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## Retinal Flavoprotein Fluorescence Correlates with Mitochondrial Stress, Apoptosis, and Chemokine Expression

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### Abstract

Oxidative stress and mitochondrial dysfunction occur before apoptosis in many retinal diseases. Under these conditions, a larger fraction of flavoproteins become oxidized and, when excited by blue-light, emit green flavoprotein fluorescence (FPF). In this study, we evaluated the utility of FPF as an early indicator of mitochondrial stress, pre-apoptotic cellular instability, and apoptosis of human retinal pigment epithelial (HRPE) cells subjected to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or monocytes (unstimulated or interferon- $\gamma$ -stimulated) *in vitro* and of freshly isolated pieces of human and rat neural retina subjected to H<sub>2</sub>O<sub>2</sub> *ex vivo*. Increased FPF of HRPE cells exposed to H<sub>2</sub>O<sub>2</sub> correlated with reduced mitochondrial membrane potential ( $\Delta\Psi$ m) and increased apoptosis in a time- and dose-dependent manner. HRPE cells co-cultured with monocytes had increased FPF that correlated in a time-dependent manner with reduced  $\Delta\Psi$ m, increased apoptosis, and early expression of pro-inflammatory chemokines, *interleukin-8 (IL8)* and *monocyte chemoattractant factor-1 (MCP1)*, which are known to be induced by oxidative stress. Increased FPF, reduced  $\Delta\Psi$ m, and upregulation of *IL8* and *MCP1* occurred as early as 1–2 hours after exposure to stressors, while apoptosis did not occur in HRPE cells until later time points. The antioxidant, N-acetylcysteine (NAC), inhibited increased FPF and apoptosis of HRPE cells subjected to H<sub>2</sub>O<sub>2</sub>. Increased FPF of human and rat neural retina also correlated with increased apoptosis. This study suggests that FPF is a useful measure of mitochondrial function in retinal cells and tissues and can detect early mitochondrial dysfunction that may precede apoptosis.

### Keywords

apoptosis; chemokines; flavoproteins; mitochondrial dysfunction; monocytes; oxidative stress; neural retina; retinal pigment epithelium

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**Declaration of Interest:** Drs. Elner and Petty have a financial interest in the presented material by having founded OcuSciences, Inc., to commercialize the clinical retinal FPF instrument.

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## 1. Introduction

Oxidant damage is believed to be a major contributor to the pathogenesis of numerous retinal diseases by participating in mitochondrial stress and apoptotic cell death of neuronal and supportive cells (Lieven et al., 2003; Sano et al., 2006; Armstrong et al., 2004; Jiang et al., 2005). Retinal cell apoptosis involves alterations in mitochondrial membrane potential ( $\Delta\Psi_m$ ) leading to the release of mediators that activate caspases, which initiate downstream damage to cellular components, including nucleic acids and proteins (Lieven et al., 2003; Sano et al., 2006; Armstrong et al., 2004; Jiang et al., 2005). However, reactive oxygen metabolites (ROM), including hydrogen peroxide ( $H_2O_2$ ), impair enzymatic complexes of the electron transport chain and reduce  $\Delta\Psi_m$  before apoptosis occurs (Long et al., 2004). Under these conditions, flavoproteins linked to these enzymes become oxidized, and when excited by blue-light, emit green flavoprotein fluorescence (FPF) (Benson et al., 1979; Chance et al., 1979). Thus, measurement of mitochondrial metabolic activity by FPF may serve as an early indicator of disease (Elner et al., 2008a; Elner et al., 2008b; Field et al., 2008; Field et al., 2009a; Field et al., 2009b).

Exogenous or endogenous ROM, including  $H_2O_2$ , induced by light, toxins, or other stimulants of apoptosis, may cause death of cells within the neurosensory retina or retinal pigment epithelium (RPE) (Jin et al., 2001; Barak et al., 2001; Kannan et al., 2004). In cultured human RPE (HRPE) cells, exogenous  $H_2O_2$  has also been shown to induce mitochondrial oxidative dysfunction and DNA damage (Ballinger et al., 1999), and elevate retinal FPF (Elner et al., 2008a). Sub-lethal or lethal concentrations of  $H_2O_2$  reduce the  $\Delta\Psi_m$  of retinal cells early in the apoptotic process (Cao et al., 1999). In addition to  $H_2O_2$ , unstimulated and interferon (IFN)- $\gamma$ -stimulated monocytes have been shown to induce generation of retinal ROM when co-cultured with mouse or human RPE cells, which subsequently may undergo apoptosis (Yoshida et al., 2003; Yang et al., 2009). ROM-induced impairment of mitochondrial electron transport results in oxidation of flavoproteins before apoptosis occurs (Long et al., 2004). Thus, FPF may serve as an early signal of cells prone to apoptosis.

ROM have also been shown to mediate production of pro-inflammatory chemokines, *interleukin-8 (IL8)* and *monocyte chemoattractant factor-1 (MCP1)*, also known as *CC motif ligand 2 (CCL2)* (Zeng et al., 2003; Kina et al., 2009), which modulate ocular inflammatory responses, including leukocyte chemotaxis and activation within the eye (Yoshida et al., 2001a). *IL8* and *MCP1* are the principal chemokines secreted by HRPE cells and have been implicated in a number of retinal diseases (Yoshida et al., 2001a; Bian et al., 2004). Direct contact between monocytes and HRPE cells upregulates the production of *IL8* and *MCP1* (Yoshida et al., 2001a; Bian et al., 2004). Thus, it is important to understand the temporal expression of monocyte adhesion-induced HRPE chemokine gene and protein expression with respect to FPF,  $\Delta\Psi_m$ , and apoptosis.

In this study, we examined the relationship of FPF,  $\Delta\Psi_m$ , and apoptosis in HRPE cells and fresh rat and human neural retina subjected to 1) lethal or sub-lethal concentrations of  $H_2O_2$ , and 2) stimulated or unstimulated monocytes to demonstrate whether FPF may serve as an early indicator of apoptosis, pre-apoptotic cellular instability, and the induction of pro-inflammatory chemokines.

## 2. Materials and Methods

### 2.1. Materials

*N*-acetyl-cysteine (NAC) and  $H_2O_2$  were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant IFN- $\gamma$  was from R&D Systems (Minneapolis, MN). Costar tissue culture 96-

well assay plates with black walls and clear bottoms were obtained from Fisher Scientific (Pittsburgh, PA).

## 2.2. HRPE cell culture

HRPE cells were isolated from donor eyes by enzymatic digestion and cultured as previously described (Elnor et al., 1990; Bian et al., 2009; Yang et al., 2011a; 2011b). The protocol adhered to the provisions of the Declaration of Helsinki for the use of human tissue in research. Briefly, the sensory retina was separated gently from the HRPE monolayer, and the HRPE cells were removed from Bruch's membrane using one hour incubation with papain. Isolated HRPE cells were grown into Falcon Primaria flasks in DMEM/F12 containing 10% fetal bovine serum (FBS), penicillin G ( $100 \text{ U ml}^{-1}$ ), streptomycin sulfate ( $100 \mu\text{g ml}^{-1}$ ), and amphotericin B ( $0.25 \mu\text{g ml}^{-1}$ ) at  $37^\circ\text{C}$  in a humidified incubator under 5%  $\text{CO}_2$ . In all experiments, parallel assays were performed on the second to fourth passaged HRPE cells. HRPE cells were seeded into tissue culture 96-well assay plates with black walls and clear bottoms (Costar) at the same time and density from the same parent cultures at a density of  $5 \times 10^3$  cells/well, and grown in phenol red-free complete DMEM/F12 for 7–14 days to 90%–100% confluence. RPE cells were placed in serum-free media for 24 hr before treatments. All experiments were repeated at least three times on three different cell lines.

## 2.3. Isolation of normal human and rat neural retina

Human neural retina was obtained from orbital exenteration specimens containing lesions not-involving the globe at the University of Michigan. The human retina was isolated from the specimens, cut into pieces and placed in Hanks' balanced salt solution (HBSS), containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , without phenol red. Specimens were examined for FPF within 30 minutes of exenteration. Human research was approved by the institutional review board (IRB) at the University of Michigan. For isolation of rat neural retina, normal, healthy Sprague-Dawley (SD) rats, less than 6 months old, were obtained from the Unit for Laboratory Animal Medicine at the University of Michigan through the Rodent Recycling Program. Animals were killed by carbon dioxide, and the eyes were removed. The rat neural retina was isolated from each eye and cut into three pieces. The fresh neural retinal pieces were washed with HBSS. Specimens were examined for FPF within 30 minutes of exenteration. Animal research was approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

## 2.4. Monocyte isolation

Human monocytes were freshly isolated from the peripheral blood of healthy volunteers, as described previously (Yoshida et al., 2001b). In brief, peripheral blood was drawn into a heparinized syringe and 1:1 diluted in 0.9% saline. Mononuclear cells were separated by density gradient centrifugation. The cells were washed and then layered onto density gradient (Fico-Lite monocytes,  $1.068 \text{ g/mL}$ ) for the enrichment of monocytes. Freshly-isolated human peripheral monocytes were preincubated with RPMI culture medium or the culture medium containing  $\text{IFN-}\gamma$  ( $500 \text{ units/ml}$ ) for 12 hr prior to co-culturing with HRPE monolayers.

## 2.5. HRPE cell and human and rat neural retina incubations

HRPE cells in 96-well plates were washed with HBSS, containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , without phenol red. HRPE cells were stimulated with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ; 0.01, 0.05, 0.1, and 0.2 mM) for 1, 3, 6, and 24 hr. For HRPE cells in monocyte co-culture, enriched unstimulated or  $\text{IFN-}\gamma$ -stimulated monocyte populations were overlaid onto near-confluent HRPE cultures for 1, 2, 4, and 24 hours. After co-culture, the monocytes were removed as

previously described (Yoshida et al., 2001b), and HRPE cells were subjected to further analyses. To evaluate neural retinal FPF, pieces of human and rat neural retina were incubated for 20 min with H<sub>2</sub>O<sub>2</sub> (0.2 mM) in the presence and absence of N-acetyl-cysteine (NAC; 1 mM). To assess apoptosis, aliquots of human and rat neural retina were exposed to 0.2mM H<sub>2</sub>O<sub>2</sub> with or without NAC for 6 hours.

## 2.6. Flavoprotein fluorescence measurement

FPF of HRPE cells was measured with a FlexStation Scanning Fluorometer (Molecular Devices, Sunnyvale, CA). Excitation and emission wavelengths were set to 436 nm and 520 nm, respectively (Shiino et al., 1998), with a cutoff of 495 nm. For human and rat neural retina, FPF acquisitions were captured from each neural retinal piece, mounted under a coverslip, using a stereomicroscope (Stereo Lumar.V12, Carl Zeiss, Oberkochen, Germany) equipped with narrow bandwidth excitation and emission filters of 465±3nm and 535±3nm, respectively (Omega Optical, Brattleboro, Vermont), and an electronmultiplying charge-coupled device camera (Photometrics, Tucson, Arizona). The images were stored as 512 × 512 pixel 16-bit grayscale TIFF files. The integrated intensity of FPF was calculated for each acquisition.

## 2.7. Mitochondrial membrane potential measurement

JC-1 dye was used to monitor  $\Delta\Psi_m$ , as previously described (Yang et al., 2009). In the undamaged mitochondria, the aggregated dye appears as red fluorescence, whereas in the apoptotic cell with altered  $\Delta\Psi_m$ , the dye remains as monomers in the cytoplasm with diffuse green fluorescence. The red/green fluorescence ratio is dependent on  $\Delta\Psi_m$ . HRPE cells were loaded with JC-1 for 1 hour, washed, and exposed to different treatments. Fluorescence intensity was measured with a FlexStation Scanning Fluorometer. For green fluorescence measurements, excitation and emission wavelengths were set to 485 nm and 530 nm, respectively; for red fluorescence measurements, excitation and emission wavelengths were set to 520 nm and 610 nm, respectively.

## 2.8. Apoptotic cell death detection

Neural retinal and HRPE apoptosis was evaluated by using a Cell Death Detection ELISA<sup>PLUS</sup> kit (Roche Applied Science, Indianapolis, IN). This photometric enzyme immunoassay provides the quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death. After various treatments, HRPE cells and human and rat neural retinas were washed, harvested, lysed, centrifuged to remove nuclei, and supernatants were collected. An aliquot of the supernatant from each sample was incubated with immunoreagents (anti-histone-biotin plus anti-DNA-peroxidase-conjugated antibody) in 96-well streptavidin-coated plates on a shaker. After three washes with incubation buffer, ABTS substrate solution was added to each well, and absorbance was read at 405 nm and 495 nm, respectively, in a microplate reader. The absorbance difference between A<sub>405nm</sub> and A<sub>490nm</sub> was standardized to wet weight of the neural retina and results normalized to control.

## 2.9. IL8 and MCP1 ELISA

The levels of antigenic *IL8* and *MCP1* in the serial dilutions of HRPE supernatants were quantitated by modification of a double-ligand ELISA method as previously described (Bian et al., 2001). Standards included 0.5 log dilutions of recombinant *IL8* and *MCP1* (R&D Systems) from 5pg to 100ng/well.

## 2.10. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from nearly confluent cultures of HRPE cells (TRIzol extraction reagent; Invitrogen) according to the manufacturer's procedure. Synthetic oligonucleotide primers based on the cDNA sequences of human *IL8*, *MCPI* and  $\beta$ -*actin* were prepared: *IL8*, 5'-AAGCTGGCCGTGGCTCTCTTG-3' and 5'-AGCCCTCTTCAAAAACCTTCTC-3'; *MCPI*, 5'-GTCATAGCAGCCACCTTCATTC-3' and 5'-GTCTTCGGAGTTTGGGTTTGC-3'; and  $\beta$ -*actin*, 5'-GTGGGGCGCCCCAGGCACCA-3' and 5'-CTCCTTAATGTCACGCACGATTTC-3'. RT-PCR was carried out in a semiquantitative manner, essentially as previously described (Yoshida et al., 1998). Linearity range of the reaction was determined running 15 to 35 cycles. DNA was denatured for 5 minutes at 94°C, followed by 28, 26, and 20 PCR cycles for *IL8*, *MCPI*, and  $\beta$ -*actin*, respectively. Each cycle included a 1-minute denaturation at 94°C, a 1-minute primer annealing at 65°C, and a 2-minute polymerization at 72°C. Each RT-PCR reaction mixture was analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The intensity of the ethidium bromide luminescence was measured by an image sensor with a computer-controlled display.

## 2.11. Northern blot analysis

Total cellular RNA was isolated from nearly confluent cultures of HRPE cells as described earlier in this manuscript. The RNA was separated by electrophoresis using 1% formaldehyde-agarose gels and transferred overnight to the positively charged nylon membranes by capillary blotting. The blots were hybridized overnight at 50°C with DIG-labeled oligonucleotide probes (25 ng/mL). Bound probes were detected with an anti-DIG Fab fragment conjugated to alkaline phosphatase with the chemiluminescent substrate CSPD (disodium 3-(4-methoxyspiro {1,2-dioxetane-3, 2'-(5'chloro) tricyclo [3.3.1.1.1] decan}-4-yl)phenyl phosphate). Specific chemokine mRNA was quantified by laser densitometry. Equivalent amounts of total RNA load per gel lane were assessed by monitoring 18s and 28s ribosomal RNA.

## 2.12. Statistical analysis

Data in the text and figure legends are expressed as the mean  $\pm$  SEM. Differences were detected using ANOVA and *t*-tests and  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. In vitro assessment of FPF, $\Delta\Psi_m$ , and apoptosis in HRPE cells subjected to oxidative stress

HRPE cells incubated with varying concentrations of sub-lethal and lethal levels of  $H_2O_2$  showed significantly increased FPF levels when compared to those of control HRPE cells after 6 hours of  $H_2O_2$  incubation, even with  $H_2O_2$  concentrations as low as 10  $\mu$ M (Fig. 1A). HRPE cells incubated with  $H_2O_2$  concentrations of 50  $\mu$ M or greater showed significantly increased FPF levels when compared to those of control HRPE cells after only 1 hour of  $H_2O_2$  incubation. Significant reductions in  $\Delta\Psi_m$  of HRPE cells were evident after 1, 3, and 6 hours of treatment with  $H_2O_2$  concentrations of 50, 100, and 200  $\mu$ M (Fig. 1B). No significant reduction in  $\Delta\Psi_m$  was detected by the JC-1 assay in HRPE cells treated with 10  $\mu$ M of  $H_2O_2$ . Apoptosis, measured by the number of oligonucleosomes released, was only significantly increased in HRPE cells incubated with 200  $\mu$ M of  $H_2O_2$  for 24 hours when compared to oligonucleosome levels of HRPE control cells (Fig. 1C). Thus, increases in FPF levels and reductions in  $\Delta\Psi_m$  occur at sub-lethal and lethal  $H_2O_2$  concentrations. In the latter, the increases in FPF levels and reductions in  $\Delta\Psi_m$  occur prior to apoptosis.



### 3.2. Ex vivo assessment of FPF and $\Delta\Psi_m$ in human and rat neural retina subjected to oxidative stress

Pieces of fresh human and rat neural retina incubated with 200  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  had significantly increased FPF levels (p-value:  $<0.001$  and  $<0.05$ ) when compared to FPF levels of control human and rat neural retina (Fig. 2A). This increase in FPF was completely blocked by co-treatment with 1mM of NAC. For rat neural retina incubated with  $\text{H}_2\text{O}_2$  + NAC, FPF levels were significantly decreased from that of the control rat neural retina. FPF of the human neural retinal pieces incubated with  $\text{H}_2\text{O}_2$  + NAC was 3.5% less than that of control pieces, but did not reach statistical significance ( $p > 0.05$ ). Apoptosis, measured by the number of oligonucleosomes released, was significantly increased in pieces of fresh human and rat neural retina incubated with 200  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  for 6 hours ( $p < 0.001$  and  $< 0.05$ ) when compared to FPF of control human and rat neural retina (Fig. 2B). This increase in apoptosis was significantly inhibited in human neural retina and completely inhibited in rat neural retina by co-treatment with 1 mM of NAC.

### 3.3. In vitro assessment of FPF, $\Delta\Psi_m$ , and apoptosis in HRPE cells subjected to unstimulated or stimulated monocytes

HRPE cells incubated with unstimulated or IFN- $\gamma$ -stimulated monocytes for 1, 2, 4, or 24 hours showed increased FPF levels and reduced  $\Delta\Psi_m$  when compared to that of control HRPE cells (Fig. 3A–B), but there was no significant differences in FPF or  $\Delta\Psi_m$  of HRPE cells co-cultured with unstimulated as compared to stimulated monocytes. Apoptosis was not present in HRPE cells co-cultured with unstimulated monocytes for 4 hours, but apoptosis did occur after co-culture for 24 hours (Fig. 3C). In HRPE cells cocultured with IFN- $\gamma$ -stimulated monocytes, apoptosis occurred at 4 and 24 hours. There was no apoptosis after 1 or 2 hours in HRPE cells co-cultured with unstimulated or IFN- $\gamma$ -stimulated monocytes (data not shown). Additionally, HRPE cells co-cultured with IFN- $\gamma$ -stimulated monocytes had significantly increased apoptosis from that of HRPE cells co-cultured with unstimulated monocytes for 4 and 24 hours.

### 3.4. Gene and protein expression of HRPE cells subjected to unstimulated or stimulated monocytes

Gene expression of *IL8* and *MCP1*, measured by northern blot analysis, was significantly upregulated after only 2 hours of co-culture with unstimulated monocytes (Fig. 4A–B). RT-PCR (Table 1) and microarray analysis (data not shown) also showed increased *IL8* and *MCP1* gene expression in HRPE cells co-cultured with unstimulated monocytes. Protein expression of *IL8*, measured by ELISA, was 1.7x, 8.8x, and 140x greater than basal levels in HRPE cells exposed to unstimulated monocytes for 1, 4, and 24 hours, respectively (Table 1). The same trend was true for *MCP1*, with increases in protein expression of 4.2x, 8.3x, and 88x over basal levels after exposure to unstimulated monocytes for 1, 4, and 24 hours, respectively. IFN- $\gamma$ -stimulated monocytes induced less HRPE *IL8* and *MCP1* gene and protein expression than unstimulated monocytes (Fig. 5, Table 1).

## 4. Discussion

Our current study substantiates FPF to be an indicator of mitochondrial dysfunction in retinal cells because increases in FPF levels in HRPE cells correlated with reductions in  $\Delta\Psi_m$ , as measured by the JC-1 assay (Figs. 1–3). With the excitation and emission wavelengths used in this study, increases or decreases in the emitted signal from baseline control should only be due to dynamic changes in the oxidation state of flavoproteins and not from other sources of inherent green emission, including advanced glycation end product (AGE) or lipofuscin autofluorescence, which do not change with acute exposure to various apoptotic stressors or treatments. Moreover, the cultured HRPE cells and rat neural retina

were too young to accumulate any significant concentrations of AGEs or lipofuscin. *Ex vivo* samples of human RPE were not analyzed in this study because of shear stress-induced damage to fresh RPE sheets. It is important to note that there are several potential sources of oxidized flavoproteins in the respiratory chain that are involved in fatty acid and carbohydrate pathways, including lipoamide dehydrogenase (LAD), NADH dehydrogenase (electron complex I), succinate dehydrogenase (electron complex II), electron transfer protein, and acyl dehydrogenase. Thus, the FPF signal is not specific to one flavoprotein source but is an amalgam of all of these sources.

In this study, apoptosis did not occur in HRPE cells until incubation with 200  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  for 24 hours, whereas increased FPF and reduced  $\Delta\Psi\text{m}$  occurred in HRPE cells after only 1 hour of incubation with 50, 100, and 200  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  (Fig. 1). The same trend was seen in HRPE cells incubated with unstimulated or IFN- $\gamma$ -stimulated monocytes, as increased FPF and reduced  $\Delta\Psi\text{m}$  was present as early as 1 hour after treatment, but there was no apoptosis at that time (Fig. 3, data not shown). Apoptosis did not occur until HRPE cells were exposed to IFN- $\gamma$ -stimulated monocytes for 4 hours or unstimulated monocytes for 24 hours, with significantly greater apoptosis occurring in response to stimulated monocytes, corroborating previous findings (Yoshida et al., 2003; Yang et al., 2009). Additionally, increased levels of apoptosis correlated with the more substantial increases in FPF levels and reductions in  $\Delta\Psi\text{m}$  seen at higher concentrations or longer incubations of  $\text{H}_2\text{O}_2$ .

In other cell types, impairment of enzymatic complexes II and IV of the electron transport chain and reductions in  $\Delta\Psi\text{m}$  occur within one hour of  $\text{H}_2\text{O}_2$  exposure, before apoptosis commences (Long et al., 2004). Interestingly, electron transport complex II is involved in the redox status of flavin adenine dinucleotide (FAD) that, when oxidized, contributes to the FPF signal. Thus, changes in the mitochondrial electrochemical gradient caused by impaired electron transport and reduced  $\Delta\Psi\text{m}$  likely result in oxidation of FAD and other flavoproteins. Therefore, FPF and  $\Delta\Psi\text{m}$  are early indicators of instability, even in cells exposed to low levels of oxidants that are insufficient to cause apoptosis, while more substantial alterations of these indicators are predictive of evolving apoptosis that appears at later time points.

Increases in FPF levels and apoptosis in HRPE cells (Elner et al., 2008a; Field et al., 2009b) and human and rat neural retina (Fig. 2A–B) subjected to lethal levels of  $\text{H}_2\text{O}_2$  were significantly inhibited by NAC-antioxidant treatment. In fact, NAC-antioxidant treatment resulted in FPF levels that were significantly below those of control rat neural retina not exposed to  $\text{H}_2\text{O}_2$  (Fig. 2A). Although not statistically significant, this trend was also seen in HRPE cell (Elner et al., 2008a; Field et al., 2009b) and human neural retina (Fig. 2A) FPF levels following NAC-antioxidant treatment. However, NAC did not reduce apoptosis levels back to those of HRPE cells (Field et al., 2009b) and human neural retina (Fig 2B) not exposed to  $\text{H}_2\text{O}_2$ . One explanation may be that  $\text{H}_2\text{O}_2$  was not completely neutralized by NAC and caused substantial cell death before the 24 hour measurements, resulting in fewer live cells to emit the FPF signal. This would imply that clinical quantitative measurement of FPF would be a more accurate indicator of retinal health in early, but not advanced disease. We also tested, but saw no significant decrease in FPF or apoptotic levels of control HRPE cells following treatment with NAC (data not shown).

In this study, we verified that HRPE cells co-cultured with monocytes induce gene and protein expression of pro-inflammatory chemokines, *IL8* and *MCPI* (Figs. 4 and 5, Table 1) (Yoshida et al., 2001a; Bian et al., 2004). Additionally, we showed that *IL8* and *MCPI* gene and protein expression increased well before apoptosis occurred, as early as 1–2 hours after HRPE cell co-culture with unstimulated monocytes, and correlated with increases in FPF levels and reductions in  $\Delta\Psi\text{m}$  (Figs 3–5). One possible explanation for prompt HRPE

chemokine induction is the high levels of ROM induced by monocyte-binding to mouse or human RPE cells (Yoshida et al., 2003; Yang et al., 2009), because oxidative stressors have been shown to induce *IL8* and *MCP1* production in other cell types (Zeng et al., 2003; Kina et al., 2009). Interestingly, production of HRPE *MCP1* was significantly inhibited by NAC while that of *IL8* was not (data not shown).

This study demonstrates that increased FPF levels in HRPE cells exposed to various stressors correlates with reductions in  $\Delta\Psi_m$  and upregulation of proinflammatory cytokines, which can all occur well before apoptosis. Current methods of clinical retinal disease assessment are only effective after a large number of cells have been lost to apoptotic cell death. However, cells exhibit disease-related mitochondrial dysfunction well before they are destined to die. Therefore, the ability to non-invasively monitor the relative health of mitochondria rapidly with FPF represents a significant opportunity to diagnose, monitor, and treat the underlying disease.

FPF has previously been used in combination with nicotinamide nucleotide (NAD(P)H) fluorescence to create a ratio that is useful for real-time *in vitro* quantification of the mitochondrial redox status of many different cell types, including corneal, pancreatic islet, and mesenchymal stem cells (Masters, 1984; Masters et al., 1989; Rocheleau et al., 2004; Tsubota et al., 1998; Reyes et al., 2006; Park et al., 2006). This ratio serves as an indicator of the mitochondrial redox status because NAD(P)H oxidation correlates with FP oxidation but the fluorescence of the two molecules is inversely related. This method may also be used for *in vivo* experimental measurements and has been used on rabbit hearts and rodent brains during surgery to assess mitochondrial functional changes due to electrical stimulation and ischemia/reperfusion injury (Reinert et al., 2004; Shibuki et al., 2003; Shino et al., 1998; Ranji et al., 2006; Rajpurohit et al., 1999). Recently, Cano et al., 2008, used this technique to show that cultured HRPE cells incubated with increasing concentrations of  $H_2O_2$  or tert-butyl hydroperoxide had declining NAD(P)H fluorescence and increased FPF, indicative of impaired oxidative phosphorylation, oxidant-induced injury, and cell death.

While measuring NAD(P)H fluorescence, in addition to FPF, is useful to assess changes in mitochondrial redox status and function, NAD(P)H fluorescence cannot be used on human eyes because the excitation ultraviolet wavelengths for NAD(P)H have phototoxic effects on ocular cells, including those of the retina. As a result, we have developed a clinical instrument that non-invasively assesses human retinal FPF. Excitation wavelengths for FPF can be phototoxic to the retina with prolonged exposures, but clinical FPF imaging uses exposures to excitatory blue light that are at least 100 times less than the International Electrotechnical Commission (IEC) maximum permissible energy safety threshold for light damage (International Electrotechnical Commission, 2007). Using this method, we have shown that FPF increases due to increased FP oxidation in eyes of humans with pseudotumor cerebri, diabetes, central serous retinopathy, age-related macular degeneration and retinitis pigmentosa (Elner et al., 2008a; Elner et al., 2008b; Field et al., 2008; Field et al., 2009a). As indicated by our previous work and our data in this study, FPF may prove to be a translational tool to safely, rapidly and non-invasively assess retinal mitochondrial function *in vitro*, *ex vivo*, and *in vivo*.

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## References

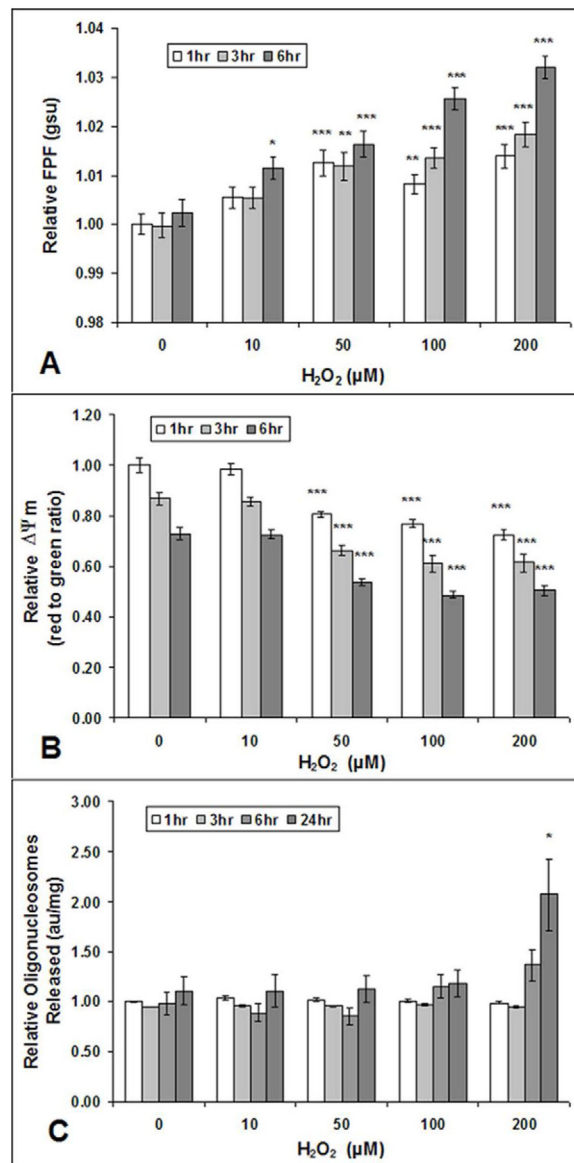
- Armstrong JS, Whiteman M, Yang H, Jones DP, Sternberg P Jr. Cysteine starvation activates the redox-dependent mitochondrial permeability transition in retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 2004; 45:4183–89. [PubMed: 15505073]
- Ballinger SW, Van Houten B, Jin GF, Conklin CA, Godley BF. Hydrogen peroxide causes significant mitochondrial DNA damage in human RPE cells. *Exp Eye Res*. 1999; 68:765–772. [PubMed: 10375440]
- Barak A, Morse LS, Goldkorn T. Ceramide: A potential mediator of apoptosis in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 2001; 42:247–254. [PubMed: 11133876]
- Benson RC, Meyer RA, Zaruba ME, McKhann GM. Cellular autofluorescence—is it due to flavins? *J Histochem Cytochem*. 1979; 27:44–48. [PubMed: 438504]
- Bian ZM, Elnor SG, Yoshida A, Elnor VM. Differential involvement of phosphoinositide 3-kinase/Akt in human RPE MCP-1 and IL-8 expression. *Invest Ophthalmol Vis Sci*. 2004; 45:1887–1896. [PubMed: 15161854]
- Bian ZM, Elnor SG, Yoshida A, Elnor VM. Human RPE-monocyte co-culture induces chemokine gene expression through activation of MAPK and NIK cascade. *Exp Eye Res*. 2003; 76:573–583. [PubMed: 12697421]
- Bian ZM, Elnor VM, Yoshida A, Kunkel SL, Elnor SG. Signaling pathways for glycated human serum albumin-induced IL-8 and MCP-1 secretion in human RPE cells. *Invest Ophthalmol Vis Sci*. 2001; 42:1660–1668. [PubMed: 11381075]
- Bian ZM, Elnor SG, Elnor VM. Dual involvement of caspase-4 in inflammatory and ER stress-induced apoptotic responses in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 2009; 50:6006–6014. [PubMed: 19643964]
- Cao W, Tombran-Tink J, Chen W, Mrazek D, Elias R, McGinnis JF. Pigment epithelium-derived factor protects cultured retinal neurons against hydrogen peroxide-induced cell death. *J Neurosci Res*. 1999; 57:789–800. [PubMed: 10467250]
- Cano MV, Reyes JM, Park CY, Gao X, Mori K, Chuck RS, Gehlbach PL. Demonstration by redox fluorometry that sulforaphane protects retinal pigment epithelial cells against oxidative stress. *Invest Ophthalmol Vis Sci*. 2008; 49:2606–2612. [PubMed: 18515589]
- Chance B, Schoener B, Oshino R, Itshak F, Nakase Y. Oxidation-reduction ratio studies of mitochondria in freeze-trapped samples. *J Biol Chem*. 1979; 254:4764–4771. [PubMed: 220260]
- Elnor SG, Elnor VM, Field MG, Park S, Heckenlively JR, Petty HR. Retinal flavoprotein autofluorescence as a measure of retinal health. *Trans Am Ophthalmol Soc*. 2008; 106:215–224. [PubMed: 19277237]
- Elnor VM, Park S, Cornblath W, Hackel R, Petty HR. Flavoprotein autofluorescence detection of early ocular dysfunction. *Arch Ophthalmol*. 2008; 126:259–260. [PubMed: 18268219]
- Elnor VM, Strieter RM, Elnor SG, et al. Neutrophil chemotactic factor (IL-8) gene expression by cytokine-treated retinal pigment epithelial cells. *Am J Pathol*. 1990; 136:745–750. [PubMed: 2183623]
- Field MG, Elnor VM, Puro DG, Feuerman JM, Musch DC, Pop-Busui R, Hackel R, Heckenlively JR, Petty HR. Rapid, non-invasive detection of diabetes-induced retinal metabolic stress. *Arch Ophthalmol*. 2008; 126:934–938. [PubMed: 18625939]
- Field MG, Elnor VM, Park S, Hackel R, Heckenlively JR, Elnor SG, Petty HR. Detection of retinal metabolic stress due to central serous retinopathy. *Retina*. 2009; 29:1162–1166. [PubMed: 19491721]
- Field MG, Elnor VM, Feuerman JM, Heckenlively JR, Petty HR. Potential Causes of Altered Autofluorescence in Diabetic Persons—Reply. *Arch Ophthalmol*. 2009; 127:943–945.
- International Electrotechnical Commission. IEC 60825-1. 2.0. 2007. Safety of laser products – Part 1: Equipment classification and requirements.
- Jiang S, Moriarty-Craige SE, Orr M, Cai J, Sternberg P Jr, Jones DP. Oxidant-induced apoptosis in human retinal pigment epithelial cells: dependence on extracellular redox state. *Invest Ophthalmol Vis Sci*. 2005; 46:1054–1061. [PubMed: 15728565]

- Jin GF, Hurst JS, Godley BF. Hydrogen peroxide stimulates apoptosis in cultured human retinal pigment epithelial cells. *Curr Eye Res.* 2001; 22:165–173. [PubMed: 11462152]
- Kannan R, Jin M, Gamulescu MA, Hinton DR. Ceramide-induced apoptosis: role of catalase and hepatocyte growth factor. *Free Rad Biol Med.* 2004; 37:166–175. [PubMed: 15203188]
- Kina S, Nakasone T, Takemoto H, Matayoshi A, Makishi S, Sunagawa N, Liang F, Phonaphonh T, Sunakawa H. Regulation of chemokine production via oxidative pathway in HeLa cells. *Mediators Inflamm.* 2009:183760. Epub 2010 Jan 27. [PubMed: 20150959]
- Lieven CJ, Vrabec JP, Levin LA. The effects of oxidative stress on mitochondrial transmembrane potential in retinal ganglion cells. *Antioxid Redox Sign.* 2003; 5:641–646.
- Long X, Goldenthal MJ, Wu GM, Marin-Garcia J. Mitochondrial  $\text{Ca}^{2+}$  flux and respiratory enzyme activity decline are early events in cardiomyocyte response to  $\text{H}_2\text{O}_2$ . *J Mol Cell Cardiol.* 2004; 37:63–70. [PubMed: 15242736]
- Masters BR. Noninvasive corneal redox fluorometry. *Curr Top Eye Res.* 1984; 4:139–200. [PubMed: 6375985]
- Masters BR, Ghosh AK, Wilson J, Matschinsky FM. Pyridine nucleotides and phosphorylation potential of rabbit corneal epithelium and endothelium. *Invest Ophthalmol Vis Sci.* 1989; 30:861–868. [PubMed: 2722442]
- Park CY, Zhu Z, Zhang C, Moon CS, Chuck RS. Cellular redox state predicts in vitro corneal endothelial cell proliferation capacity. *Exp Eye Res.* 2006; 83:903–910. [PubMed: 16806172]
- Rajpurohit R, Mansfield K, Ohyama K, Ewert D, Shapiro IM. Chondrocyte death is linked to development of a mitochondrial membrane permeability transition in the growth plate. *J Cell Physiol.* 1999; 179:287–296. [PubMed: 10228947]
- Ranji M, Kanemoto S, Matsubara M, et al. Fluorescence spectroscopy and imaging of myocardial apoptosis. *J Biomed Opt.* 2006; 11:064036. [PubMed: 17212559]
- Reinert KC, Dunbar RL, Gao W, Chen G, Ebner TJ. Flavoprotein autofluorescence imaging of neuronal activation in the cerebellar cortex in vivo. *J Neurophysiol.* 2004; 92:199–211. [PubMed: 14985415]
- Reyes JM, Fermanian S, Yang F, et al. Metabolic changes in mesenchymal stem cells in osteogenic medium measured by autofluorescence spectroscopy. *Stem Cells.* 2006; 24:1213–1317. [PubMed: 16439616]
- Rocheleau JV, Head WS, Piston DW. Quantitative NAD(P)H/flavoprotein autofluorescence imaging reveals metabolic mechanisms of pancreatic islet pyruvate response. *J Biol Chem.* 2004; 279:31780–1787. [PubMed: 15148320]
- Ryu SY, Peixoto PM, Teijido O, Dejean LM, Kinnally KW. Role of mitochondrial ion channels in cell death. *BioFactors.* 2010; 36:255–263. [PubMed: 20623547]
- Sano Y, Furuta A, Setsuie R, Kikuchi H, Wang YL, Sakurai M, Kwon J, Noda M, Wada K. Photoreceptor cell apoptosis in the retinal degeneration of Uchl3-deficient mice. *Am J Pathol.* 2006; 169:132–141. [PubMed: 16816367]
- Shibuki K, Hishida R, Murakami H, et al. Dynamic imaging of somatosensory cortical activity in the rat visualized by flavoprotein autofluorescence. *J Physiol.* 2003; 549:919–927. [PubMed: 12730344]
- Shiino A, Matsuda M, Handa J, Chance B. Poor recovery of mitochondrial redox state in CA1 after transient forebrain ischemia in gerbils. *Stroke.* 1998; 29:2421–2425. [PubMed: 9804657]
- Tsubota K, Laing RA, Chiba K, Hanninen LA, Kenyon KR. Non-invasive metabolic analyses of preserved rabbit cornea. *Arch Ophthalmol.* 1998; 106:1713–1717. [PubMed: 3196214]
- Yang D, Elnor SG, Lin LR, Reddy VN, Petty HR, Elnor VM. Association of superoxide anions with retinal pigment epithelial cell apoptosis induced by mononuclear phagocytes. *Invest Ophthalmol Vis Sci.* 2009; 50:4998–5005. [PubMed: 19458341]
- Yang D, Elnor SG, Clark AJ, Hughes BA, Petty HR, Elnor VM. Activation of P2X Receptors Induces Apoptosis in Human Retinal Pigment Epithelium. *Invest Ophthalmol Vis Sci.* 2011a; 52:1522–1530. [PubMed: 21071745]
- Yang D, Elnor SG, Chen X, Field MG, Petty HR, Elnor VM. MCP-1-activated monocytes induce apoptosis in human retinal pigment epithelium. *Invest Ophthalmol Vis Sci.* 2011b in press. 10.1167/iovs.10-7023

- Yoshida A, Elnor SG, Bian ZM, Kindezeliskii AL, Petty HR, Elnor VM. Activated monocytes induce human retinal pigment epithelial cell apoptosis through caspase-3 activation. *Lab Invest.* 2003; 83:1117–1129. [PubMed: 12920241]
- Yoshida A, Elnor SG, Bian ZM, Kunkel SL, Lukacs NW, Elnor VM. Differential chemokine regulation by TH2 cytokines during human RPEmonocyte coculture. *Invest Ophthalmol Vis Sci.* 2001; 42:1631–1638. [PubMed: 11381071]
- Yoshida A, Elnor SG, Bian ZM, Kunkel SL, Lukacs NW, Elnor VM. Thrombin regulates chemokine induction during human retinal pigment epithelial cell/monocyte interaction. *Am J Pathol.* 2001; 159:1171–1180. [PubMed: 11549610]
- Yoshida A, Yoshida S, Khalil AK, Ishibashi T, Inomata H. Role of NF-kappaB- mediated interleukin-8 expression in intraocular neovascularization. *Invest Ophthalmol Vis Sci.* 1998; 39:1097–1106. [PubMed: 9620068]
- Zeng X, Dai J, Remick DG, Wang X. Homocysteine mediated expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human monocytes. *Cir Res.* 2003; 93:311–320.

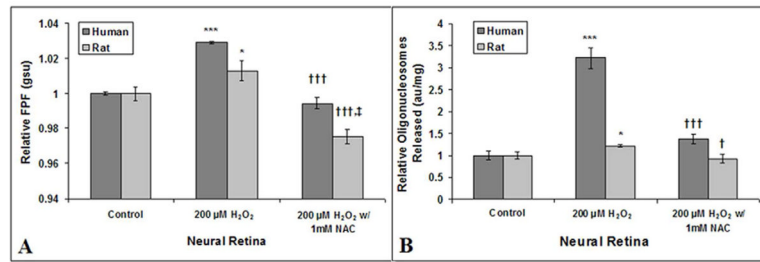
### Highlights

- Flavoprotein fluorescence is an indicator of mitochondrial dysfunction
- Oxidative stress and monocytes increase retinal flavoprotein fluorescence
- Increased flavoprotein fluorescence correlates with increased chemokine expression
- Flavoprotein fluorescence is an early indicator of cells prone to undergo apoptosis



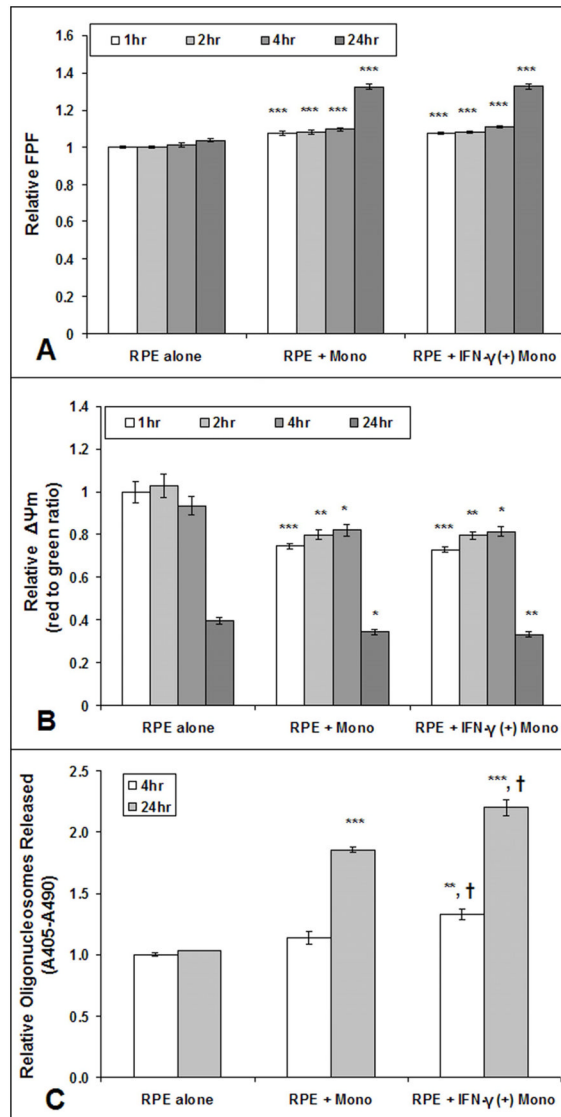
**Fig. 1. FPF,  $\Delta\Psi_m$ , and apoptosis in cultured human RPE cells after exposure to H<sub>2</sub>O<sub>2</sub>**  
 Increased FPF (A) and reduced  $\Delta\Psi_m$  (B) at 1, 3, and 6 hours after cultured human RPE cell exposure to various H<sub>2</sub>O<sub>2</sub> concentrations. Human RPE cells did not show increased levels of apoptosis (released mono- and oligonucleosomes) measured by Cell Detection ELISA, until 24 hours after exposure to 200 μM H<sub>2</sub>O<sub>2</sub> (C). Values are mean (standard error). \*P<.05, \*\*\*P<.001, compared with corresponding control (0 μM H<sub>2</sub>O<sub>2</sub>). Each experiment was conducted on 3 different cell lines, n=9.





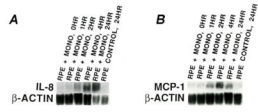
**Fig. 2. Ex vivo FPF assessment of human and rat neural retina mitochondrial stress and correlation with apoptosis**

FPF (A) and apoptotic cell death (B) of human (n=4) and rat neural retina (n=12) incubated with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), with or without N-acetylcysteine (NAC). Values are mean (standard error). \*P<.05, \*\*\*P<.001, compared with control; †<.05, ††<.001, compared with H<sub>2</sub>O<sub>2</sub>-treated cells; ‡<.01, compared with control; gsu indicates grayscale units; au, arbitrary units.

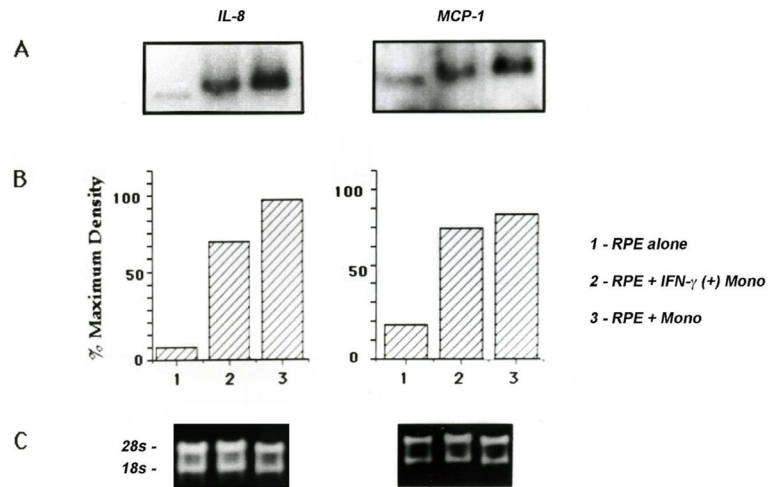


**Fig. 3. FPF,  $\Delta\Psi_m$ , and apoptosis in cultured human RPE cells after exposure to unstimulated or IFN- $\gamma$ -stimulated (+) monocytes**

Increased FPF (A) and reduced  $\Delta\Psi_m$  (B) at 1, 2, 4 and 24 hours after cultured human RPE cell exposure to unstimulated or IFN- $\gamma$ -stimulated (+) monocytes. Human RPE cells did not show increased levels of apoptosis (released oligonucleosomes) measured by Cell Detection ELISA, until 4 hours after exposure to IFN- $\gamma$ -stimulated (+) monocytes or until 24 hours after exposure to unstimulated monocytes(C). Values are mean (standard error). \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , compared with control RPE alone. † $P < .05$ , compared with RPE + unstimulated monocytes. Each experiment was conducted on 3 different cell lines,  $n=9$ .



**Fig. 4. *IL8* and *MCP1* gene expression in HRPE cells exposed to unstimulated monocytes**  
Northern blot analysis of HRPE *IL8* (A) and *MCP1* (B) mRNA expression in HRPE cells exposed to unstimulated monocytes for 0, 1, 2, 4, and 24 hours. Unstimulated HRPE cells were used as control. The data are representative of experiments performed 3 times on each of 3 different cell lines, n=9.



**Fig. 5. *IL8* and *MCP1* gene expression in HRPE cells exposed to unstimulated or IFN- $\gamma$ -stimulated monocytes**

Northern blot analysis of HRPE *IL8* and *MCP1* mRNA expression in HRPE cells exposed to unstimulated or IFN- $\gamma$ -stimulated monocytes for 24 hours (A, B). Unstimulated HRPE cells were used as control. Equivalent total cellular RNA loading per lane is shown by electrophoretic profile of 18s and 28s RNA (C). The data are representative of experiments performed 3 times on each of 3 different cell lines, n=9.

**Table 1**

Gene (RT-PCR) and protein (ELISA) expression in HRPE cells exposed to monocytes.

	Cell Culture	IL-8	MCP-1
RT-PCR <sup>a</sup>	RPE + MONO, 4HR	15.3	6.1
	RPE + MONO, 24HR	86.2	9.5
ELISA <sup>b</sup>	RPE + MONO, 0HR	0.73 ± 0.13	0.3 ± 0.02
	RPE + MONO, 1HR <sup>c</sup>	1.23 ± 0.02 (p=0.019)	1.26 ± 0.14 (p=0.0022)
	RPE + MONO, 4HR <sup>c</sup>	6.45 ± 0.62 (p=0.00082)	2.49 ± 0.06 (p=0.000047)
	RPE + MONO, 24HR <sup>c</sup>	99.80 ± 8.54 (p=0.00021)	26.29 ± 0.69 (p=0.00040)
	RPE + IFN-γ (+) MONO, 24 HR <sup>c</sup>	48.61 ± 3.32 (p=0.00032)	15.80 ± 4.04 (p=0.000029)

<sup>a</sup>Fold increase is compared to RPE + MONO, 0HR

<sup>b</sup>Values are mean concentrations (ng/mL) ± standard error

<sup>c</sup>p-value is compared with control RPE + MONO, 0HR

The data are representative of experiments performed 3 times on each of 3 different cell lines, n=9.