Current Eye Research
2001, Vol. 23, No. 1, pp. 11–19

0271-3683/01/2301-011$16.00

Rod outer segments mediate mitochondrial DNA damage and apoptosis in human retinal pigment epithelium

Gui-Feng Jin, John S. Hurst and Bernard F. Godley

Department of Ophthalmology and Visual Sciences, University of Texas Medical Branch, Galveston, USA

Abstract

Purpose. To investigate the interrelationships between DNA damage, mitochondrial activity, and apoptosis in retinal pigment epithelial cells (RPE) after exposure to rod outer segments (ROS).

Methods. After incubation of cultured human RPE with ROS, mitochondrial redox function was evaluated from MTT reduction. Mitochondrial (mt) and nuclear (n) DNA damage were determined by quantitative polymerase chain reactions (QPCR). Apoptotic RPE cells were detected by binding of annexin V to phosphatidyl serine (PS) using fluorescence microscopy. The expression of the pro-apoptotic proteins, p53 and $p21^{waf\text{-}1}$, and DNA repair enzymes, apurinic/apyrimidinic endonuclease ($APE^{ref\text{-}1}$) and DNA polymerase β (β-pol) were quantitatively determined by Western blotting analysis.

Results. Mitochondrial function decreased by 20 ± 5% and annexin V immunofluorscent binding was enhanced after exposure of cells to physiological levels of ROS ($3.8 \times 10^6\,cm^{-2}$) for 4 h. MtDNA was preferentially damaged after exposure to ROS with increased lesion frequencies of 1.49 ± 0.37 and 2.2 ± 0.14 per 10 kb base pairs (bp), respectively after 5 and 7 h contact, compared to untreated controls (zero class damage). $APE^{ref\text{-}1}$ expression increased more than 340% above controls after exposure to ROS for 7 and 24 h. The expression of β-pol in cultures increased 110% above controls after 24 h contact with the ROS. The expression of p53 and p21 in cells increased 100 and 38% above controls after 24 h exposure to the ROS.

Conclusions. Exposure of ROS to ROS induced mtDNA damage and dysfunction and activated nDNA repair pathways, which did not prevent apoptosis.

Keywords: DNA damage; mitochondrial dysfunction; oxidative stress; retinal pigment epithelium; rod outer segments

Introduction

In developed societies age-related macular degeneration (ARMD) predominates, as the cause of irreversible profound vision loss in people aged over 60 years, and in the future will consume a major portion of finite health resources as the aging population expands. It is a multifactorial syndrome with genetic and environmental contributions. It is hypothesized that lifelong chronic cumulative oxidative stress constitutes a significant risk factor.[1–5] The pathobiological changes that culminate in the loss and/or dysfunction of macular RPE cells are believed to be central to the development and progression of this disease.

The RPE regulates nutrient and metabolite exchange to the neural retina including the photoreceptors and is essential to their survival. Consequently degeneration of the postmitotic RPE cells will result in the death of neighboring photoreceptors. Each RPE cell contacts 8–10 photoreceptor cells, and each photoreceptor cell sheds approximately 20–30 of its outer segment discs resulting in the daily phagocytosis by each RPE cell of more than 2000 photoreceptor outer segment discs.[6] The retinal environment favors the generation of reactive oxygen intermediates (ROI) as photoreceptors and RPE cells are metabolically highly reactive. The neural retina, especially the photoreceptor outer segment membranes, are enriched in polyunsaturated fatty acids, such as docosahexaenoic, which are susceptible to attack and degradation by ROI. In addition, the retina is continuously exposed to sunlight, especially hazardous blue light, has high

Received: January 22, 2001
Accepted: June 15, 2001

Correspondence: Bernard F. Godley, MD, PhD, FACS, Department of Ophthalmology and Visual Sciences, University of Texas Medical Branch, Galveston, TX 77555-0787, USA. Tel: (409) 772-28107, Fax: (409) 772-28106, E-mail: bgodley@utmb.edu

levels of molecular photosensitizers, and is subjected to high oxygen tension. Thus phagocytosis of ROS could augment oxidative stress to the RPE cells.

During photoreceptor outer segment phagocytosis, the RPE generate ROI such as superoxide[7] and hydrogen peroxide (H_2O_2).[8] It has been suggested that increased phagocytic and metabolic burden imposed upon the RPE in the accumulation of age-related lipofuscin, derived in part from oxidatively damaged lipids and proteins, are implicated in RPE and photoreceptor death.[9]

Previously we have shown that hydrogen peroxide preferentially damages mtDNA, disrupts mt metabolic function[10] and promotes apoptosis in RPE cells (submitted). To study the interaction of the RPE with photoreceptor outer segments, cultured human RPE cells were exposed ROS for varying times. The effects upon RPE mt and nDNA damage were reported by sensitive QPCR assays. As aging and ROI are known to damage DNA,[11–13] especially mtDNA[14–16] by forming adducts, and generating mutagenic abasic sites, it would also be important to study the effects of ROS on the expression of the DNA base excision repair enzymes (BER), apurinic/apyrimidinic endonuclease ($APE^{ref\text{-}1}$) and DNA polymerase β (β-pol), as it has been reported that these enzymes are upregulated as an adaptive response to oxidative stress[17,18] and cooperate in DNA repair.[19] We wanted to study potential interactions between factors promoting DNA repair and apoptosis in RPE cells, to determine if BER upregulation followed exposure of the cells to ROS, and also whether this could influence their survival. $APE^{ref\text{-}1}$ and β-pol expression was analyzed by Western blotting. Mitochondrial redox function was quantitated by 3-[4,5-dimethyliazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reduction. Apoptosis was qualitatively determined from phosphatidyl serine (PS) externalization as suggested by increased immunofluorescent binding of annexin V to PS, and the expression of the pro-apoptotic and/or cell cycle growth arrest nuclear transcription factors p53 and $p21^{waf\text{-}1}$ were analyzed by Western blotting.

Materials and methods

Reagents

Electrophoretic-grade reagents: sodium chloride, sodium deoxycholate, sodium dodecyl sulfate (SDS), ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), NP-40, polyacrylamide, Tris, Tween-20, and non-fat dry milk were obtained from Biorad Laboratories (Hercules, CA). Nitrocellulose membranes were obtained from Micron Separations (Westborough, MA). Antibodies raised to $p21^{waf\text{-}1}$ (monoclonal), and p53 (polyclonal), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies raised to β-pol, and $APE^{ref\text{-}1}$ were the gifts of Dr. S. H. Wilson, Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, TX. The electrochemiluminescence (ECL) Western Blotting kit including horse-radish peroxidase-conjugated secondary antibodies were obtained from Amersham-Pharmacia Biotechnology (Piscataway, NJ). Goat anti-mouse and anti-rabbit antibodies were used respectively with primary monoclonal and polyclonal antibodies. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Promega (Madison,WI). Diethylenediaminetetraacetic acid (EDTA) dimethyl sulfoxide (DMSO) and sucrose were purchased from Sigma-Aldrich Co. (St. Louis, MO). Bradford reagent was purchased from Biorad (Hercules, CA). ApoAlert® Kits were obtained from Clontech Laboratories, Inc., Palo Alto, CA.

RPE cell cultures

SV40-transformed fetal male human RPE cells (RPE 28 SV4, Coriell Institute, Camden, NJ) were cultured as previously described.[10] The rationale for using this established and well-documented cell line was as follows. A disadvantage with primary cell cultures derived from different donors is that the cellular response to H_2O_2 may lack consistency, which would not be a concern with a well-defined cell line, such as the one used in our studies. These transformed cells exhibit epithelioid morphology and retain physiological functions characteristic of primary human RPE cells, such as polarity and the ability to phagocytize rod outer segments. Additional studies also indicated that both primary and transformed cell lines respond similarly to oxidative stress.[20–23]

Isolation of rod outer segments (ROS)

ROS were aseptically isolated from fresh bovine eyes obtained locally (Dereck and sons, Inc., Santa Fe, TX) using a discontinuous sucrose density gradient.[24] Ocular globes were sectioned and the neural retinae were removed into 43% sucrose dissolved in 10 mM TRIS-buffered saline (TBS, pH 7.0) containing 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, and 0.1 mM EDTA and thoroughly vortexed and centrifuged at 5000 g for 5 min. The supernatants were diluted with TBS and centrifuged at 27 000 g for 15 min. The supernatants were discarded and the precipitates were suspended in TBS containing 33% sucrose. The sucrose content of the suspensions were determined by refactometry and was adjusted to 32.5% with added 45% sucrose solution. The sucrose was overlaid with TBS to create a gradient and the samples were centrifuged at 16 500 g for 2 h. The interphases between the two layers were collected by syringe aspiration and the ROS were counted using a hemocytometer chamber and were stored at –80°C till use.

MTT assay

The MTT reduction assay was used as the index for mitochondrial redox function. Cells were seeded onto 96-well plates at a density of 12 000 cells per well. The medium was replaced 48 h later with one that was serum-free containing varying numbers of ROS ($0.95–15 \times 10^6\,cm^{-2}$). Cells were

exposed to ROS for 4 h, then the media containing the ROS were removed and the cells were washed with phosphate buffered saline (PBS). The cells were then incubated with serum-free medium containing 4.83 µM MTT for 4 h at 37°C. Then 150 µl DMSO was added to each well and the plates were shaken for 10 min. The supernatants were removed into fresh multi-well plates and the optical densities of the solutions were read at 590 nm[25] using a scanning multi-well spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ). Absorbance values were converted to MTT reduction as previously described.[10] Values were normalized with respect to the untreated control cultures (100% mitochondrial redox function). Determinations were performed in duplicate and experiments were repeated (n = 4).

DNA damage

QPCR was used to determine the average lesion frequency per DNA strand in specified sequences for both template strands.[10,26,27] Only intact DNA sequences were amplified and lesions including strand breaks, apurinic sites and base modifications would prevent amplification. To control for template copy number differences, and damage unrelated to experimental conditions, DNA sequences of approximately 200 kb base pairs were quantitatively amplified. Total RPE cellular DNA was isolated using a genomic tip 20G kit (Quiagen, Inc., Valencia, CA) according to the instructions. Total DNA concentrations were determined by ethidium bromide fluorescence using an A4-filter fluorimeter with an excitation band pass filter at 360 nm and emission cut-off filter at 600 nm (Optical Devices, Elmsford, NY) using λ/Hind III DNA as standard. Sample quality was assessed by QPCR of a 222 base pair mtDNA fragment and an 84 base pair fragment of the β-globin gene as previously described.[10] QPCR was performed in a GeneAmp PCR system 9600 with the GeneAmp XLPCR kit (Perkin-Elmer, San Jose, CA). Reaction mixtures contained 15 ng genomic DNA as template. The composition and sequences of the primers and the PCR conditions employed for the 16.2 kb mtDNA, 13.4 kb β-globin, 12.2 kb β-pol and the 10 kb hprt products were as previously described.[10] A quantitative control using 7.5 ng genomic template was included in every PCR series. PCR products were electrophoretically resolved on 1% agarose gels (dissolved in TBE buffer, Amresco, Solon, OH) at 80–90 v for 4 h. Dried gels were exposed to phosphor screens for 12 h and quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). DNA lesion frequencies (λ) were calculated as the amplification of damaged DNA relative (A_d) to non-damaged controls (A_0), where $\lambda = -\ln A_d/A_0$. Experiments to assess DNA damage in genomic and mtDNA were determined 4 times in duplicate.

Phosphatidyl serine exposure

RPE cells were placed on a microscope slide and exposed to $3.8 \times 10^6\,cm^{-2}$ ROS for 4 h. Cells were washed in PBS and then the binding buffer supplied with the ApoAlert® kit. 5 µl annexin V conjugated to fluoroisothiocyanate (FITC) and 10 µl propidium iodide were added and the cells were covered with a glass coverslip and incubated with the dyes for 10–15 min in the dark. The cells were viewed under a fluorescence microscope, using a dual filter set for FITC and rhodamine. Apoptotic RPE cells stained with FITC-Annexin V fluoresced green, whereas necrotic cells stained with propidium iodide fluoresced red. The experiment was repeated (n = 2).

Western blot analysis

Cultured RPE cells (~90% confluent) exposed to $3.8 \times 10^6\,cm^{-2}$ for different times were lysed by RIPA buffer (1 mM Tris-HCl, sodium deoxycholate, NaCl, EGTA, 1% NP-40.[28] Cell lysates corresponding to 50 µg protein, determined according to Bradford,[29] and pre-stained molecular weight markers were loaded onto 1% SDS, 10–15% polyacrylamide gels under reducing conditions. Following electrophoretic separation, the proteins were transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk and washing in 10 mM Tris-buffered saline, pH 7.5, containing, 0.1% Tween-20, (T-TBS) the membranes were incubated with the appropriately diluted antibodies to, $APE^{ref\text{-}1}$, β-pol, $p21^{waf\text{-}1}$ or p53 for 3 h at room temperature. The primary antibodies were decanted and the membranes were washed with TBS. The membranes were then incubated for 2 h at room temperature with horseradish peroxidase conjugated secondary antibodies (goat anti-mouse for the monoclonal p53 and goat anti-rabbit for the polyclonal $APE^{ref\text{-}1}$, β-pol, P53, and $p21^{waf\text{-}1}$ antibodies) in T-TBS diluted to the same concentrations as the primary antibodies. The membranes were washed with TBS and incubated with the ECL substrate for 1 min and then exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY). The processed gel images were analyzed using a phosphoimager with the supplied ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Analyses for each protein were repeated (n = 2).

Statistical analysis

Data for the QPCR and MTT are presented as the means ± standard deviation (SD). The Students *t*-test was used for statistical evaluation between experimental groups. When the *p* values were <0.05, values were considered significant.

Results

Mitochondrial dysfunction

Mitochondrial redox function as assayed by the MTT test is generally considered an index for cellular survival. Mitochondrial MTT reduction in cells treated for 4 h with 3.8, 7.6, and $15 \times 10^6\,cm^{-2}$ ROS was decreased by 20, 36, and 46%,

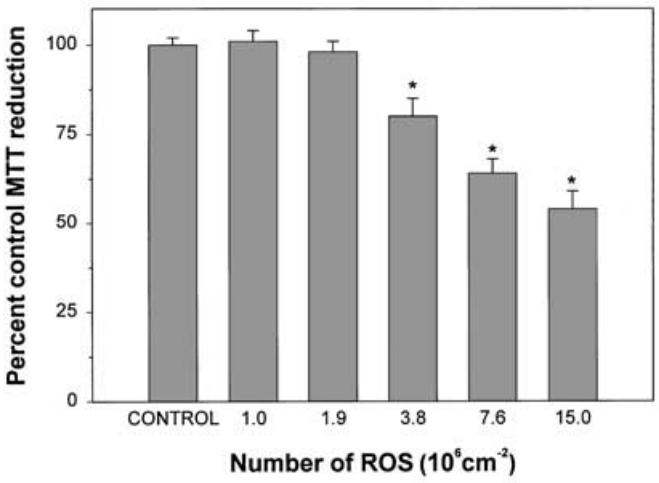

Figure 1. Effect of rod outer segments on mitochondrial redox function in RPE cells.

Histogram showing the effects of different concentrations of rod outer segments (ROS) on RPE mitochondrial redox function assayed by MTT reduction. RPE were exposed to the indicated concentrations of bovine ROS for 4 h at 37°C. After exposure, cells were rinsed with PBS and incubated with serum-free media containing 4.83 µM MTT. Media were removed, the cells lysed, and absorbance determined at 570 nm. MTT reduction was determined as a percentage of untreated controls. Data are expressed as means ± SD from 4 experiments. Each experiment was performed in duplicate.

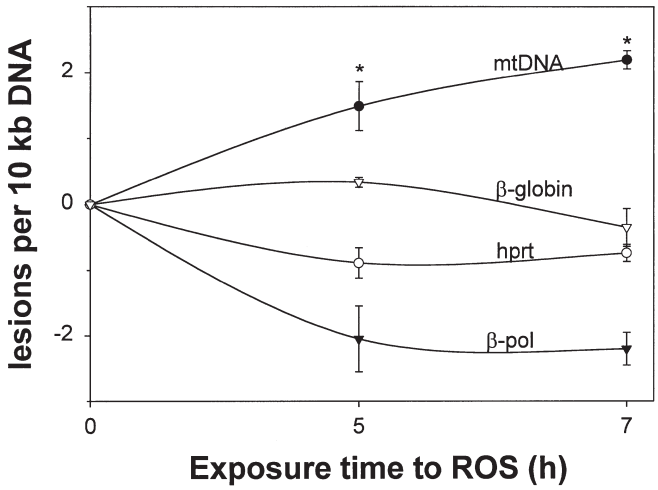

Figure 2. Time course of DNA damage in RPE cells after exposure to rod outer segments.

RPE cells were exposed to $3.8 \times 10^6\,cm^{-2}$ ROS for indicated times. Lesions per 10 kb base pairs nuclear (n) and mitochondrial (mt) DNA were determined by quantitative polymerase chain reactions (QPCR), using the Poisson equation. Untreated controls were considered zero class damage. Data are expressed as the means ± SD from 4 experiments. Each experiment was performed in duplicate.

nDNA is represented by genes coding for β-globin, hprt and DNA polymerase β (β-pol).

respectively (Fig. 1). This indicated a loss of mt redox function or dysfunction as a possible consequence of mtDNA damage after exposure to ROS.

Mitochondrial DNA damage

RPE cells were treated with $3.8 \times 10^6\,cm^{-2}$ ROS to determine whether ROS exposure could compromise either mt or nDNA integrity in the RPE cells. This was assessed from lesion frequency in the relative amplification of mt and nDNA sequences. MtDNA damage significantly increased with increased exposure time to ROS. After 5 and 7 h treatment, mtDNA lesion frequencies increased from 0 ± 0.0015 (control RPE cells not exposed to ROS) to 1.49 ± 0.39 and 2.2 ± 0.14 per 10 kb base pairs, respectively (Fig. 2). Whereas nDNA, represented by the three genomic gene clusters, β-globin, β-pol, and hprt was resistant to ROS-induced damage and did not suffer increased lesion frequency (Fig. 2). Moreover, lesion frequency in β-pol was less after 5 and 7 h exposure to ROS, suggesting an increased rate of repair of pre-existing lesions.

PS exposure

RPE cells were treated with $3.8 \times 10^6\,cm^{-2}$ ROS for 4 h to determine if PS externalization, an apoptotic marker, followed. Annexin V binding to exposed PS was detected by fluorescence microscopy[30] as shown in Figures 3A and B. Low levels of Annexin V and propidium iodide fluorescence are shown in control cells not exposed to ROS (Fig. 3A). Whereas Annexin V labeling as shown by green fluorescence was noticeably increased in cells treated with ROS (Fig. 3B), suggesting an increase in apoptotic death after interaction with the ROS.

Effect of ROS exposure on levels of BER genes

Oxidative stress is known to upregulate levels of BER enzymes, such as APE[ref-1], and β-pol. Therefore experiments were conducted to determine if levels of these enzymes increased in RPE cells after exposure to ROS. Levels of APE[ref-1] were increased in RPE cells after 5, 7, and 24 h exposure times to $3.8 \times 10^6\,cm^{-2}$ ROS (Figs. 4A and B). After 5 h, there was a greater than twofold increase above control values. After 7 h, expression was 344% above control values. A similar increase (360% above control values) was also observed in cells treated with ROS for 24 h. The Western blot is representative of 2 determinations from different cell cultures.

Levels of β-pol was increased twofold above control values in cells exposed to $3.8 \times 10^6\,cm^{-2}$ ROS for 24 h (Figs. 5A and B). The Western blot is representative of two determinations from different cell cultures.

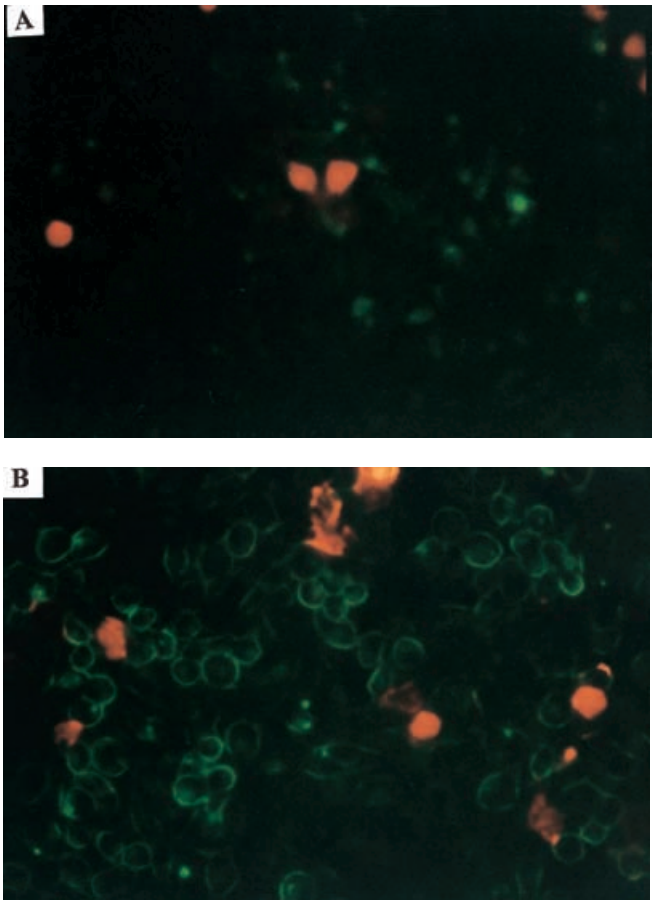

Figure 3. Phospatidyl serine exposure in RPE cell plasma membranes after exposure to ROS.

Photomicrographs showing phosphatidyl serine (PS) externalization in cultured RPE cells exposed to $3.8 \times 10^6\,cm^{-2}$ ROS for 4 h. Cells were immunofluorescently-labeled with propidium iodide, PI, (orange, necrotic cells) and annexin V (green, PS-labeled apoptotic cells). A Untreated control cells, with isolated necrotic and apoptotic cells are shown in (A). ROS-exposed cells with higher levels of apoptotic cells, as indicated by increased green immunofluorescent binding of Annexin V to exposed PS are shown in (B). Photomicrographs are representative of 2 experiments. Magnification was 200×.

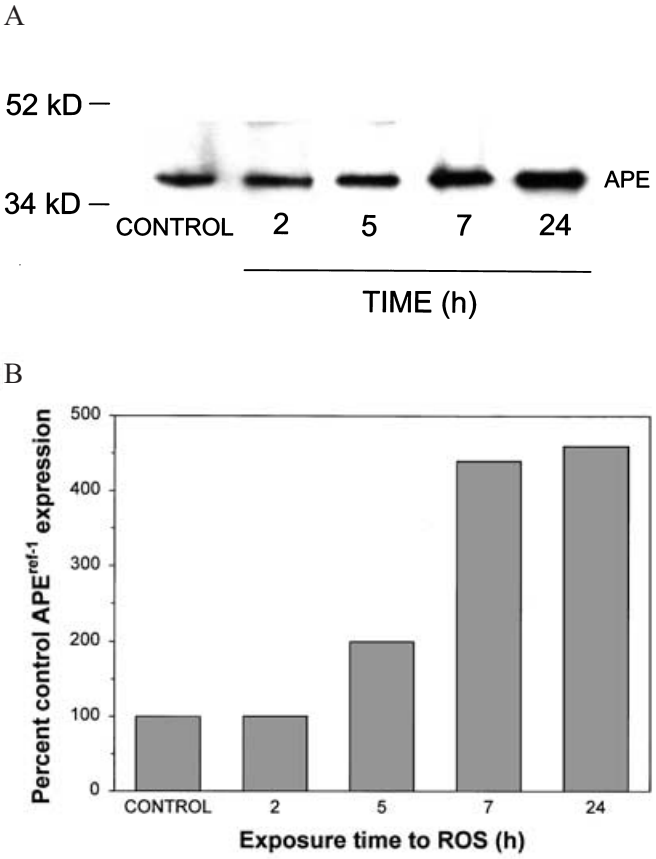

Figure 4. Time course of $APE^{ref\text{-}1}$ expression in RPE cells exposed to ROS.

RPE cells were exposed to $3.8 \times 10^6\,cm^{-2}$ ROS for the indicated times. Protein expression was analyzed by Western blot. Each gel lane (A) was loaded with 50 µg protein. Control lanes represent protein expression in untreated cells. The densitometric analysis of (A) is shown in (B) and represents the mean of 2 experiments.

P53 and $p21^{waf\text{-}1}$ expression

Increases in levels of p53 and $p21^{waf\text{-}1}$ are associated with apoptosis, therefore the influence of ROS exposure on the levels of these proteins in RPE cells was studied. The expression of p53 (Figs. 6A and B) and p21 (Figs. 7A and B) after exposure to $3.8 \times 10^6\,cm^{-2}$ were followed in cultured RPE cells as a function of time. Expression of p53 and $p21^{waf\text{-}1}$ was only affected after 24 h of exposure, when it increased 100 and 38% respectively, above the control values, suggesting that extracellular contact of RPE cells with ROS increased levels of both proteins and that p53 did not apparently induce $p21^{waf\text{-}1}$ synthesis. The Western blots for both proteins were performed twice for each time-point and the data shown represents the averaged values obtained from two different experiments.

Discussion

Oxidative stress is proposed as a major contributory factor in the degeneration of aging RPE cells.[1,4] It is also believed that an increased rate of apoptotic cell death in the RPE layer could mediate and hasten the onset of ARMD.[31] This hypothesized mechanism as an etiological factor for ARMD has recently been supported by the confirmed presence of apoptotic RPE cells in retinal specimens of ARMD patients.[32] Mitochondria are recognized as pivotal mediators of apoptotic signaling[33] and mtDNA is more susceptible to damage from ROI than nDNA because it lacks non-coding introns and protective histones, but especially because it is in close proximity to high levels of ROI generated in the mt membranes. The relatively low capacity to repair mtDNA has been correlated with apoptosis[34] and oxidant-induced damage to mtDNA is at least three times more extensive and more sus-

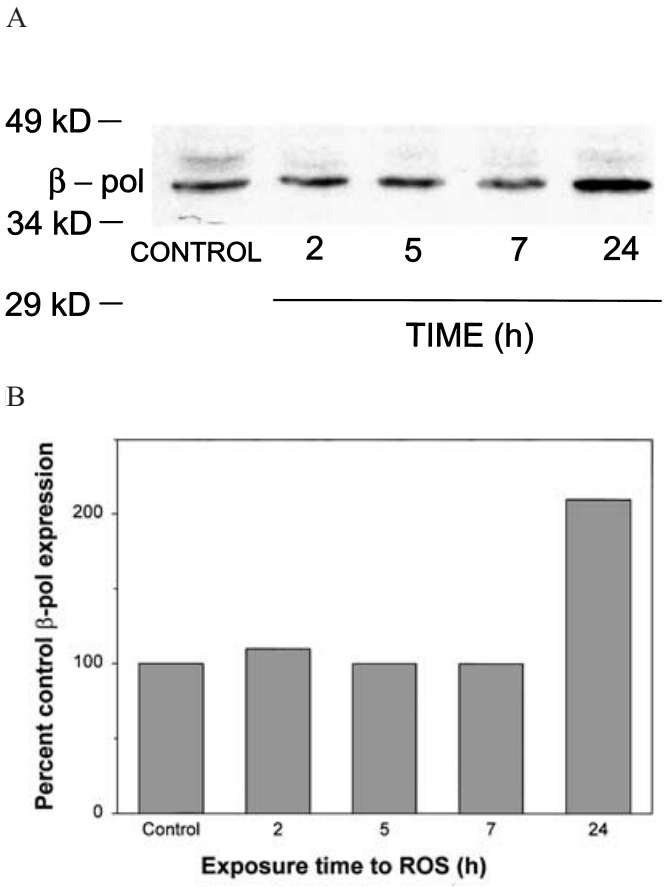

Figure 5. Time course of DNA polymerase β (β-pol) expression in RPE cells exposed to ROS.

RPE cells were exposed to $3.8 \times 10^6\,cm^{-2}$ ROS for the indicated times. Protein expression was analyzed by Western blot. Each gel lane (A) was loaded with 50 µg protein. Control lanes represent protein expression in untreated cells. The densitometric analysis of (A) is shown in (B) and represents the mean of 2 experiments.

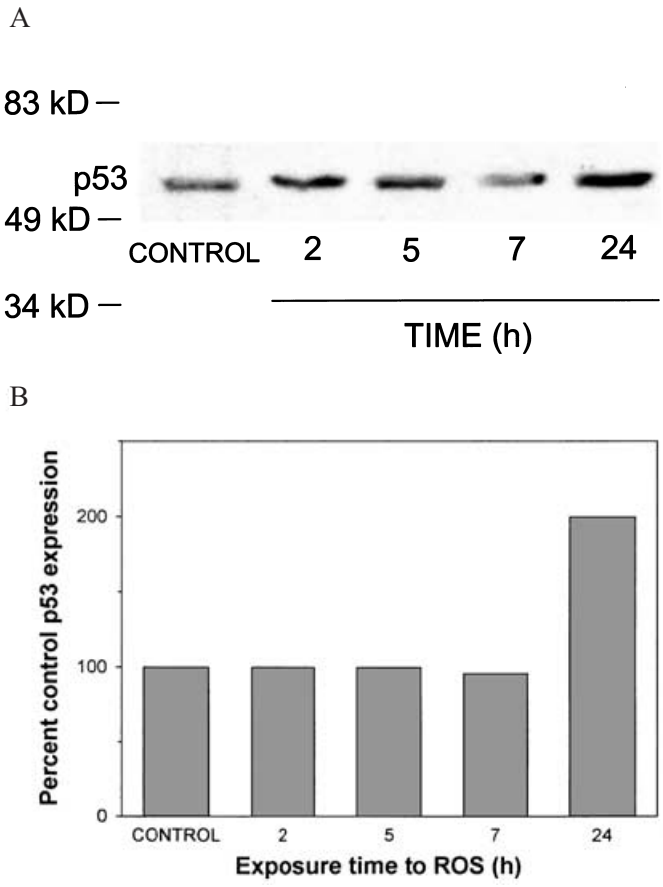

Figure 6. Time course of p53 expression in RPE cells exposed to ROS.

RPE cells were exposed $3.8 \times 10^6\,cm^{-2}$ ROS for the indicated times. Protein expression was analyzed by Western blot. Each gel lane (A) was loaded with 50 µg protein. Control lanes represent protein expression in untreated cells. The densitometric analysis of (A) is shown in (B) and represents the mean of 2 experiments.

tained than that to nDNA.[35] Our previous studies reported mitochondrial redox dysfunction with consequential mtDNA damage[10] and apoptosis in RPE cells in response to the prooxidant, H_2O_2 (unpublished data).

The daily phagocytosis of spent photoreceptor outer segment discs, enriched in polyunsaturated fatty acids, by the RPE has been identified as a major source of ROI, such as superoxide[7] and H_2O_2.[8] The present studies indicate that this interaction of cultured RPE with ROS could also trigger mitochondrial metabolic dysfunction that correlates with mtDNA damage and also with apoptosis, as suggested by the QPCR assays and the enhanced immunofluorescent binding of annexin V to externalized PS. The increased expression of the pro-apoptotic and cell cycle arrest-growth proteins, p53 and $p22^{waf\text{-}1}$, in the RPE cells further support the evidence for apoptosis after contact with ROS. However, despite some similarities in responses by the RPE cells at the molecular level to both H_2O_2 and ROS, there were also significant qualitative and quantitative differences. For example, the response time courses in the RPE cells differed with H_2O_2 or ROS treatments. Generally, a delay was observed in cells exposed to ROS when compared to the effects of H_2O_2. Mitochondrial redox activity in RPE cells exposed to ROS was inhibited by 20% after 4 h, which was similar to that in cells exposed to 50–100 µM H_2O_2 after only 1 h.[10] The lesion frequency in the RPE mtDNA with the ROS treatments after 5 or 7 h exposure was greater than that previously reported for 200 µM H_2O_2 after 1 h[10] and the compensatory response in the transcriptionally active gene β-pol was also increased.

Low levels of the ROI, hypochlorite and H_2O_2, have been previously reported to activate and induce $APE^{ref\text{-}1}$ in hamster ovarian and HeLa cells after 3 to 9 h lag periods, suggesting involvement of multiple signaling intermediates.[17,36] Our data also showed that $APE^{ref\text{-}1}$ was also activated/induced in RPE cells after exposure to ROS for 7 and 24 h. $APE^{ref\text{-}1}$ is a multifunctional protein that not only repairs DNA but also regulates the DNA-binding of redox-regulated transcription

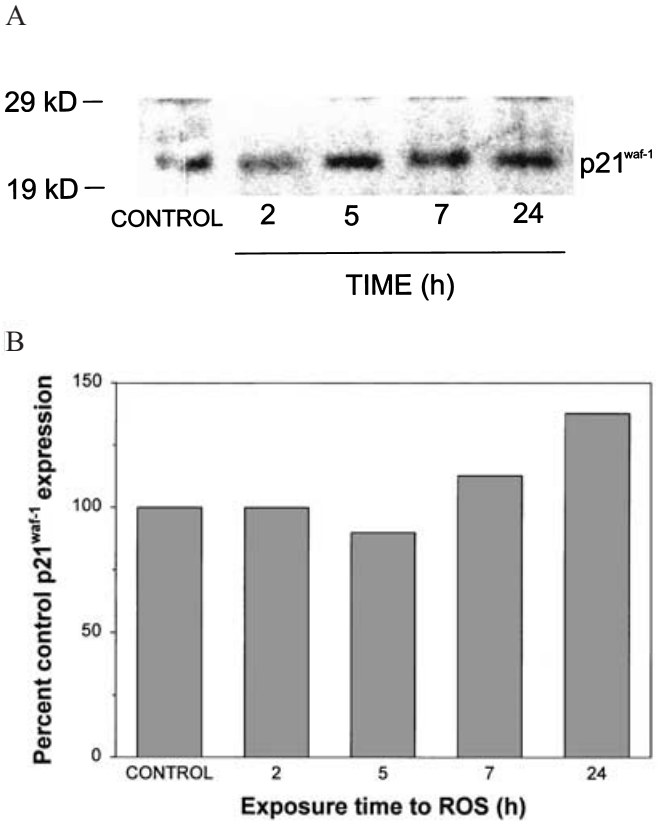

Figure 7. Time course of $p21^{waf-1}$ expression in RPE cells exposed to ROS.

RPE cells were exposed to $3.8 \times 10^6\,cm^{-2}$ ROS for the indicated times. Protein expression was analyzed by Western blot. Each gel lane (A) was loaded with 50 µg protein. Control lanes represent protein expression in untreated cells. The densitometric analysis of (A) is shown in (B) and represents the mean of 2 experiments.

factors, such as nuclear transcription factor κβ, Fos, Jun, $p21^{waf-1}$ and Bax, etc.[37] and also modulates the activation of p53.[38] In addition, β-pol was also activated/induced after 24 h ROS treatment. Previous studies such as those of Chen *et al.* have reported the upregulation of β-pol in murine monocytes and fibroblasts in response to oxidative-stress-inducing agents including H_2O_2.[18] Regarding this observation therefore, it may be significant that the reported specific interaction of APE^{ref-1} and β-pol is coordinated to assemble the polymerase onto potentially mutagenic abasic nDNA sites to facilitate base excision repairs.[19] However, despite these late adaptive responses to oxidative stress in the cultured RPE cells after exposure to ROS, the putative early pro-apoptotic reactions such as cytochrome *c* and PS migrations already committed some susceptible cells to an activated apoptotic program from which they could not be rescued. Interestingly, Chen *et al.* recently showed that oxidized low density lipoprotein (LDL) down-regulates β-pol in murine monocytes.[39] Oxidized lipids and proteins including LDL are believed to be present in the ROS daily phagocytized by the RPE and constitute part of the drusen and other extracellular deposits that accumulate with age and may be implicated in ARMD progression. The upregulation of β-pol seen in the RPE cultures exposed to ROS may represent a short-term adaptation to oxidative stress and may not reflect the long-term chronic *in vivo* response which might be different and may result in β-pol down regulation with consequently accelerated cell death.

PS exposure, detected as enhanced fluorescent labeling, was evident in cultures treated for only 1 h with 50–200 µM H_2O_2, whereas it was apparent only after 4 h exposure with ROS. This implies that the oxidative stress that followed ROS contact was delayed compared to direct H_2O_2 exposure, and/or that H_2O_2 release during ROS phagocytosis could only in part account for the resultant apoptosis.

Expression of p53, known to be an potent cell death effector after activation by mtDNA damage, was increased in RPE cultures above constitutively-expressed levels only after protracted exposure (24 h) to ROS, unlike the rapid increase observed after 1 h H_2O_2 treatments (unpublished data). It is possible that this represents the previously demonstrated stimulation of p53 activity by redox-dependent and independent activities of APE^{ref-1} in HeLa cell.[38] Therefore, APE^{ref-1} may function as a critical sensor of genotoxic oxidative stress in RPE and other cells and may play an important role in switching the cell cycle program from survival to apoptosis. In the ROS model of oxidative stress, increased p53 activity did not induce $p21^{waf-1}$ expression, as it is unlikely that the modest increases in $p21^{waf-1}$ levels (38% above control values) could be attributed to upregulation of its gene activity by p53 protein. This contrasted with the ninefold increase in $p21^{waf-1}$ protein expression observed in RPE cells, which correlated with raised p53 protein levels after treatment with 200 µM H_2O_2 (unpublished data). Evidently therefore, apoptosis induced by ROS in the RPE cells was largely a $p21^{waf-1}$-independent process. Experimental evidence from studies with the $P21^{waf-1}$ null-mutant mouse demonstrate that p53-promotes apoptosis in the absence of $p21^{waf-1}$ [40,41] support our hypothesis that the apoptosis of RPE cells induced by p53 is not mediated via the p21 pathway. The mechanisms by which p53 is believed to induce apoptosis independent of the p21 pathway is by stimulation of a burst of ROI on the mitoichondrial membrane that releases the triggers of the effector pathway of apoptosis.[42] β-pol is reported to be upregulated by $p21^{waf-1}$.[43] However, it is unlikely that the induction of β-pol was due to the modest increase in $p21^{waf-1}$ levels seen in our study.

The marked contrasts in the modulation of apoptotic activity by H_2O_2 and ROS most probably reflect different ROI species. H_2O_2 participates in ROS-induced apoptosis of RPE cells, but from our studies may not be the major promoter. It is very possible that ROS lipid hydroperoxides and their aldehydic end products, such as 4-hydroxynonenal (HNE) and 4-hydroxyhexenal, are more actively involved. Arachidonate-derived lipid hydroperoxides, such as the-15 isomer are potent inducers of apoptosis in cell lines derived from T lymphoctytes[44] and human retinoblastoma cells[45] and

also prevent inhibition of apoptosis by Bcl-2 overexpression.[44] Mitochondrial dysfunction and apoptosis in PC12 and neuronal cells have been reported as consequences of 4-HNE exposure.[46]

In conclusion, our studies show that upregulation of nDNA repair enzymes in response to oxidative stress is correlated with the increased expression of nuclear transcription factors that promote apoptosis rather than arrested cell growth.

Acknowledgments

This study was supported by the Paul Beeson Physician Aging Research Program, the Research to Prevent Blindness, Inc., and by Grant EY12850-01 from the National Institutes of Health.

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