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Unfolded Protein Response is Activated in Aged Retinas

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Abstract

An unfolded protein response (UPR) in addition to oxidative stress and the inflammatory response is known to be activated in age-related ocular disorders, such as macular degeneration, diabetic retinopathy, glaucoma, and cataracts. Therefore, we aimed to investigate whether healthy aged retinas display UPR hallmarks, in order to establish a baseline for the activated UPR markers for age-related ocular diseases. Using western blotting, we determined that the hallmarks of the UPR PERK arm, phosphorylated (p) eIF2a, ATF4, and GADD34, were significantly altered in aged vs. young rat retinas. The cleaved pATF6 (50) and CHOP proteins were dramatically upregulated in the aged rodent retinas, indicating the activation of the ATF6 UPR arm. The UPR activation was associated with a drop in rhodopsin expression and in the NRF2 and HO1 levels, suggesting a decline in the anti-oxidant defense in aged retinas. Moreover, we observed down-regulation of anti-inflammatory IL-10 and IL-13 and upregulation of pro-inflammatory RANTES in the healthy aged retinas, as measured using the Bio-plex assay. Our results suggest that cellular homeostasis in normal aged retinas is compromised, resulting in the concomitant activation of the UPR, oxidative stress, and inflammatory signaling. This knowledge brings us closer to understanding the cellular mechanisms of the age-related retinopathies and ocular disorders characterized by an ongoing UPR, and highlight the UPR signaling molecules that should be validated as potential therapeutic targets.

Introduction

The cellular mechanisms of age-related ocular disorders, such as age-related macular degeneration (AMD) [3, 4, 14, 25], diabetic retinopathy [19, 23, 41], glaucoma [1, 9, 11], and cataracts [5, 37], are tightly associated with oxidative stress, inflammation, and the unfolded protein response (UPR), suggesting cross talk between these cellular signals. Oxidative stress has been found to play a deteriorating role in aged retinas by producing reactive oxygen species (ROS) that are known to trigger different retinopathies, causing damage to the mitochondrial DNA. The generation of ROS is part of the normal metabolism in a biological system; however, free radicals (extremely reactive species), once formed, can

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begin a series of reactions that are harmful to the cell. Normally present, different cellular defense mechanisms have been launched to combat the consequences of acute oxidative stress. However, if the stress becomes chronic, and the cell cannot overcome the consequences, the pathological events in the retinas could be detrimental. ROS are also capable of lipid peroxidation, protein misfolding, and nucleic acid damage [18].

Aging is characterized by increased low-grade chronic pro-inflammation markers that circulate in the body, such as cytokines, C-reactive proteins (CRPs), and mannose-binding lectin (MBL) [13]. Among the factors that chronically trigger the immune system in the elderly, the epigenetic factor, viral CMV and HHV5 infections, and post-translational macromolecule modifiers are present in aged tissue. All of these antigens can provoke the innate immune system to secrete pro-inflammatory mediators and launch the inflammatory response [13].

Another cellular signal involved in the pathology of many age-related ocular disorders is the UPR. The UPR is comprised of a set of signaling pathways that collectively adjust the cell's ER protein folding capacity according to need. Three independently activated UPR signals, RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), activating transcriptional factor 6 (ATF6), and inositol requiring kinase 1 (IRE1), are known to be activated upon stress. The main function of the UPR is to recognize misfolded proteins that occur, with the help of the immunoglobulin protein (BiP), and to lower the protein load via the phosphorylation of eukaryotic initiation factor 2α (peIF2α). The transcription factors, x-box binding protein 1 (Xbp1) and activating transcription factors 4 and 6, act to reestablish the equilibrium of the endoplasmic reticulum (ER) [15]. Oxidative stress and inflammation [35] have been highlighted for their associations with normal aging; however, the UPR and its role in the aged cell receiving internal or external insult has not yet been established. Moreover, the proteomics of normal aged retinas have not been investigated in detail, suggesting that ocular geriatrics must move forward to advance our knowledge and the treatment of age-related ocular disorders.

A great deal of published literature suggests that there is a critical need to establish a baseline for activated UPR markers in aged retinas, which would bring us closer to understanding the cellular mechanisms of age-related retinopathies and ocular disorders characterized by ongoing UPR. This has motivated us to initiate a study to analyze the UPR markers in aged retinas, creating a parallel between the UPR activation and the expression of the cellular markers of oxidative stress and inflammation. This knowledge would allow not only the design of common therapeutic treatments for age-related ocular disorders, but also the creation of a “fountain of youth” to delay aging in humans.

Materials and Methods

Animals

The samples consisting of 4-month and 24-month-old enucleated F344 rat eyes were obtained from the National Institute of Aging tissue bank. The one-year C57BL6 mouse retinas were a generous gift from Dr. Daniel Smith Jr. (University of Alabama at Birmingham).

Western Blot Analysis

For the protein extraction, whole retinas were isolated from the enucleated eyes using surgical excision. The total protein was extracted via sonication in a protein extraction buffer containing 970 uL of RIPA, 10 uL of 100 mM PMSF, 10 uL of 100 mM EGTA, and a mixture of protease inhibitors (PMSF, TLCK, aprotinin, leupeptin, and pepstatin). The protein concentrations were determined using BioRad Protein Assays and based on the Bradford method of protein quantitation. Next, the proteins (30–120 ug) were separated in 4–20% Criterion Precast gels and 5% polyacrylamide gels (BioRad), transferred to a polyvinylidene difluoride (PVDF) membrane using the Trans-Blot Turbo Transfer System (BioRad), and incubated with primary antibodies overnight at 4°C under agitation. Goat anti-rabbit (1:10,000, #926-68021), donkey anti-goat (1:10000, #926-32214), and donkey anti-mouse (1:10000, #926-32210) secondary antibodies were used (LI-COR Odyssey). In addition, β-actin, GAPDH, or Tubulin was used as the gel loading control, and was detected using an anti-β-actin antibody (1:5000, Sigma-Aldrich, #A1978) or anti-GAPDH antibody (1:1000, Abcam, #ab9485). Finally, the developed membrane was imaged using the LI-COR Odyssey Quantitative Fluorescence Imaging System.

Fixation of the Retinal Sections

The F344 enucleated rat eyes were fixed overnight in 4% paraformaldehyde, freshly made in phosphate-buffered saline (PBS). Then, the retinal cryosections were rinsed in PBS and blocked in 2% normal goat serum and 0.3% Triton X-100 in 0.01% BSA in PBS for 1 hour at room temperature. The sections were stained with primary anti-GFAP antibodies (Sigma Aldrich, C9205) which were diluted in PBS with 0.1% Triton X-100 and 1% BSA, and incubated overnight at 40°C. The Cy2-labeled anti-IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:500 in PBS was applied at room temperature for 1 hour; then, the sections were mounted using Vectashield Mounting Medium (Vector Lab) and cover slipped. Images were taken using a confocal microscope (Leica SP1 UV Confocal Laser Scanning Microscope).

Cytokine Detection

The retina lysates (900 mg in 1 mL) were prepared using the Bio-Plex Cell Lysis Kit (BioRad 171-304011). The IL-10, IL-13, and RANTES cytokine levels were determined in the lysate according to the manufacturer's instructions, using the Bio-Plex set, Bio-Plex reader, and Bio-Rad L600013F25 machine.

Results

A review of the current literature has suggested that the UPR is involved in age-related ocular pathologies [15]. Recently, we have demonstrated that the UPR, once persistently activated, can also promote retinal degeneration [30]. These data together indicate that the UPR could affect the initiation and the progression of ocular disease in the elderly; therefore, we began our experiment with a determination of the UPR markers in 24 month old F344 retinas. The choice of 24 months was based on published data correlating rat age with human age [33] that would equate 24 months of rat age to 60 years of human age.

UPR Markers are Elevated in Aged Retinas

The activation of the UPR was measured using a western blot analysis of the retinal protein lysates from 4-month or 24-month-old F344 rats, quantifying the BiP, cleaving pATF6, peIF2α, ATF4, CHOP, and GADD34 (Figs. 1A and B, Table S1). The binding of the immunoglobulin protein GRP78 showed a slight decrease by 1.2 fold ($p = 0.19$) in the 24-month-old retinas, compared to the young retinas, confirming our previous findings in the nigrostriatal system in which the Grp78 was lower in the aged rat brains vs. the young (2 months) animals [32]. However, the markers of the ATF6 and PERK pathways were significantly modified in the aged retinas. Thus, we found that the phosphorylated (p) ATF6 (90 kD) was slightly decreased in the aged retinas ($p < 0.05$), while its active form, cleaved pATF6 (50 kD), was significantly increased by 1.8 fold ($p < 0.001$), suggesting the activation of the ATF6 UPR arm in the aged retinas.

Another UPR marker, the PERK kinase substrate eukaryotic initiation factor 2α (peIF2α), was dramatically decreased in the old retinas, and a 1.8 fold reduction in the peIF2a level was found in the old retinas when compared to the young rats ($p < 0.001$). Interestingly, this decrease was in agreement with the level of the growth arrest and DNA damage inducible protein 34 (GADD34), which is known to be a subunit for a peIF2α phosphatase. A 2.3-fold increase between 4 months and 24 months was found in the retinal extracts ($p < 0.01$), suggesting that this increase could be responsible for reducing the level of peIF2a in aged rat retinas. As shown in Figure 1A, the UPR downstream transcriptional factors, activating transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP) dimer were increased by 1.6 and 5.6-fold ($p < 0.001$ and $p < 0.0001$, respectively). The CHOP monomer, known to act as a pro-apoptotic transcription factor [15], was found to be elevated by 1.9 fold in the aged rat retinas ($p < 0.05$), proposing an increase in the level of apoptosis in aged retinas.

To verify whether or not the UPR activation in aged animals is associated with the inability of the retinas to produce melanin in albino retinas, making them vulnerable to light damage [29], we performed an analysis of one-year-old C57BL6 pigmented mouse retinas and found up-regulation of the CHOP and cleaved pATF6 UPR markers, suggesting that UPR activation is a common feature in aged retinas.

Rhodopsin Expression is Reduced in Aged Retinas

We have previously demonstrated that the rhodopsin protein and mRNA levels are significantly down regulated in the mouse model of inherited retinal degeneration with UPR activation [20, 27]. Therefore, we became interested in analyzing the RHO in aged retinas with ongoing UPR, and found that the protein level in the aged retinas was significantly two-fold down regulated (Figs. 1B and C, Table S1). This suggests either an impairment in the RHO expression machinery or upregulation of the E3 ubiquitin ligases responsible for rhodopsin degradation in aging photoreceptors.

Oxidative Stress and Inflammation Increases with Age

Numerous reports suggest that oxidative stress ubiquitously increases with age. We analyzed the antioxidant proteins nuclear factor erythroid-derived factor 2 (NRF2), superoxide

dismutase 1 (SOD1), heme oxygenase 1 (HO1), and serine 15 phosphorylated protein 53 (p-p53 ser15) via western blot analysis (Figs. 1B and D, Tables S1). The NRF2 shows a 1.9 fold downregulation with age ($p = 0.002$), confirming the previously found decline in the NRF2 transcriptional binding activity with age [36]. Both the HO1 and SOD1 are antioxidant-responsive element (ARE) genes [10, 16] found to be modified in aged retinas. Surprisingly, the HO1, as shown in Figures 1B and C, was decreased by 1.3-fold ($p = 0.05$) while the SOD1 was increased by 1.8 fold ($p < 0.01$). The other key coordinator of oxidative stress and aging, protein 53, was increased in its active phosphorylated form at serine 15 [22] by 2.1 fold ($p = 0.05$). The phosphorylation of Ser at 15 is known to occur during ultraviolet light-induced DNA damage and/or hydrogen peroxide damage [2, 7, 34]; therefore, this data points out the fact that light and ROS can damage normally aged retinas.

Retinal age-related diseases such as diabetic retinopathy have been characterized by an increase in inflammation [17, 38]. Therefore, we analyzed inflammatory markers during the normal aging process by measuring the interleukin 10 (IL-10), interleukin 13 (IL-13), and chemokine ligand 5 (CCL5/RANTES) cytokines using magnetic bead flow cytometry (Fig. 1E, Table S2). We found that the IL-10, an anti-inflammatory cytokine, was decreased by 2.1 fold with age ($p = 0.027$), while another anti-inflammatory marker, the IL-13 cytokine, was found to be decreased by 1.8 fold ($p = 0.036$). Given that the human recombinant IL-13 significantly inhibits ocular inflammation in the LPS-induced rat model of endotoxin-induced uveitis, results in diminished TNFa, pro-inflammatory IL-6, and IL-1b [21], this suggests that the anti-inflammatory defense in aged retinas is weakened. In support of this hypothesis, we found a distinct increase in the pro-inflammatory marker RANTES, which was elevated 5 fold in the aged retinas, compared to the young retinas ($p = 0.024$).

Next, we confirmed glial activation in the aged retinas via immunohistochemistry (Fig. 1F). The microglial activation marker glial fibrillary acidic protein (GFAP) was found to be dramatically increased in aged vs. young retinas, supporting the findings of the distinct rises in the pro-inflammatory signals between 4 and 24 months of age.

Discussion

It has been demonstrated that the UPR is implicated not only in inherited retinal degenerative disorders, but also in age-associated ocular diseases [15]. Our findings suggest that the normal aging process is marked by activation of the UPR, dysregulation of antioxidant response proteins, and disruption of the anti and pro-inflammatory markers towards a pro-inflammatory state. They also indicate that, when launched in aged retinas, these cellular pathways must be taken into consideration while investigating the cellular mechanisms of age-associated eye disorders.

PERK signaling is activated in aged retinas, and both the ATF4 and CHOP proteins are significantly elevated, pointing out pro-apoptotic events overcoming anti-apoptotic. For example, it is known that the CHOP significantly affects the ratio of the BCL2 anti-apoptotic vs. pro-apoptotic events, shifting the balance towards apoptosis. In addition, the CHOP could promote cell death by the induction of miR-708, which is known to regulate the expression of many genes, including the rhodopsin. Perhaps, the miR-708 is elevated in

aged retinas, and might be responsible for the reduction in the RHO found in aged retinas in addition to age-related photoreceptor cell loss [31] and potential epigenetic changes [28, 40].

In our experiments, we found that during persistently activated UPR the peIF2α level becomes reduced, suggesting that deregulated protein and chaperone synthesis are age-associated events. The phosphorylation of eIF2a is known to inhibit the GTP-GDP exchange, affecting protein translation in general. Thus, we speculate that aged retinas undergo translational dysregulation that plays a deleterious role in aging retinas. However, to answer whether lowered peIF2a affects, for example, photoreceptor specific proteins in aged retinas, additional experiments need to be conducted in the future.

Our data also indicate an increase in protein phosphatase 1 activity in aged retinas; the GADD34 expression was elevated, in agreement with a study by Naidoo et al. [26]. These authors have demonstrated that aged mice do not display an increase in BiP expression, while a decline in eIF2α phosphorylation and higher levels of the GADD34 and pro-apoptotic CHOP protein were found in the aged mouse cerebral cortex. This study also proposed that young animals possess an efficient ER adaptive response that declines with aging [26].

An interesting finding was the decrease in the NRF2 expression in aged retinas. One logical explanation for this discovery could be the negative regulation of NRF2 by either KEAP1 or HRD1 E3 ligases that are known to promote Nrf2 degradation [6, 8, 39, 42]. Perhaps, the expression or activities of these ligases are altered with aging in the retina, leading to more pronounced NRF2 degradation. In support of this hypothesis, a study with NRF2 knockout mice has demonstrated that these mice undergo accelerated aging by developing age-related RPE and choroidal degeneration resembling the cardinal features of human AMD, exhibiting extensive hair loss and a decreasing body weight [42]. Altogether, these facts point to NRF2 as a promising therapeutic target to slow down aging.

A decrease in the NRF2 can explain the trend in the reduction of the HO1 protein encoded by the HO1 ARE-containing protein. However, the other ARE-dependent SOD1 gene encoding the SOD1 protein was increased in the aged retinas. Perhaps increased glial proliferation in aged retinas detected via GFAP contributes to SOD1 over-expression, indicating that in aged retinas there are mechanisms responsible for natural cytoprotection. In addition, altered miRNA expression or deficiency in Gp78 ER-associated E3 ligase (known to promote SOD1 degradation) might occur in aged retinas as well.

The role of P53 in aging remains controversial; for example, it has recently been proposed that P53 hyperactivity can accelerate aging through the insulin/IGF-1 pathway [24]. Therefore, it is not surprising that we found an increase in p-P53 in the aged retinas. Other studies have suggested that P53 plays a role as a longevity regulator through its tumor-suppressive function, giving an example of wild-type mice having a maximal life span of 3 years versus P53-null mice that succumb to tumors within several months [12]. Nevertheless, our results indicated that because retinal cells are highly metabolically active and aged retinas have a greater tendency for ROS production, P53 phosphorylation at Ser15

is increased. It would not be surprising to find that the insulin is also elevated in aged retinas, and additional studies on insulin level in healthy aged retinas are necessary.

Activated UPR and oxidative stress in aged retinas occur concomitantly with inflammation. The dramatic increases in the RANTES, chemotactic cytokines, and macroglial marker GFAP, commonly seen as hallmarks of glial activation after damage to retinal cells, are evidence that aged retinas experience stress. We have recently demonstrated that sustained UPR in wild-type mouse retinas can promote retinal degeneration [30]; therefore, it would be interesting to determine whether inflammation arises in healthy aged retinas due to oxidative stress, compromised redox potential, and activated UPR.

Finally, we showed an increase in the basal levels of the UPR, dysregulation of oxidative stress response, and elevation of inflammatory markers during the normal aging process of the retina, which might be helpful in the assessment of mechanisms responsible for age-related ocular dysfunction. Our study also pointed to UPR markers, which should be validated in order to postpone aging in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We demonstrated activation of the Unfolded Protein Response in aged retinas
- We found that the NRF2 level is dramatically reduced in aged retinas
- Oxidative stress and inflammation are concomitantly activated in aging retina with ongoing UPR.

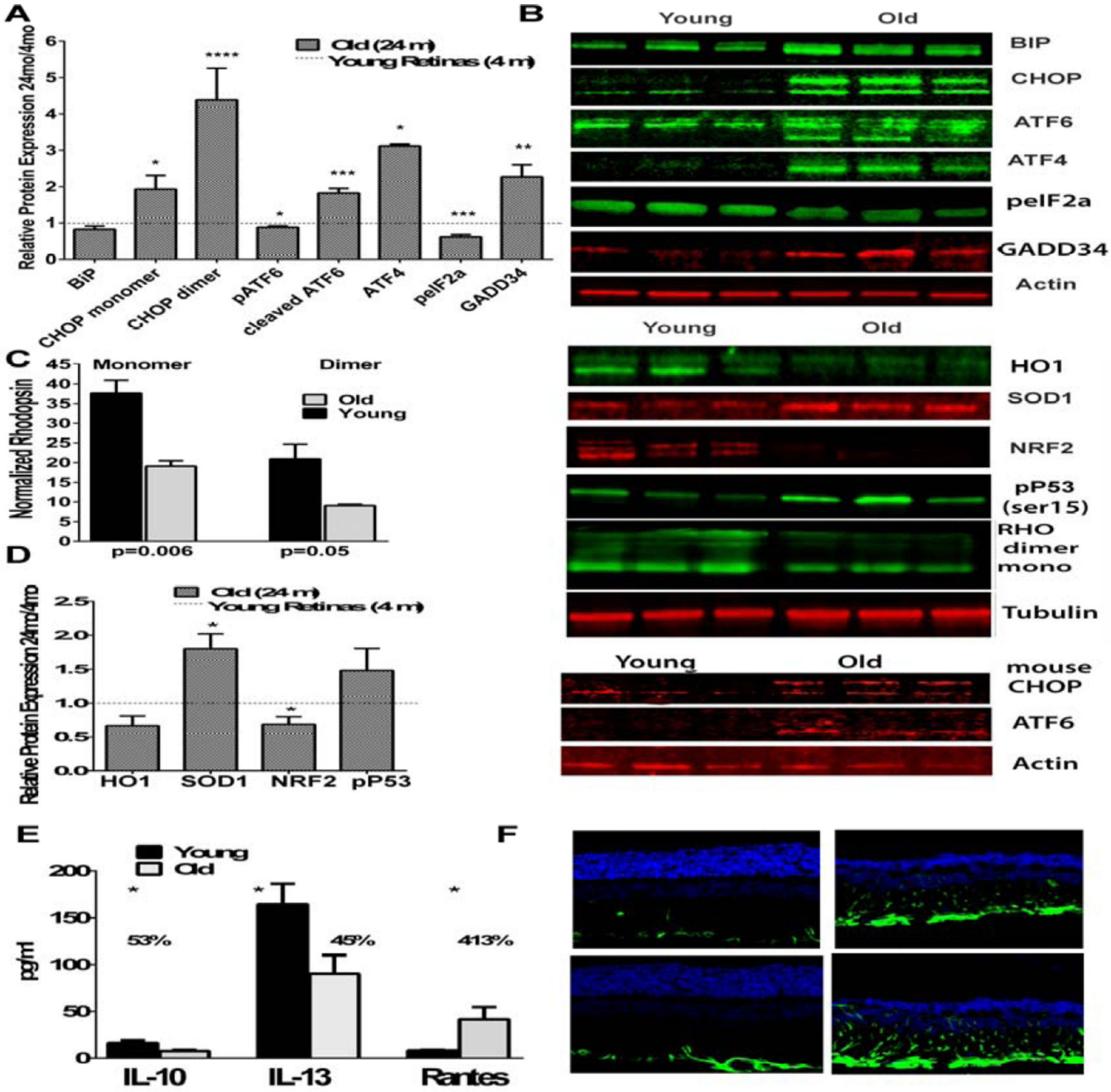

Figure 1.

The unfolded protein response is activated in aged rat retinas concomitantly with oxidative stress and inflammatory signaling (N=5). **A.** The hallmarks of an activated UPR are significantly modified in aged retinas. **B.** Images of representative western blots probed with antibodies against UPR, oxidative stress markers, and the rhodopsin protein. Red or green coloration indicates use of a 700 or 800 channel imaged secondary, respectively. **C.** The expression of the rhodopsin protein was significantly downregulated (N=5). **D.** The expression of the oxidative stress markers was significantly modified (N=5). **E.** The activation of the UPR and impaired oxidative stress defense in aged retinas were associated

with a decrease in the anti-inflammatory cytokines and increase in the pro-inflammatory RANTES cytokine (N=4). **F.** Images of young and old retinas treated with GFAP demonstrating immunoreactivity of the glia with aging.