspases, JNK: c-Jun N-terminal Kinase, Keap1: Kelch-like ECH-associated protein 1, MAPK: mitogen activated protein kinases, MDM2: mouse double minute 2, MMP: matrix metalloproteinase, MPT: mitochondrial permeability transition, mTOR: mammalian target of rapamycin, NFkB: nuclear factor '*kappa*-light-chain-enhancer' of activated *B*-cells, NO: nitric oxide, NPD1: Neuroprotectin D1, Nrf2: nuclear factor erythroid-2 related factor 2, p66Shc: 66 kDa proto-oncogene Src homologous-collagen homologue protein, p70S6K: p70 S6 kinase, PI3K: phosphatidylinositide 3-kinase, POS: photoreceptor outer segments, PP2A: protein phosphatese 2A, $PtInP_3$: phosphatidylinositol-3,4,5-trisphosphate, PUFA: polyunsaturated fatty acids, H_2O_2: hydrogen peroxide, HSF-1: heat shock factor protein 1, Hsp: heat shock protein, ROS: reactive oxygen species, RPE: retinal pigment epithelium, SOD: superoxide dismutase, t-BH: tert-butylhydroperoxide, VEGF: vascular endothelial growth factor, VEGF-R: vascular endothelial growth factor receptor, XIAP: X-linked inhibitor of apotosis

Key Words: AMD, Akt, apoptosis, MAPK, Nrf2, ERK1/2, oxidative stress, RPE, VEGF, Review

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Black arrows indicate activation, red arrows indicate inhibition. Arrows that are crossed out by two black parallel lines indicate that the pathway is inhibited by an upstream effector. A “p” in a green circle indicates activating phosphorylation, a “p” in a red circle indicates inhibitory phosphorylation.

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