Published in final edited form as:

Adv Exp Med Biol. 2010 ; 664: 447–456. doi:10.1007/978-1-4419-1399-9_51.

Oxidative Stress and the Ubiquitin Proteolytic System in Age-Related Macular Degeneration

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Abstract

AMD is a leading cause of irreversible vision loss in people over 60 years of age. Although the pathogenesis of this disease is multifactorial, clinical studies have revealed that oxidative damage is a significant etiological factor. The ubiquitin proteolytic system (UPS) plays a major cytoprotective role in the retina. It accomplishes this largely by degrading oxidatively-damaged proteins to prevent their toxic accumulation. In this review, we discuss numerous features of the UPS in the retina and propose various ways that components of the UPS can be harnessed for therapeutic intervention in AMD. We discuss published work describing the distribution of various UPS enzymes in different retinal cell types and present new findings describing the localization of the class III ubiquitin conjugating enzymes. These enzymes are functional homologues of a pair of yeast enzymes that mediate the degradation of misfolded and oxidatively-damaged proteins. We also discuss recent work showing that only newly synthesized proteins which have incurred oxidative damage are targeted for degradation by the UPS whereas the turnover of oxidatively-damaged, long-lived proteins is largely unchanged. Additionally, we review recent work describing how polyubiquitylation influences the sorting of damaged proteins into one of two novel intracellular compartments. Finally, we discuss how the UPS modulates the stability and activity of Nrf2, the major anti-oxidant transcription factor in the retina.

51.1 Oxidative Stress and Age-Related Macular Degeneration

AMD is a leading cause of irreversible vision loss in people over 60 years of age. The pathogenesis of this disease is multifactorial and appears to involve a combination of environmental, metabolic, and genetic inputs. Morphological changes and disruptions to the numerous cell types in the macular region accompany the onset and progression of AMD. Among the cells and structures that become altered are the retinal pigment epithelium (RPE), the photoreceptors, the choriocapillaris, and Bruch's membrane. The two types of AMD, wet and dry, are distinguishable. Wet (or exudative) AMD is characterized by neovascularization in the choriocapillaris and fluid leakage in the subretinal macular region whereas the hallmark of dry (or atrophic) AMD is degeneration of photoreceptors and the RPE monolayer. Both types are characterized by irregular and/or loss of pigmentation from the RPE. In addition, although dry AMD is more prevalent, it can progress to the wet form thus putting the patient at risk for more severe vision loss (all reviewed in Nowak 2006).

Oxidative stress is widely held to be a significant pathological factor in AMD progression. Clinical evidence supporting this comes from numerous studies, most notably, the Age-Related Eye Disease Study (AREDS) (2001). This study showed that high doses of anti-

oxidants and zinc slowed wet AMD progression and vision loss. Biochemical evidence implicating oxidative stress stems from the observation that the toxic, lipid-rich, granules that become deposited between the RPE monolayer and Bruch's membrane are largely composed of oxidized proteins and lipids and are a hallmark of AMD pathology. These aggregates grow in size to become drusen and trigger a cascade of pro-inflammatory processes involving the complement system, acute phase proteins, and cytokines. This in turn promotes new blood vessel formation, fluid leakage, and macular scarring, leading to visual loss (reviewed in Ehrlich et al. 2008). Compelling experimental evidence that oxidative damage is a root cause of AMD comes from recent work by Hollyfield et al. (2008). These investigators demonstrated that major pathological hallmarks of dry AMD could be reconstituted in mice immunized with carboxyethylpyrrole (CEP)-derivatized albumin. CEP is a unique oxidation product of docosahexaenoic acid, the major polyunsaturated fatty acid in the photoreceptor outer segments of most vertebrates (Benolken et al. 1973). Importantly, CEP is adducted to proteins in drusen deposits (Crabb et al. 2002) and CEP-adducted protein levels are elevated in the plasma of AMD patients (Gu et al 2003).

51.2 The Ubiquitin Proteolytic System (UPS) and Oxidative Stress in the Retina

Ubiquitin (Ub) is a highly conserved, 76 amino acid polypeptide that gets post-translationally attached to target proteins by the coordinated actions of a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2) and a Ub protein ligase (E3) (Fig. 51.1). Ub is first activated in an ATP-dependent manner by the E1. The activated Ub is then transferred to the active site cysteine of an E2 in a transesterification reaction. A third enzymatic component, an E3 protein ligase, cooperates with the E2 to transfer Ub to substrates. Substrate selection and specificity are conferred primarily through the pairing of particular E2–E3 combinations. After transfer of the first Ub to a target lysine, subsequent Ubs are attached sequentially to a lysine of the previously added Ub. When lysine 48 is utilized for polyUb chain assembly, the resulting polyUb structure signals delivery of the modified target to the 26S proteasome, a macromolecular assembly of proteases, for destruction. In contrast, polyUb chains constructed through other lysines (e.g., K63) of Ub typically result in non-proteolytic outcomes. Protein targets can also be regulated in non-proteolytic ways by monoubiquitylation. Balance in the Ub system is achieved by a set of deubiquitylating isopeptidases that cleave Ub off of substrates (reviewed in Fang and Weissman 2004).

Efforts to characterize the UPS in the retina have revealed that multiple retinal cell types have distinct subsets of UPS components. For example, four different Ub conjugating enzymes (E2₁₄ K, E2₂₀ K, E2₂₅ K, and E2₃₅ K) have been identified in bovine rod outer segments (Obin et al. 1996). PGP 9.5, a Ub carboxy-terminal hydrolase, is only present in retinal ganglion and horizontal cells (Bonfanti et al. 1992), but the Ub hydrolase, UCH-L3, is enriched in photoreceptor inner segments (Sano et al. 2006). We have observed that a subset of highly conserved Ub conjugating enzymes, the class III E2s, are differentially expressed in the mouse retina (Fig. 51.2). These three enzymes are functional homologues of a pair of yeast E2s, Ubc4 and Ubc5, that play essential roles in mediating the degradation of

misfolded and oxidatively-damaged proteins (Kaganovich et al. 2008; Matuschewski et al. 1996; Medicherla and Goldberg 2008). The mouse versions of these enzymes are called UbcM2, UbcM3, and UBE2E2, and each is identical to its human counterpart. These enzymes are distinguished from one another by unique N-terminal extensions of 40–60 residues (Matuschewski et al. 1996).

To analyze the distribution of the class III E2s in the retina, we raised rabbit polyclonal antibodies against the unique N-terminal extensions and labeled paraffin-embedded eye sections from 8-month old, SvEv129 mice. α -UbcM2 immunolabeling was most prominent in ganglion cells (Fig. 51.2, panel c) as well as the inner nuclear layer. In addition, a subpopulation of photoreceptors was labeled (Fig. 51.2, panel c, small arrowheads) as were the nuclei in the RPE layer. α -UbcM3 yielded a punctate pattern of immunolabeling at the interface between the inner and outer segments of the photoreceptors (Fig. 51.2, panel a). We speculate that this may represent labeling of Mueller cells. α -UBE2E2 faintly but specifically immunolabeled retinal ganglion cells and a subpopulation of nuclei of the inner nuclear layer (Fig. 51.2, panel b). Retinal substrates for the above mentioned UPS enzymes remain to be determined.

The retina is exquisitely susceptible to oxidative damage due to its robust oxygen consumption, exceptionally high content of polyunsaturated fatty acids, and exposure to bright light. Collectively, these factors create an environment of redox flux in which proteins, DNA, and lipids become oxidatively damaged. Removal and/or reversal of these oxidatively damaged biomolecules is required to prevent the toxicity that can result from their accumulation. Such accumulation is a hallmark of numerous neurodegenerative disorders including AMD (reviewed in Sas et al. 2007). Recent work has shed new light on the mechanisms by which cells process, sequester, and eliminate misfolded and aggregated proteins. In an elegant series of experiments using both yeast (i.e., *S. cerevisiae*) and mammalian cells, two novel, cellular ‘compartments’ for sequestering misfolded proteins were characterized (Kaganovich et al. 2008). One is a juxtanuclear quality control compartment, named JUNQ, which is enriched with chaperone proteins and proteasomes. This inclusion accumulates soluble misfolded proteins that can either be processed for Ub-dependent degradation or alternatively, refolded. In contrast, insoluble, aggregated proteins are sequestered in an inclusion termed the IPOD (insoluble protein deposit). The IPOD is localized peripherally (perivacuolar in yeast) and accumulates non-diffusing aggregates that cannot be salvaged. Numerous autophagic marker proteins co-localize with the IPOD. Importantly, the IPOD is the site of accumulation of disease-associated, amyloidogenic proteins such as prion proteins and Huntington’s protein. Intriguingly, polyubiquitylation is a critical factor in determining the solubility of a misfolded protein and thus whether the protein gets sorted to the JUNQ or the IPOD. These findings imply that the UPS could potentially be harnessed (e.g., over-expression of particular UPS enzymes) to direct oxidatively damaged and misfolded proteins to the JUNQ for destruction and thereby decrease the kinetics with which toxic aggregates accumulate in IPODs within the retinal cells of AMD patients.

The UPS plays a primary role in destroying misfolded and damaged proteins (e.g., oxidant-induced) and this function is conserved from yeast to man (reviewed in Ross and Pickart

2004). Multiple lines of evidence implicate a critical function for the UPS in countering oxidative stress in the retina. The Taylor laboratory has had a long-standing interest in the interplay between oxidative stress and the UPS in various ocular tissues. These investigators have produced a body of work supporting the notion that the UPS selectively degrades oxidatively damaged proteins in the retina and lens and that inhibition of the UPS, either by pharmacological means or with mutant Ub, leads to the deleterious accumulation of oxidized proteins (e.g., Dudek et al. 2005; Shang et al. 2001).

Despite the widely held notion that the UPS indiscriminately disposes of oxidatively damaged proteins to prevent their toxic accumulation, Medicherla and Goldberg have recently demonstrated that in *S. cerevisiae*, only newly synthesized proteins that have incurred oxidative damage are targeted for degradation by the UPS. In contrast, the turnover of oxidatively-damaged, long-lived proteins (~ 60 min post-synthesis) is largely unchanged (Medicherla and Goldberg 2008). The authors interpret these findings to indicate that nascently-synthesized proteins which undergo oxidative damage are prevented from folding properly and this unfolding, and/or denaturation, is what triggers their degradation by the UPS. In contrast, because 'older' proteins are already in their final conformation and in complexes with their binding partners, oxidative damage does not drive their unfolding. The 'older' proteins are thus more resistant to being denatured by oxidants and subsequently less susceptible to degradation by the UPS. It remains to be determined whether this scenario holds true for human cells but if so, it could provide new insights into the etiology of AMD and other neurodegenerative disorders. For example, it implies that the protein aggregates that accumulate in AMD patients are derived mainly from newly-synthesized, oxidant-damaged proteins. As a corollary, it suggests that enhancing the capacity of the UPS to degrade this class of compromised proteins may be a legitimate therapeutic strategy for treating AMD. Therefore, it should be a high priority to validate the findings of Medicherla and Goldberg in mammalian retinas and also to determine the fate of oxidatively-damaged 'older' proteins, many of which are likely being inactivated, though not denatured, in the highly oxidizing environment of the retina.

51.3 The UPS and the Cytoprotective Transcription Factor, Nrf2

Not only does the UPS mediate the degradation of misfolded and damaged proteins, but it also functions to counter the deleterious effects of oxidative stress in other ways. Perhaps most importantly for the retina is the role that the UPS plays in regulating the stability of nuclear-factor-E2-related factor 2 (**Nrf2**). Nrf2 is a critical anti-oxidant transcription factor that binds to a cis-acting regulatory element, called the antioxidant response element (ARE), embedded in the promoters of phase 2 genes. Phase 2 genes encode detoxification enzymes and other factors responsible for eliminating reactive oxygen species (ROS). Thus, in response to an oxidative stress, Nrf2 induces the expression of an anti-oxidant defense system that re-establishes redox homeostasis (all reviewed in Li and Kong 2009).

The degradation of Nrf2 by the UPS is directly coupled to cellular redox state (Nguyen et al. 2003). That is, in the absence of an oxidative insult, Nrf2 is constitutively destroyed by the UPS. Oxidative stress, however, arrests this degradation, and the stabilized Nrf2 is now able to translocate into the nucleus and induce phase 2 gene transcription (Fig. 51.3). This control

of Nrf2 stability can be readily observed in vivo by comparing the steady levels of Nrf2 in the presence and absence of either a proteasome inhibitor (e.g., MG132) or chemical inducers of oxidative stress (e.g., tert-butylhydroquinone (tBHQ)) (Fig. 51.4). Nrf2 degradation during redox homeostasis is mediated by a specific UPS E3 ligase called CUL3^{Keap1} (Kobayashi et al. 2004). CUL3^{Keap1} is comprised of three core proteins-cullin 3 (CUL3), Keap1, and ROC1 (Fig. 51.5). CUL3 is a scaffold. Through its C-terminal domain, it binds the RING-finger protein, ROC1. ROC1 recruits and coordinates an Ub-charged E2 into the complex. The N-terminal domain of CUL3 binds the substrate adaptor, Keap1. Keap1, in turn, recruits Nrf2 into the complex. Ub-modified Nrf2 is subsequently degraded by the 26S proteasome. The capacity of CUL3^{Keap1} to conjugate polyUb chains to Nrf2 is regulated by a small Ub-like modifier called Nedd8. Nedd8 is covalently attached and then removed from CUL3 by a dedicated set of enzymes and this dynamic cycling is required for Nrf2 ubiquitylation (all reviewed in Bosu and Kipreos 2008).

Strategies to potentiate the cytoprotective activity of Nrf2 have centered on stabilizing the transcription factor by repressing CUL3^{Keap1} function. CUL3^{Keap1} repression in vivo can be accomplished through multiple mechanisms all of which culminate in the dissociation of the Keap1:Nrf2 complex from CUL3. The best characterized of these mechanisms is predicated on modifying redox-sensitive cysteine residues within Keap1 (Dinkova-Kostova et al. 2002; Levonen et al. 2004). In particular, Cys151 of Keap1 has been definitively shown to function as a redox sensor that mediates the binding of Keap1 to CUL3 (Eggler et al. 2007; Zhang et al. 2004). Current models support the notion that oxidant-induced modification of Cys151 dissociates Keap1:CUL3 binding and results in Nrf2 stabilization. As predicted for a redox sensor, mutation of this cysteine to serine blocks the capacity of Keap1 to detect alterations in intracellular redox status and to promote the dissociation of Keap1:Nrf2 from CUL3 in response to oxidative stress (Zhang and Hannink 2003; Zhang et al. 2004). Thus, Nrf2 is not efficiently stabilized in cells expressing Keap1 (C151S) following an oxidative insult (Yamamoto et al. 2008) This same cysteine is also targeted by the dietary anti-oxidant, sulforaphane, an isothiocyanate found in broccoli and other cruciferous vegetables (Zhang et al. 2004). In this way, sulforaphane-modified Keap1 functionally mimics an oxidant stress and results in the stabilization and activation of Nrf2 in homeostatic cells.

The central role of Nrf2 in protecting and preserving retinal health and function has been revealed through recent studies exploring the therapeutic potential of sulforaphane. Sulforaphane administration has been demonstrated to protect against photoreceptor degeneration in rodent models of experimental light stress (e.g., Tanito et al. 2005; Kong et al. 2007). In addition, the Talalay laboratory (the discoverers of sulforaphane) (Zhang et al. 1992) has shown that the compound can protect cultured retinal pigment epithelial cells from photooxidative damage (Gao and Talalay 2004). These studies and others support the idea that sulforaphane could have potential clinical utility in preventing and/or slowing the progress of retinal degeneration in AMD patients. Furthermore, they provide a rationale for further pursuing strategies to enhance Nrf2 stabilization by manipulating the UPS. Unlike dietary anti-oxidants, which require high doses, do not specifically target the retina, and require frequent administration, ocular gene therapy-based approaches targeting particular UPS enzymes in either the RPE or photoreceptors could potentially overcome these shortcomings and greatly advance efforts to prevent AMD and/or retard its progression.

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Fig. 51.1.

Cartoon of the enzyme cascade that attaches Ub to substrates. The types of enzymes that cooperate to transfer Ub to substrates are illustrated. Of note, E3 ligases can be single polypeptides or alternatively, multi-subunit complexes

Fig. 51.2.

Differential expression of class III E2s in the mouse eye. Paraffin-embedded sections from 8-month old SvEv129 mice were immunolabeled with the indicated antibodies. The *black arrowheads* highlight specific labeling in each panel. Serial sections from the same eye were labeled individually with each antibody

Fig. 51.3.

The stability and activity of Nrf2 are redox-sensitive. Nrf2 is constitutively degraded by CUL3^{Keap1} and the 26S proteasome in the absence of stress but is stabilized in response to oxidative stress. Stabilized Nrf2 induces the expression of a battery of anti-oxidant genes

Fig. 51.4.

Nrf2 stability is controlled by cellular redox status and by the UPS. a-Nrf2 western blot demonstrating that endogenous Nrf2 in RPE-1 cells is stabilized by oxidative stress (TBHQ) or by proteasome inhibition (MG132). ETOH and DMSO are the vehicles for TBHQ and MG132, respectively. The *asterisk* marks a non-specific band

Fig. 51.5.

Nrf2 ubiquitylation is mediated by the multi-subunit E3 ligase CUL3^{Keap1}. Step 1: Ub is activated by the E1 enzyme in an ATP-dependent manner. Step 2: The activated Ub is transferred to an E2 enzyme. Step 3: The Ub-charged E2 is recruited to the ROC1 component of CUL3^{Keap1} and directly transfers Ub to Nrf2. Nrf2 is recruited to CUL3^{Keap1} by the Keap1 substrate adaptor. The small ball on CUL3 is Nedd8, a Ub-like protein that regulates the activity of CUL3-based E3 ligases