Published in final edited form as:

Invest Ophthalmol Vis Sci. 2008 August ; 49(8): 3622–3630. doi:10.1167/iovs.07-1559.

THE PROTEASOME IS A TARGET OF OXIDATIVE DAMAGE IN HUMAN RETINA PIGMENT EPITHELIAL CELLS

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Abstract

Purpose—Dysfunction of the ubiquitin-proteasome pathway (UPP) is associated with several age-related degenerative diseases. The objective of this study is to investigate the effect of oxidative stress on the UPP in retina pigment epithelial cells.

Methods—To mimic physiological oxidative stress, ARPE-19 cells were exposed to continuously generated H₂O₂ or A2E-mediated photo-oxidation. The proteasome activity was monitored using fluorogenic peptides as substrates. The ubiquitin conjugation activity was determined by the thioester assay. Levels of ubiquitin and ubiquitin conjugates were determined by Western blotting.

Results—Exposure of ARPE-19 cells to 40–50 μM H₂O₂ for 4 h resulted in a 30–50% reduction in all the three peptidase activities of the proteasome. Similarly, exposure of A2E loaded ARPE-19 cells to blue light resulted in a 40–60% reduction in proteasome activity. Loading of A2E or exposure to blue light alone had little effect on proteasome activity. In contrast, exposure of ARPE-19 to low levels of H₂O₂ (3–20 μM) stimulated ubiquitin conjugation activity. Loading of A2E, with or without blue light exposure, up-regulated the levels of ubiquitin activating enzyme and increased conjugation activity. Exposure to H₂O₂ or A2E-mediated photo-oxidation also resulted in a 2–3 fold increase in levels of endogenous ubiquitin conjugates

Conclusion—These data show that the proteasome in ARPE-19 is susceptible to oxidative inactivation whereas activities of the ubiquitin conjugating enzymes are more resistant to oxidative stress. Oxidative inactivation of the proteasome appears to be one of the mechanisms underlying stress-induced accumulation of ubiquitin conjugates in the cells.

Introduction

The ubiquitin-proteasome pathway (UPP) is the major non-lysosomal proteolytic pathway within cells.^{1–3} In this pathway, proteins destined for degradation must be conjugated with a ubiquitin chain in order to be recognized and degraded by a large protease complex called proteasome. The proteasome complex consists of a 20S proteolytic core and typically two regulatory 19S caps. The 19S cap recruits ubiquitin conjugates to the proteasome, then it cleaves ubiquitin moieties from the substrate, unfolds the polypeptide and feeds it through the narrow channel of the proteolytic chamber of the 20S core.⁴ Ubiquitin conjugation, or ubiquitination, is a highly ordered process in which a ubiquitin-activating enzyme (E1) first activates and transfers ubiquitin to a ubiquitin-conjugating enzyme (E2), which then acts in

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concert with one of a large family of ubiquitin protein ligases (E3) to transfer ubiquitin to a lysine residue on the target substrate^{2, 5}. In most cases, multiple ubiquitins are conjugated to the initial ubiquitin moiety to form polyubiquitin chains. A chain of at least four ubiquitin moieties is often required for substrate recognition by the 26S proteasome complex.⁶⁻⁸

The UPP is an important protein quality control system,⁹⁻¹¹ which selectively degrades mutant, misfolded, or damaged proteins.¹²⁻¹⁴ Timely removal of abnormal or damaged proteins by the UPP is essential for the cells to withstand and recover from various environmental stresses.^{15, 16} However, the UPP itself is also a target of such stresses. All of the three classes of ubiquitination enzymes (E1, E2 and E3) have a cysteine in their active sites and therefore the activities of these enzymes are subject to redox regulation.^{17, 18} In addition, other types of modifications, such as S-nitrosylation can also inactivate these enzymes.¹⁹ Reactive oxygen species and reactive lipid peroxidation products, such as 4-hydroxynonenal (HNE), also impair the function of the proteasome.^{10, 20-24}

Similar to most other types of cells, retina pigment epithelial (RPE) cells have an active UPP.^{16-18, 25, 26} In a cell-free system, RPE cell supernatant is capable of degrading a variety of substrates for the UPP, such as histone 2A, oxidized RNase, transducin, and beta-lactoglobulin.^{25, 27} In contrast to many other types of cells, RPE have limited levels of free ubiquitin. Thus, addition of exogenous ubiquitin to RPE supernatant generated significantly higher levels of ubiquitin conjugates and also enhanced the proteasome-dependent proteolysis.^{16, 25, 27}

Chronic exposure to light and the high metabolic rate constantly generate reactive oxygen species in the retina. In addition, A2E, a major fluorophore of lipofuscin, acts as a photosensitizer in RPE to generate intracellular reactive oxygen species.^{28, 29} Therefore, the RPE is under continuous oxidative stress.

The presence of an active ubiquitin proteasome system together with the constant exposure to oxidative stress makes RPE cells a relevant model in which to assess the effect of oxidative stress on components of the ubiquitin-proteasome pathway. Previous studies showed that extensive oxidative stress can reversibly inhibit the ubiquitin conjugation process in RPE.^{17, 18} In this work we assessed the effects of physiologically relevant levels of oxidative stress on the UPP in cultured RPE cells. The data indicate that the proteasome is more susceptible to either H₂O₂- or photo-oxidation-induced inactivation than the ubiquitin conjugating enzymes. The preferential inactivation of the proteasome by oxidative stress indicates a mechanism for the commonly observed the accumulation of endogenous ubiquitin conjugates in response to oxidative stress in various cell types and tissues.

Material and methods

Materials

N-ethyl-maleimide (NEM) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). 4-(2-Aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF) was obtained from Calbiochem-Novabiochem Corp (La Jolla, CA, USA). HNE was purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-rabbit-HRP antibody was from Jackson ImmunoResearch (West Grove, PA, USA). ¹²⁵I (NaI) was purchased from PerkinElmer (Boston, MA, USA). SDS-PAGE reagents were from BIORAD (Hercules, CA, USA). Antibodies to ubiquitin and ubiquitin-activating enzymes were produced in rabbits as described previously.^{30, 31} Dulbecco's Modified Eagle Medium (DMEM) was purchased from Mediatech, Inc. (Herndon, VA) or Invitrogen Corporation (Carlsbad, CA). Fetal calf serum, nonessential amino acid solution and antibiotics for cell cultures were purchased from Invitrogen Corporation (Carlsbad, CA). A2E was synthesized as described.³² All other chemicals were obtained from Sigma and were of the highest purity available.

Exposure to H₂O₂

Confluent ARPE-19 cells were incubated in serum-, pyruvate-, and phenol red-free DMEM containing 4.5g/L glucose in the presence or absence of 15 or 40 milliunits/ml of glucose oxidase. Levels of H₂O₂ in the medium were monitored by a colorimetric method.¹³

Exposure to A2E and blue light

ARPE-19 cells known to be devoid of endogenous lipofuscin²⁸ were grown to confluence and then cultured in DMEM with 10% heat-inactivated fetal calf serum and 0.1 mM nonessential amino acid solution with or without 10 μ M A2E for 2 weeks. The media were changed twice a week. With this protocol, A2E accumulated in the lysosomal compartment of the cells.²⁸ For blue light exposure, cell cultures were transferred to PBS with calcium, magnesium and glucose and were exposed to 430 nm light delivered from a tungsten halogen source (430 nm $\pm$ 20; 15 minutes; 2.62 mW/cm²). The cells were then incubated for an additional 6 hours in DMEM with 1% fetal calf serum and harvested by scraping on ice. Controls included cultures that had neither accumulated A2E nor been exposed to blue light, cell cultures that accumulated A2E only, or cells exposed to blue light only. All of the control cells were treated in the same manner as the cells that were exposed to A2E and blue light.

De novo ubiquitin conjugation and thiol-ester assay

To determine the ability to form ubiquitin conjugates, cells were lysed in 50 mM Tris-HCl, pH 7.6, containing 1 mM DTT. The conjugation activity was determined using endogenous enzymes and substrates with exogenous ¹²⁵I-ubiquitin. Briefly, the assay was carried out in a final volume of 25 μ l, containing (final concentrations) ~10 mg/ml cell supernatant, 50 mM Tris buffer, pH 7.6, 2 mM ATP, 1 mM DTT, 5 mM MgCl₂, 4 μ M ¹²⁵I-labeled ubiquitin. The mixture was incubated at 37° C for 20 min, the reaction was then stopped by addition of 25 μ l 2 $\times$ SDS-PAGE loading buffer. To determine the thioesters of E1 and E2s, the reaction was stopped by addition of 25 μ l 2 $\times$ SDS-PAGE loading buffer without DTT. After boiling at 100 °C for 3 min, aliquots of the mixture were resolved by SDS-PAGE. The *de novo* formed ubiquitin conjugates and E1~ubiquitin or E2~ubiquitin thioesters were visualized by autoradiography.

Detection of endogenous ubiquitin conjugates, E1 and HNE-modified proteins

Levels of endogenous ubiquitin conjugates, E1 and HNE-modified proteins in cell lysates were determined by Western blotting as described previously.^{14, 30, 33} Briefly, proteins were resolved by SDS-PAGE on 12% gels and transferred to nitrocellulose. The blots were probed with antibodies to ubiquitin, E1 or to HNE-modified proteins. The specifically bound antibody was detected by chemiluminescence using the Super Signal kit (Pierce) after incubating with HRP-conjugated anti-rabbit secondary antibodies.

Proteasome activity assay

ARPE-19 cells were lysed in 25 mM Tris-HCl buffer, pH 7.6. All of the three peptidase activities of the proteasome were determined using fluorogenic peptides as described.³⁴ Succinyl-Leu-Leu-Val-Tyr-amidomethylcoumarin (LLVY-AMC) was used for the chymotrypsin-like activity, *N*-*t*-butyloxycarbonyl-Leu-Ser-Thr-Arg-amidomethylcoumarin (LSTR-AMC) was used for the trypsin-like activity, and benzyloxycarbonyl-Leu-Leu-Gluamidomethylcoumarin (LLE-AMC) was used for the peptidylglutamyl-peptide hydrolase activity. The mixture, containing 20 μ g of cell supernatant in 25 mM Tris-HCl, pH 7.6, was incubated at 37 °C with the appropriate concentrations of peptide substrate (LLVY-AMC at 25 μ M, LLE-AMC and LSTR-AMC at 40 μ M) in a buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 3 mM NaN₃ and 0.04% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS). The final volume of the assay was 200 μ l.

Enzymatic kinetics was measured with a temperature-controlled microplate fluorometric reader. Excitation/emission wavelengths were 380/440 nm. Proteasome activity was defined as the peptidase activity in the cell extracts that was inhibited by 20 μ M *N*-Cbz-Leu-Leu-leucinal (MG132), a potent proteasome inhibitor.

Results

Continuous generation of H₂O₂ by glucose/glucose oxidase in the presence of ARPE-19 cells

The high metabolic rate and high oxygen consumption of the retinal tissue continuously generate reactive oxygen species. In addition, ocular inflammation increases the production of H₂O₂ and other reactive oxygen species. As high as 50 μ M H₂O₂ can be detected in the vitreous of human eyes.³⁵⁻³⁷ To mimic the chronic oxidative stress, we used a glucose/glucose oxidase system to continuously generate relatively constant levels of H₂O₂ in the presence of ARPE-19 cells. Data in Fig. 1 indicate that RPE cells are able to scavenge H₂O₂ from the medium. In the absence of cells, the levels of H₂O₂ in the medium increased in a dose- and time- dependent manner upon addition of glucose oxidase (Fig. 1A). In the presence of ARPE-19 cells, levels of H₂O₂ in the medium were much lower than in the absence of cells. The H₂O₂ levels reached 20 μ M by 1 h after addition of 15 mU/ml glucose oxidase, but decreased to 3 μ M by 3 h (Fig. 1A), indicating that the cells increased capability to scavenge H₂O₂ in adaptation to mild oxidative stress. In contrast, addition of 40 mU/ml glucose oxidase maintained the levels of H₂O₂ in the medium at a relatively constant concentration of 40-50 μ M (Fig. 1A).

A2E-mediated photo-oxidation increases levels of hydroxynonenal (HNE)-modified proteins in ARPE-19 cells

In vivo, there is an age-dependent accumulation of lipofuscin, which in turn acts as a photo-sensitizer in RPE, leading to an increased production of reactive oxygen species in response to short wavelength light.³⁸⁻⁴¹ To mimic the *in vivo* photo-oxidation, ARPE-19 cells were loaded with A2E, a major fluorophore and a photo-sensitizing component of lipofuscin, and were subsequently exposed to blue light. In control ARPE-19 cells, there were two bands that were recognized by the antibody to HNE-modified proteins (Fig. 1B). Several new HNE-reactive bands were detected following A2E and blue light exposure (Fig. 1B, indicated by arrows). The majority of HNE-positive proteins were of high mass, which barely entered the resolving gel (Fig. 1B). In contrast, exposure to A2E or blue light alone did not significantly increase the levels of HNE-modified proteins in the cells (Fig. 1B). These data are consistent with our previous work⁴² and confirmed that A2E is capable of triggering photo-oxidation in ARPE-19 cells.

Oxidative stress results in accumulation of ubiquitin conjugates in ARPE-19 cells

Previous work indicates that the level of endogenous ubiquitin conjugates is a sensitive marker of oxidative stress.^{31, 33, 43-48} To study the effect of physiologically relevant levels of oxidative stress on the UPP, we first determined levels of endogenous ubiquitin conjugates in RPE cells. As reported in other cell types, the majority of ubiquitin conjugates detected in ARPE-19 cells were of high mass (Fig. 2). Levels of endogenous ubiquitin conjugates increased ≥ 2 fold when the cells were exposed to H₂O₂ in the range of 3-20 μ M (10 μ M in average) for 4 hours (Fig. 2A). Exposure to 50 μ M H₂O₂ increased the levels of endogenous ubiquitin conjugates only slightly further. A2E-mediated photo-oxidation also resulted in a significant increase in the levels of endogenous ubiquitin conjugates in the cells (Fig. 2B). Exposure to blue light alone had little effect on the levels of endogenous ubiquitin conjugates, whereas accumulation of A2E alone resulted in a modest increase in levels of endogenous conjugates (Fig. 2B). The dramatic accumulation of endogenous ubiquitin conjugates when the RPE were exposed to physiologically relevant levels of oxidative stress indicates that the ability of the cells to process ubiquitin-conjugates was impaired by oxidative stress.

Physiologically relevant levels of oxidative stress stimulate ubiquitin conjugating activity in RPE cells

The steady state levels of endogenous ubiquitin conjugates are the net balance between the rate of ubiquitin conjugation and the rate of degradation of the conjugates by the proteasome or deubiquitination by isopeptidase. In order to search for the mechanism whereby physiologically relevant oxidative stress increases the levels of endogenous ubiquitin conjugates, we evaluated the effect of oxidative stress on ubiquitin conjugating activity. As shown in Fig. 3A (left panel), exposure to 10 μM H_2O_2 increased the ubiquitin conjugating activity. Exposure to higher levels of H_2O_2 (50 μM), also increased the conjugating activity, but not as much as when cells were exposed to 10 μM H_2O_2 . Thiolester assays showed that the level of E1~ubiquitin thiolester did not change significantly following exposure to the oxidative insult (Fig. 3A, right panel). However, levels of E2s~ubiquitin thiolesters increased upon exposure to 10 μM , but not 50 μM , H_2O_2 . These results indicate that low levels (non-toxic levels), but not high levels (toxic levels), of oxidative stress stimulate some E2s of the ubiquitin conjugating machinery.

We further assessed the effect of A2E-mediated photo-oxidation on the ubiquitin conjugating activity. We found that accumulation of A2E, regardless of blue light exposure, enhanced the ability of cell lysates to form *de novo* ubiquitin conjugates (Fig. 3B). The enhanced ubiquitin conjugating activity appeared to correlate with the significant increase in the levels of E1~ubiquitin and E2~ubiquitin thiolesters (Fig. 3B, right panel). Western blotting assays showed that levels of E1, particularly E1A, ³⁰ increased dramatically upon accumulation of A2E in the cells (Fig. 3C). Exposure to blue light had no detectable effect on the levels of E1 in the cells. These data indicate that the up-regulation of the ubiquitin-conjugating system in response to mild oxidative stress may partially explain the accumulation of endogenous ubiquitin conjugates in RPE cells.

Physiologically relevant levels of oxidative stress inactivate proteasome in ARPE-19 cells

The upregulation of ubiquitin conjugating activity in response to mild oxidative stress can only partially explain the oxidation-induced increase in levels of endogenous ubiquitin conjugates. To further explore the causes of the elevated levels of endogenous ubiquitin conjugates, we evaluated the effect of physiological oxidative stress on three peptidase activities of the proteasome. Whereas exposure to 10 μM H_2O_2 had no detectable effect on any peptidase activity of the proteasome, treatment with 50 μM H_2O_2 resulted in ~50% inhibition of chymotrypsin-like and trypsin-like activities. The peptidylglutamyl peptide hydrolase activity of the proteasome was inhibited by ~30% under these conditions (Fig. 4A). The decrease in the peptidase activities of the proteasome was not due to loss of cell viability, since there was no significant loss of cell viability in this time frame (4 hours) (Fig. 4C). However, prolonged exposure (>8 hours) to 40-50 μM H_2O_2 resulted in >60% loss of cell viability (Fig. 4C). To further test the effect of physiologically relevant oxidation on the activity of the proteasome, we evaluated the effect of A2E-mediated photo-oxidation on the proteasome activity in ARPE-19 cells. We found that exposure to blue light or accumulation of A2E alone had little effect on proteasome activity (Fig. 4B). In contrast, accumulation of A2E and exposure to blue light together resulted in a 40-60% decrease in the three peptidase activities of the proteasome. The chymotrypsin-like activity and trypsin-like activity of the proteasome were preferentially affected by photo-oxidation. The data indicate that, as in other cell types, the proteasome in RPE is susceptible to oxidative stress and that it can be inactivated by physiologically relevant levels of oxidative stress.

H₂O₂ and HNE, but not oxidized proteins, inhibit the proteasome in supernatant of ARPE-19 cells

The H₂O₂- or photo-oxidation -induced inactivation of the proteasome may be mediated either by the reactive oxygen species, or by lipid peroxidation products, such as HNE. It has been shown that HNE can inhibit the proteasome.^{22, 49} Additionally, data in Fig. 1B also showed that cellular proteins were modified by HNE upon A2E-mediated photo-oxidation. Oxidation-induced formation of protein aggregates could also inhibit the proteasome. It has been reported that protein aggregates inhibit the proteasome mediated degradation of several typical substrates in intact cells^{50, 51} or in cell free systems.^{24, 49, 52} To determine the mechanism of oxidative inactivation of proteasome, we directly tested the effect of H₂O₂, HNE and oxidized proteins on the chymotrypsin-like activity of the proteasome in the supernatants of ARPE-19 cells. We found that addition of as much as 100 μ M H₂O₂ to the supernatant had little effect on the proteasome activity (Fig. 5A). However, addition of 500 μ M or higher concentrations of H₂O₂ to the supernatant significantly inhibited the proteasome (Fig. 5A). Similarly, addition of 500 μ M or higher concentrations of HNE to the supernatant of ARPE-19 cells also inhibited the proteasome (Fig. 5B). To determine the effect of oxidized proteins on proteasome activity, we prepared the proteasome-free supernatant of ARPE-19 cells by centrifugation at 100,000 $\times$ g for 5 hours.^{16, 53} A fraction of the proteasome-free supernatant was treated with 2 mM H₂O₂ at 37° C for 2 hours and followed by dialysis to remove residual H₂O₂. Another fraction the proteasome-free supernatant was treated identically, but without H₂O₂. Protein oxidation was monitored by determining levels of protein carbonyls in the supernatant.^{13, 15} As shown in Fig. 5C, treatment of the supernatant with 2 mM H₂O₂ resulted in a significantly increase in protein carbonyls, an indicator of protein oxidation. We added the non-oxidized or oxidized proteasome-free supernatants to the proteasome-enriched fraction of ARPE-19 cells and incubated at 37° C for 30 min and then measured the chymotrypsin-like activity of the proteasome. We found that addition of non-oxidized proteasome-free supernatant to the proteasome-enriched fraction had no effect on proteasome activity (Fig. 5D). Addition of oxidized proteasome-free supernatant to the proteasome-enriched fraction slightly stimulated the chymotrypsin-like activity of the proteasome (Fig. 5D). These data indicate that H₂O₂ and HNE, but not oxidized proteins, inhibit proteasome activity.

Discussion

The UPP plays important roles in a vast number of cellular functions, including protein quality control and signal transduction.³ A functional UPP is required for the cells to cope with various stresses, including heavy metals,^{54, 55} amino acid analogs and oxidation^{15, 16}. However, an extensive oxidative insult is also likely to damage or impair the function of critical components of the UPP.^{17-23, 56} RPE *in vivo* are exposed to chronic oxidative stress due both to high oxygen consumption and to lipofuscin-sensitized photo-oxidation. In this work we evaluated the effects of physiologically relevant levels of oxidative stress on the UPP in cultured human RPE. We found that the UPP can withstand 3-20 μ M H₂O₂ without suffering major functional damage. In fact, these levels of H₂O₂ even stimulated the ubiquitin-conjugating activity (Fig. 3A). However, prolonged exposure to 40-50 μ M H₂O₂ inhibited all of the three peptidase activities of the proteasome. Furthermore, A2E-mediated photo-oxidation also inhibited the proteasome activity. However, exposure to the same oxidizing agents for the same dosage did not inhibit the ubiquitin conjugating activity. These data indicate that the proteasome is more susceptible to oxidative stress than the ubiquitin conjugating enzymes.

To determine the potential mechanism by which oxidative stress inactivates the proteasome, we directly tested the effect of H₂O₂ and HNE on the proteasome activity in RPE supernatants. We found that both H₂O₂ and HNE are capable of inactivating the chymotrypsin-like activity

of the proteasome (Fig. 5). However, relatively high levels ($>100\ \mu\text{M}$) of H_2O_2 and HNE were required to inactivate the proteasome in the cell supernatants. The resistance to a single bolus of H_2O_2 or HNE may be related to the rapid detoxification of H_2O_2 or HNE by the active antioxidant system in these cells, such as high levels of glutathione. Consistent with this idea, we also found that the RPE cells are resistant to a single bolus of H_2O_2 . Exposure of ARPE-19 cells to single bolus of $200\ \mu\text{M}$ H_2O_2 for 1 hour only marginally decreased proteasome activity (data not shown). Data in Fig. 1 also indicate that significant fraction of H_2O_2 in the medium was decomposed in the presence of ARPE-19 cells. To test whether the proteasome in ARPE-19 cells could be inhibited by oxidized proteins, we oxidized the proteasome-free supernatant of ARPE-19 cells with $2\ \text{mM}$ H_2O_2 for 1 hour and then tested its effects on proteasome activity in the proteasome-enriched fraction of ARPE-19 cells. We found that oxidized proteins did not inhibit the peptidase activity of the proteasome. These data suggest that reactive oxygen species, or lipid peroxidation products, such as HNE, play a major role in the inactivation of the proteasome upon physiologically relevant levels of oxidative stress.

Consistent with the observation that the proteasome in ARPE-19 cells is susceptible to oxidative stress, oxidative inactivation of the proteasome has been reported in many cell types. In K562 human hematopoietic cells, the ATP-independent degradation of the fluorogenic peptide suc-LLVY-MCA was not affected by H_2O_2 concentrations of up to $5\ \text{mM}$, the ATP-stimulated degradation of suc-LLVY-MCA by the 26S proteasome began to decline at $400\ \mu\text{M}$ and was completely abolished at $1\ \text{mM}$ H_2O_2 , indicating that the 26S proteasome is more susceptible to oxidative stress than the 20S proteasome.⁵⁷ In a motor neuron cell line (NSC-19 cells), exposure to FeSO_4 resulted in a dose- and time-dependent increase in reactive oxygen species formation and a dose- and time-dependent decrease in proteasome activity. All of the three peptidase activities decreased within 6 h by $1\ \mu\text{M}$ FeSO_4 .⁵⁸ Exposure of NSC-19 cells to HNE, a lipid peroxidation product, also resulted in a time- and dose-dependent decrease in proteasome activity.⁵⁸ Modifications and inactivation of the proteasome by HNE were also detected in other cell type and tissues.^{22, 59} Oxidative inactivation of the proteasome was also observed upon ischemia-reperfusion injury,^{34, 60} UVA or UVB radiation⁵² and chronic exposure to high concentrations of oxygen.²¹ In addition to reactive oxygen species and lipid peroxidation products, protein aggregates, including oxidized or HNE-modified proteins, may also inhibit the proteasome directly or indirectly.^{24, 49-52, 61} However, data presented here indicated that the proteasome was inhibited by reactive oxygen species or lipid peroxidation products, but not by oxidized proteins under these mild oxidative conditions (Fig. 5D). However, our data do not rule out the possibility that proteins that are extensively oxidized will inhibit the proteasome in the supernatant of ARPE-19 cells. Comparison to most other studies,^{24, 49, 52} we used relatively mild oxidative condition in this experiment. The oxidative inactivation of proteasome may be responsible for age-related decline in proteasome activity in the retina.^{62, 63}

Increased levels of endogenous ubiquitin conjugates have been reported in many types of cells and tissues in response to oxidative stress.^{13, 15, 31, 33, 45-48, 64-66} Since oxidized proteins are preferred substrates for the UPP, the increase in substrate availability may be one of the mechanisms for the accumulation of ubiquitin conjugates.³¹ However, this work indicates that another mechanism, oxidative inactivation of the proteasome, could also contribute to the accumulation of ubiquitin conjugates in response to oxidative stress.

The UPP plays an important role in protein quality control. Many forms of damaged proteins, including oxidized proteins are degraded by the proteasome.^{10, 13, 15, 67-70} Oxidative stress may not only increase the generation of oxidized cellular proteins, it may also impair the machinery which degrades oxidized proteins. Thus, the age-related decline in proteasome activity may be responsible for the accumulation of damaged proteins in various tissues, particularly for the postmitotic cells.^{58, 70-73} Since accumulation of oxidized or otherwise

damaged proteins is associated with many age-related diseases, protecting the proteasome from oxidative inactivation appears to be essential for prevention of age-related accumulation of damaged proteins and the onset and progress of age-related diseases. ^{10, 74, 75}

In addition to protein quality control, the UPP plays important roles in signal transduction. Various transcription factors, such as hypoxia-inducible factor (HIF), ^{76, 77} p53 ^{78, 79} and stat-1, ^{80, 81} are the substrates of the UPP. The activity of NF-κB is also regulated by proteasome-mediated degradation of the inhibitor of NF-κB (I-κB). ⁸²⁻⁸⁵ Our previous work demonstrated that the inhibition of the UPP in ARPE-19 cells altered the signal transduction cascade. ⁸⁶ We have shown that inhibition of the proteasome resulted in accumulation of HIF-1α and increased the expression and secretion of vascular endothelial growth factor (VEGF). ⁸⁶

Age-related macular degeneration (AMD) is the leading cause of blindness in industrialized countries. Elevated levels of VEGF in the eye are associated with the development of AMD, particularly the wet form of AMD. ^{87, 88} A growing body of literature demonstrates that oxidative stress is one of the risk factors for AMD. ⁸⁹ Previous work also showed that oxidative stress, including A2E-mediated photo-oxidation stimulates the expression and secretion of VEGF by RPE cells. ^{42, 90-92} The data presented in this paper suggest that oxidative inactivation of the proteasome may be responsible, at least in part, for the oxidation induced expression and secretion of VEGF by RPE cells. Consistent with this hypothesis, the age-related decline in proteasome activity in the retina is associated with increased levels of HNE-modified proteins in the retina. ⁶² Taken together, the data indicate that the proteasome is a target of oxidative stress and that inhibition of the proteasome may account, at least in part, for the accumulation of ubiquitin conjugates in experimental oxidative insult as well as in pathologies related to oxidative damage. Given the important roles of the UPP in various cellular functions, oxidative inactivation of the UPP is likely to have physiological consequences. For example, oxidative inactivation of the proteasome in RPE could be one of the mechanistic steps contributing to development of AMD. Therefore, protecting the proteasome from oxidative inactivation may be a valid strategy for the prevention of AMD.

Acknowledgements

This work is supported by NIH grant EY11717 (to FS), EY 13250 (to AT), EY12951 (to JRS), USDA CRIS 1950-51000-060-01A (to AT), and a grant from the Portuguese Foundation for Science and Technology POCI/SAU-OBS/57772/2004 (to PP). AF is a recipient of a Fellowship from the Portuguese Foundation for Science and Technology (SFRH/BD/19039/2004).

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Fig. 1. Exposure of ARPE19 cells to H_2O_2 or A2E-mediated photo-oxidation

(A) Glucose oxidase was added into 10 ml of serum-, pyruvate-, and phenol red-free DMEM in the presence or absence of a monolayer of confluent ARPE-19 cells and incubated at 37° C. Aliquots of the medium were taken at the indicated time points and levels of H_2O_2 in the medium were determined colorimetrically. (B) The ARPE-19 cells were treated with blue light alone, A2E alone, or with both A2E and blue light. Levels of HNE-modified proteins in the cells were determined by Western blotting with an antibody specific for HNE-modified proteins. Arrows indicate the HNE-modified proteins that were detected only when the cells were exposed simultaneously to A2E and blue light.

Figure 2. Oxidative stress results in an accumulation of ubiquitin conjugates

(A) ARPE-19 cells were exposed to 10 or 50 μM H_2O_2 for 4 hours and levels of endogenous ubiquitin conjugates were determined by Western blotting. (B) ARPE-19 cells were treated with blue light alone, A2E alone, or both A2E and blue light as described. Levels of endogenous ubiquitin conjugates were determined by Western blotting. Levels of β -actin were used as protein loading controls.

Figure 3. Physiological levels of oxidative stress stimulate ubiquitin conjugating activity in ARPE-19 cells

(A) The ARPE19 were exposed to 10 and 50 μM H₂O₂ for 4 hours. Ubiquitin conjugating activity, E1 activity and E2 activities were determined by thiolester assays. (B) The ARPE-19 cells were treated with blue light alone, A2E alone, or A2E together with blue light. Ubiquitin conjugation activity, E1 activity and E2 activity were determined thiolester assays. (C) The ARPE-19 cells were treated with blue light alone, A2E alone, or A2E together with blue light and levels of E1 were determined by Western blotting.

Figure 4. Physiologically relevant levels of oxidative stress inactivate the proteasome in ARPE-19 cells

Panel A: ARPE-19 cells were exposed to a continuously generated H_2O_2 for 4 hours. Panel B: The ARPE-19 cells were exposed to blue light alone, accumulated A2E alone, or accumulated A2E and exposed to blue light as indicated in Fig. 1. The cells were harvested and proteasome activities were determined using fluorogenic peptides as substrates, Panel C. ARPE-19 cells were exposed to a continuously generated H_2O_2 for 4 and 8 hours, respectively. Cell viability was determined with MTS assay using the kits from Promega. The proteasome activity and viability of the cells that were not treated with H_2O_2 were arbitrarily defined as 100% and the rest were expressed as a relative activities normalized with controls.

Figure 5. H₂O₂ and HNE, but not oxidized proteins, inhibit the proteasome in supernatant of ARPE-19 cells

ARPE-19 cells were homogenized in lysis buffer (50 mM sodium phosphate buffer, pH 7.4) and centrifuged at $30,000 \times g$ for 20 min. The resulting supernatants (1 mg protein/ml) were incubated with indicated concentrations of H₂O₂ (panel A) or HNE (panel B) for 1 hour at 37°C. The chymotrypsin-like activity of the proteasome in the supernatants was measured as described in Figure 4. A fraction of ARPE-19 cell supernatant was centrifuged at $100,000 \times g$ for 5 hours. The resulting supernatant was devoid of proteasome and was defined as proteasome-free supernatant. The pellet was resuspended with the lysis buffer and was defined as the proteasome-enriched fraction. The proteasome-free fraction was treated with/without 2 mM H₂O₂ at 37°C for 1 hour and followed by dialysis against the lysis buffer overnight to remove any unreacted H₂O₂. Protein oxidation was monitored by determining levels protein carbonyls in the supernatant (panel C). The non-oxidized and oxidized proteasome-free supernatants were added into the proteasome-enriched fraction and incubate for 30 min at 37°C and the chymotrypsin-like activity of the proteasome was determined as described (panel D).