Oxidative Stress Affects the Junctional Integrity of Retinal Pigment Epithelial Cells

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PURPOSE. Oxidative stress has been implicated in the pathogenesis of age-related macular degeneration. The cell line ARPE-19 was therefore examined for response to oxidative stress and its effect on stress protein induction and junctional integrity.

METHODS. ARPE-19 cell viability after 1 week or 5 weeks in culture was assessed in response to different concentrations of hydrogen peroxide. The response to sublethal doses was assessed by examination of heme oxygenase (HO)-1, Hsp27 and Hsp70 by immunofluorescence and Western blot analysis. Immunofluorescence was used to investigate the localization of the junctional proteins zona occludens (ZO)-1, occludin, and N-cadherin, and β-catenin. Subcellular fractionation was used to assess any redistribution of β-catenin. Monolayer integrity was examined by measurement of flux of rhodamine-conjugated dextrans from the apical to basal aspect of cells.

RESULTS. ARPE-19 cells cultured for 5 weeks were less sensitive to chronic oxidative stress induced by hydrogen peroxide than those cultured for 1 week. The more differentiated ARPE-19 cells had higher steady state levels of Hsp27 and Hsp70. The response to stress also differed with time in culture. The localization of junctional proteins, which became strongly peripheral after 5 weeks in culture, became disrupted after oxidative stress, and cytosolic β-catenin increased. Chronic oxidative stress also increased paracellular flux across the monolayer.

CONCLUSIONS. Increased resistance to chronic oxidative stress with differentiation in ARPE-19 cells correlated with higher steady state levels of Hsp27 and Hsp70. Oxidative stress disrupted RPE cell junction and barrier integrity, which may contribute to the pathogenesis of diseases related to RPE through disruption of the blood–retinal barrier. (Invest Ophthalmol Vis Sci. 2004;45:675–684) DOI:10.1167/iovs.03-0351

The retinal pigment epithelium (RPE) is responsible for the regulated exchange of nutrients and metabolites between the retina and the choriocapillaris, as well as for aspects of the maintenance of photoreceptor cells. There is currently much interest in the possibility that oxidative stress brings about changes in the RPE and subretinal layers associated with aging of the retina and age-related macular degeneration (AMD). 1,2 High blood flow through the choriocapillaris and the phagocytosis of spent photoreceptor components by the RPE results in the generation of hydrogen peroxide: 3 This and the potentially photosensitizing properties of RPE lipofuscin contribute to what may be a sustained source of oxidative stress throughout life; a burden that may be enhanced by acute or chronic pathologic insults. Direct evidence for oxidative damage in the vicinity of RPE during aging has come from studies demonstrating an accumulation of advanced glycation end products with age in Bruch’s membrane. 5

Several studies have used RPE cell lines to investigate the response of these cells to oxidative stress. Specifically, treatment with hydrogen peroxide or tert-butyl hydroperoxide (TBH) has been shown to affect the expression of Hsp70 6,7 metallothionein and catalase, 8 FGF2, 9 and FGF receptors. 9 Hydrogen peroxide treatment has also been shown to result in decreased expression of the RPE markers RPE-65 and cellular retinaldehyde-binding protein (CRALBP). 9

The formation of the blood–retinal barrier (BRB) by the RPE is dependent on the function of tight junctions to create a restricted diffusion barrier to nontransported solutes. The localization of the tight junction proteins ZO-1 and occludin at apical membranes has been demonstrated in the RPE of late-stage chick embryos. 11 A near continuous belt of ZO-1 around the apical aspect of the cells has also been described in ARPE-19 and other RPE cultured cell lines. 11 ZO-1 also has functions additional to those in tight junctions, such as associations with adherens junctions, and therefore permeability of tight junctions cannot be predicted on the pattern of expression of ZO-1 alone. 12 Adherens junctions facilitate intercellular adhesion, a process that also plays a key role in maintaining tissue integrity and the normal morphology of RPE cells. 13 In cultured RPE, N-cadherin is the major cadherin responsible for mediating development of an epithelial phenotype. 14

A variety of factors have been described as affecting integrity and function of tight junctions in epithelial cells. For example, hepatocyte growth factor (HGF) treatment of RPE results in rapid disassembly of both adherens and tight junctions through loss and redistribution of their protein components. 15 Rapid loss of ZO-1 was found to occur before loss of occludin and claudins and was associated with rapid tyrosine phosphorylation. HGF has also been shown to disrupt the association between adherens junction proteins to allow mesenchymal transformation of RPE. 15 The mechanism by which this occurs in RPE is unknown. In Caco-2 cells, the disruption of junctional proteins by oxidative stress is mediated through kinases such as c-Src, which are activated and translocated to the cell membrane, accompanying tyrosine phosphorylation of ZO-1 and β-catenin in conjunction with their translocation to the cytoplasm. 16 This suggests that factors affecting tight junctions are likely to affect adherens junctions through a pathway that results in disruption of monolayer integrity.

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Supported by the Community Fund and Fight For Sight.

Submitted for publication April 7, 2003; revised September 29, 2003; accepted October 19, 2003.

Disclosure: T.A. Bailey, None; N. Kanuga, None; I.A. Romero, None; J. Greenwood, None; P.J. Luthert, None; M.E. Cheetham, None

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Oxidative stress has been suggested as being a contributory factor in AMD, and therefore it seems likely that the generation of hydrogen peroxide through phagocytosis of spent retinal components under certain predisposing conditions may contribute to pathogenesis through disruption of the posterior BRB provided by RPE. Hydrogen peroxide treatment has been used as a source of oxidative stress with other cell types, such as bovine pulmonary artery endothelial (BPAE) cells, and has been shown to induce gap formation and internalization of cadherins. Similarly, rearrangement of occludin and its dissociation from ZO-1 by peroxide treatment of human umbilical vein endothelial cells (HUVECs) has been demonstrated. Therefore, we sought to examine in RPE cells the disruptive effect of hydrogen peroxide exposure on proteins of tight and adherens junctions, as has been seen in other cell types. The effect of hydrogen peroxide on stress protein induction was also investigated. We studied both acute (30 minute) and chronic (24 hour) oxidative stress and investigated the differential vulnerability of cells that had been cultured for 1 week or 5 weeks. We hypothesized that cells grown for a longer period would be less vulnerable to both short- and long-term stress and that there might be discernible reasons for this variation.

**Materials and Methods**

**Cell Culture**
All experiments were performed using ARPE-19, a human diploid RPE cell line, which is in many ways similar to RPE in vivo. All cell culture media were obtained from Life Technologies (Paisley, UK) and the other reagents from Sigma-Aldrich (Poole, UK), unless otherwise stated. Before experimentation, cells were seeded at approximately $0.5 \times 10^5$/cm² and maintained in culture for 1 week or 5 weeks before seeding, unless otherwise stated. All cultures were fed twice weekly with Dulbecco’s modified Eagle’s medium (DMEM); nutrient mixture F12, plus 10% (vol/vol) fetal bovine serum, 2 mM l-glutamine, and penicillin-streptomycin to 100 IU/mL. Cultures were maintained at 37°C in 5% (vol/vol) CO₂. For oxidative stress experiments, hydrogen peroxide was added to the culture medium, with acute treatment being for 30 minutes in absence of serum before replacement with fresh medium containing serum, and chronic treatment being for 24 hours in medium containing serum. Cell viability was assessed by counting cells excluding trypan blue. For prior serum-withdrawal experiments, cells were grown in serum-free medium for 48 hours treated with hydrogen peroxide treated and cell viability measured as described. As a positive control for response to stress, cells were treated with hydrogen peroxide treated and cell viability measured as described.9 As a positive control for response to stress, cells were treated with hydrogen peroxide treated and cell viability measured as described.9 As a positive control for response to stress, cells were treated with hydrogen peroxide treated and cell viability measured as described.9 As a positive control for response to stress, cells were treated with hydrogen peroxide treated and cell viability measured as described.9 As a positive control for response to stress, cells were treated with hydrogen peroxide treated and cell viability measured as described.9 As a positive control for response to stress, cells were treated with hydrogen peroxide treated and cell viability measured as described.9 As a positive control for response to stress, cells were treated with hydrogen peroxide treated and cell viability measured as described.9 As a positive control for response to stress, cells were treated with hydrogen peroxide treated and cell viability measured as described.9 As a positive control for response to stress, cells were treated with hydrogen peroxide treated and cell viability measured as described.9 As a positive control for response to stress, cells were treated with hydrogen peroxide treated and cell viability measured as described.9 As a positive control for response to stress, cells were treated with hydrogen peroxide treated and cell viability measured as described.9

**Epithelial Permeability**
Cells were seeded on to 0.4 µM pore polyester membrane filters (Transwell; Corning Costar, Acton, MA) at approximately 0.5 $\times 10^5$/cm² and maintained in culture for 1 week or 5 weeks, changing the media above and below the filters twice weekly. Filters without cells were also maintained as the control. Rhodamine isothiocyanate (RITC)-dextran (RD70) was dissolved in serum-free medium to a concentration of 1 mg/mL. For assessment of epithelial permeability in response to oxidative stress, cells were first incubated with the desired concentration of hydrogen peroxide 2 hours before addition of RITC-dextran. Basal medium was replaced with serum-free medium, and medium with RITC-dextran was then added to the apical side containing hydrogen peroxide, as indicated. At each time point, 50 µL of medium was removed from the basal side of the filter and diluted in PBS before measurement of fluorescence with a fluorometer at an excitation wavelength of 570 nm and emission wavelength of 595 nm (Safire; Tecn, Mannedorf, Switzerland). Controls included medium from cell-free filters, RITC-dextran alone, and cells treated with RITC-dextran without hydrogen peroxide. At each time point, medium removed from the basal side of the filter was replaced with fresh serum-free medium to maintain volume.

**Reverse Transcription–Polymerase Chain Reaction**
Total RNA was isolated from cells with an extraction kit (RNeasy; Qiagen, Crawley, UK), according to the manufacturer’s instructions, and was quantified by spectrophotometry. RT-PCR was performed with a reverse transcription system (Promega, Madison, WI), using conditions recommended by the manufacturer. PCR was stopped in the exponential phase of the reaction to allow semiquantitative comparison of product levels. Samples were checked for integrity and equalized by use of RT-PCR with primers for β-actin with 25 cycles to detect product in the exponential phase. For RT-PCR for RPE-65, 35 cycles of PCR were necessary to detect product in the exponential phase. The primers and annealing temperatures were identical to those previously used by our group.11

**Antibodies**
Primary antibodies used for Western blot analysis and immunofluorescence were mouse monoclonals for HO-1, OSA-110; Hsp-27, SPA-800; Hsp70, SPA-810 (Stressgen, San Diego, CA); β-tubulin, Tub 2.1 (mouse ascites; Sigma-Aldrich); rabbit polyclonal for ZO-1, 61-7300: occludin, 71-1500 (Zymed Laboratories Inc., S. San Francisco, CA); and mouse monoclonals for N-cadherin, 610920; β-catenin, 610653; (Transduction Laboratories, Lexington, KY); and CRALBP, B2 (a kind gift of John C. Saari, University of Washington, Seattle, WA). FITC-conjugated secondary antibodies (Dako, Cambridge, UK) were used for immunofluorescence, and horseradish peroxidase (HRP)-conjugated antibodies (Sigma-Aldrich) were used for Western blot analysis.

**Western Blot Analysis**
Cells were lysed in 20 mM Tris-HCl (pH 8.0), 4 M NaCl, 0.5 M EDTA, 0.5 mM CHAPS (3-[3-cholamidopropyl] dimethylammonio)-l-propanesulfonate; Sigma-Aldrich) and sheared with a fine-gauge needle. Cytosolic and membrane fractions were produced according to a previously published method.20 Protein content was quantified with a protein assay (Bio-Rad, Hercules, CA) with a bovine serum albumin (BSA) standard. Electrophoresis by SDS-PAGE was followed by transfer to nitrocellulose membranes (Schleicher and Schuell, Dassell, Germany). Membranes were blocked in 5% (wt/vol) low-fat milk powder (Marvel; Premier Brands UK Ltd., Moreton, UK) in PBS plus 0.1% (vol/vol) Tween 20 (PBST; Sigma-Aldrich) before addition of primary antibody in blocking solution for 1 hour at room temperature or overnight at 4°C. Secondary antibodies were also diluted into blocking solution. PBST was used for washing between steps. Detection was with chemiluminescence (ECL plus; Amersham Biosciences, Little Chalfont, UK). Immunoreactive bands were quantified and relative sum intensity of bands compared using image analysis software (Kodak Digital Science, 1D Image Analysis Software, Eastman Kodak Co., Rochester, NY). For comparison of Western blot results, the mean result of four experiments was taken, with results from heat shock or hydrogen peroxide treatment being normalized to the control.

**Immunofluorescence**
Cells were fixed and permeabilized by incubation with ice-cold methanol for 20 minutes, and blocked by 30 minutes with 3% (wt/vol) BSA (Sigma-Aldrich) in PBS which included 1% (vol/vol) serum from the animal in which the appropriate secondary antibody was raised. Primary and secondary antibodies were also diluted in this blocking solution. For visualization of actin, Alexa Fluor 594 or Oregon Green 488 phalloidin (Molecular Probes, Leiden, The Netherlands) was used at a 1:200 dilution. PBS was used for washing between steps. Cells
were mounted with fluorescent mounting medium (Dako). Images were acquired by confocal microscope (LSM 510; Carl Zeiss Meditec, Oberkochen, Germany).

RESULTS

Effect of Oxidative Stress on ARPE-19 Cell Viability

ARPE-19 cells that had been maintained in culture for 1 week or 5 weeks were treated with a range of concentrations of hydrogen peroxide, for 30 minutes in the absence of serum (acute treatment) or for 24 hours in the presence of serum (chronic treatment), and cell viability was measured after 24 hours by trypan blue exclusion counts (Fig. 1A). After 24-hour chronic treatment, the viability of 1-week cells was dramatically reduced, and few viable cells remained at concentrations above 3 mM hydrogen peroxide. Observations were also made at 4 hours, at which time some viable cells remained in the 1-week cultured cells at up to 5 mM of chronic treatment with hydrogen peroxide (data not shown). Acute treatment of 1-week cells had a much lesser effect on cell viability, with more than 85% of cells viable after 24 hours at 5 mM hydrogen peroxide. After 5 weeks in culture, more than 60% of the ARPE-19 cells were still viable, even after 24 hours of chronic treatment with 5 mM hydrogen peroxide, and viability was not significantly reduced at any treatment point. The data from these experiments were used to select 0.5 mM hydrogen peroxide for use in further experiments as a sublethal chronic dose that had little effect on cell viability at either time point of cell culture.

To assess the rate of loss of cell viability with chronic oxidative stress, cells at 1 week after seeding were treated with 5 mM hydrogen peroxide for a range of times, and viability counts were performed at 24 hours. These data showed that the chronic effect manifested when cells were treated for 2 hours or longer (Fig. 1B).

These data appeared to be contradictory to those obtained in a previous study by Alizadeh et al.,9 which showed that 3-month cultures of ARPE-19 cells were more sensitive to a 30-minute exposure to hydrogen peroxide than were 3-day cultures. One of the main differences between our experimental paradigm and that of Alizadeh et al. was that they included a period of serum withdrawal before oxidative stress. Therefore, we examined the effect of prior serum withdrawal on the response to acute hydrogen peroxide treatment. Serum withdrawal increased the sensitivity of both 1-week and 5-week cultures to oxidative stress, but this effect was much more pronounced in the 5-week cultures (Fig. 1C). Serum withdrawal significantly reduced viability in the 5-week cultures (P < 0.01) to levels similar to that previously reported for 3-month cultures,9 suggesting that there was a synergistic effect between serum withdrawal and hydrogen peroxide treatment that is enhanced by increased differentiation of ARPE-19 cells.

**P < 0.01 for serum withdrawal versus serum. (**

Figure 1. (A) ARPE-19 cells were cultured for 1 week or 5 weeks after seeding before incubation with medium containing hydrogen peroxide for 24 hours (chronic treatment) or for 30 minutes in the absence of serum, after which medium was replaced with fresh medium without hydrogen peroxide for 24 hours (acute treatment). Viability was assessed using trypan blue exclusion counting and compared with an untreated control. Data are expressed as the mean ± SEM (n = 3). (B) ARPE-19 cells cultured for 1 week after seeding were incubated with medium containing 5 mM hydrogen peroxide and cell viability assessed after 0.5, 1, 2, 4, 8, and 24 hours of treatment. (C) Viability of 1-week and 5-week cells in response to 2.0 mM hydrogen peroxide treatment, with or without a period of prior serum withdrawal. **P < 0.01 for serum withdrawal versus serum. (D) RT-PCR of total RNA extracted from ARPE-19 cells after 1 week or 5 weeks for semiquantitative analysis of RPE-65. (E) Western blot for CRALBP of 1-week and 5-week ARPE-19 cell lysates.
Assessment of ARPE-19 Differentiation Status with Time in Culture

To assess whether differences in viability correlated with the acquisition of features characteristic of differentiated RPE, we performed RT-PCR for RPE-65 and Western blot for CRALBP on total RNA and cell lysates obtained from cells cultured for 1 week or 5 weeks after seeding. Some studies suggest that expression of RPE-65 and CRALBP correlates with differentiation in RPE cells.\textsuperscript{21,22} RT-PCR yielded a 390-bp product for RPE-65, which was faint in 1-week control cells, but more intense in the 5-week control cells (Fig. 1D). Similarly, Western blot of CRALBP demonstrated higher levels of the CRALBP in the 5-week than in the 1-week sample (Fig. 1E).

Characterization of Stress Protein Response by ARPE-19

As we had observed differential sensitivity to chronic oxidative stress between the 1- and 5-week cultures with respect to viability, we investigated whether there were differences in the steady state levels of stress proteins or whether there was a differential stress response of ARPE-19 at different stages of cellular differentiation. Western blot analysis was used to monitor the expression of HO-1 as a marker of oxidative stress. HO-1 could be detected only in 1-week cells exposed to 0.5 and 1 mM hydrogen peroxide (Fig. 2A). Cells treated with 0.5 mM hydrogen peroxide were also subjected to immunofluorescence using antibodies to HO-1. It was observed that there were very low expression levels in control cells at 1 week (Fig. 3A; compared with a negative control with no primary antibody, not shown), which could not be seen at 5 weeks in control cells (Fig. 3D). Although cells at both culture time points demonstrated an increase in HO-1 staining after heat shock (for 1 hour with a 2-hour recovery) or 24 hours of exposure to 0.5 mM hydrogen peroxide, the response differed, in that all 1 week cells could be seen to express HO-1 after oxidative stress (Fig. 3C), whereas at 5 weeks only a few cells were positive for HO-1 immunostaining after a 24-hour exposure to hydrogen peroxide (Fig. 3F), such that the increase in expression could not be detected by Western blot analysis (Fig. 2A).

Hsp27 and Hsp70 were used as markers of a general stress response. In each case, there was an upregulation of the constitutive levels after extended culture of the cells. This was particularly pronounced for the Hsp27 with a more than threefold increase in the 5-week as opposed to the 1-week cells (Fig. 2B). After oxidative stress, a small increase in Hsp27 was seen in the poorly differentiated (1 week) cultures with chronic exposure to hydrogen peroxide, although this was less than the increase in response to heat shock, which doubled the amount of protein detected at the same time point (Fig. 2C). No significant upregulation was observed for Hsp70 after oxidative stress in 1-week cultures (Fig. 2E). The well-differentiated (5-week cultures) showed no increase in either Hsp27 or Hsp70 after either 0.5 or 1.0 mM hydrogen peroxide, although heat shock stimulated an increase in each case, typically with at least a 50% increase after the 24-hour recovery (Figs. 2D–F).

For Hsp27, a differential response to heat shock was also demonstrated by immunofluorescence. In the poorly differentiated cells, nucleolar spots were detected (Fig. 3H) that were not seen in the more differentiated cultures, although a perinuclear immunostain appeared to be increased in some cells (Fig. 3K). In a manner similar to Hsp27, localization of Hsp70 differed between the poorly and well-differentiated cells in response to heat shock. In the poorly differentiated cells, heat shock resulted in a strong nuclear immunostain for Hsp70 (Fig. 3N), whereas at 5 weeks localization was diffuse through the nucleus (Fig. 3Q). We observed a small increase in Hsp27 staining in the poorly differentiated cells in response to 0.5 mM hydrogen peroxide (Fig. 3I), but no differences in the localization of Hsp27 were observed in the more differentiated cells (Fig. 3L). Hsp70 was unchanged in response to hydrogen peroxide at either time point (Figs. 3O, 3R).

Localization of Tight Junction Proteins in Response to Hydrogen Peroxide

To investigate the effect of hydrogen peroxide on tight junctions ZO-1 and occludin localization were assessed by immunofluorescence. One-week cells were treated with 0.5 mM hydrogen peroxide (Fig. 4) and 5-week cells were treated with 0.5 and 2.0 mM hydrogen peroxide (Fig. 5). Actin staining was used to define the general morphology of the cells. At 1 week, the cells showed some cortical actin staining accompanied by stress fibers (Fig. 4A), and by 5 weeks in culture, cortical staining predominated (Fig. 5A). Treatment with hydrogen peroxide resulted in an increase in stress fiber staining (Figs. 4B, 5B) with a concomitant reduction in cortical actin staining that was particularly pronounced in the 5-week cells (Fig. 5C). In poorly differentiated cultures, ZO-1 was becoming localized at the cell periphery (Fig. 4C, arrow), although the immunostaining was interrupted in some places and there was also some intracellular staining (Fig. 4C, asterisk). After treatment with 0.5 mM hydrogen peroxide, a treatment that has little effect on cell viability, peripheral ZO-1 became more disorganized and more intracellular (Fig. 4D). In untreated, well-differentiated cells, the localization of ZO-1 was much more strongly peripheral. Treatment with hydrogen peroxide led to clear breaks in the cell periphery staining pattern (Figs. 5E, 5F, arrow) and an increase in intracellular staining (Fig. 5E, asterisk). The breakdown in the peripheral staining pattern was more pronounced at the 2.0 mM treatment (Fig. 5F).

Immunofluorescence for occludin also showed an increase in peripheral localization with increasing time in culture (Fig. 4E compared with Fig. 5G). Again, disruption of junctional occludin staining (Figs. 4F, 5H, 5I, arrow) occurred in a dose dependent manner with hydrogen peroxide treatment, the effect being clearer in the better-differentiated cells (Figs. 5I, 5L), because the cells had a much more defined junctional staining pattern before treatment.

Localization of Adherens Junction Proteins and Response to Hydrogen Peroxide

To assess the effect of oxidative stress on adherens junctions, we also examined N-cadherin and β-catenin by immunofluorescence. In a similar manner to that observed for the tight junction proteins, localization of N-cadherin and β-catenin to the cell periphery increased with increasing differentiation (Figs. 5J, 5M compared with 4G, 4I). There was also disruption of the peripheral localization in 5-week cells on hydrogen peroxide treatment (Figs. 5K, 5L, 5N, 5O; examples of peripher-
eral breaks are denoted by arrows and intracellular staining by asterisks).

To confirm the apparent redistribution of β-catenin observed by immunocytochemistry we performed Western blot analysis of the soluble cytosolic (C) and insoluble membrane (M) fractions from 5-week cultures in response to hydrogen peroxide (Fig. 6). This demonstrated a shift in the β-catenin from being predominantly in the membrane fraction to a majority in the soluble cytosolic fraction ($P < 0.05$) after treatment with 2.0 mM hydrogen peroxide (Fig. 6).

**Integrity of the Epithelial Monolayer and Response to Hydrogen Peroxide**

To assess the relationship between disruption of tight junction proteins observed by immunofluorescence and loss of functional integrity of the epithelial monolayer, transepithelial flux assays were performed. In well-differentiated cells, the baseline level of flux of fluorescently labeled dextran (molecular weight [MW] 70,000) was much less than that in less well-differentiated control cells, although 1-week cells had formed a confluent monolayer. A mean relative flux of approximately 7,000 units compared with 11,000 units after 6 hours suggested that the differentiated cells have formed a tighter barrier against the flux of large molecules, which correlated with the increased order observed for the junctional proteins (Figs. 4, 5).

A dose-dependent increase of dextran flux through the differentiated cell layer was observed with increasing concentrations of hydrogen peroxide ($P < 0.05$ at 1.0 mM, $P < 0.01$ for 2.0 mM treatment compared with control in 5-week cells). At the maximum tested level of 2.0 mM hydrogen peroxide, there was a mean relative fluorescence of approximately 12,500 units after 6 hours (Fig. 7). This rate of flux in well-differentiated cells was similar to that observed in poorly differentiated cells without treatment, which correlated with the enhanced breakdown of junctional integrity and redistribution of junctional proteins observed at the higher doses of hydrogen peroxide. In poorly differentiated cells, flux increased dramat-
ically at concentrations above 0.5 mM hydrogen peroxide to rates of flux similar to that seen in the absence of cells (21,000 units vs. 26,000; data not shown), suggesting that at these concentrations there is a significant breach of the cellular monolayer, probably as a result of cell death (Fig. 1).

**DISCUSSION**

Oxidative stress is considered to be an important component of many disorders and has been studied in a variety of cell models of disease. Oxidative stress in RPE cells has been implicated in the pathogenesis of AMD and other retinal conditions. In the current study, we examined both acute and chronic treatment with hydrogen peroxide and investigated its effect on cell viability and investigated the effect of chronic treatment on the stress response and on proteins responsible for the integrity of the posterior BRB.

Differential responses to oxidative stress and heat shock were observed in RPE cells after 1 week or 5 weeks in culture. Our results suggest that there is an increased resistance of RPE cells to chronic oxidative stress with increased time in culture, with no significant difference in response to acute oxidative stress. By introducing a period of serum withdrawal before oxidative stress, however, the more differentiated cells were sensitized to oxidative stress, suggesting a synergistic effect between serum withdrawal and oxidative stress. The mechanism by which this occurred is not clear and requires further investigation, but it may relate to growth factor signaling cascades. Studies of FGF1 in RPE suggest that there is a survival-enhancing effect in aged cells due to endogenous FGF1-induced activation of the ERK2 cascade, and Alizadeh et al. have related changes in RPE viability and differentiation status to changes in the FGF receptor.

Between 1 week and 5 weeks, the cells become more differentiated as indicated by the increased expression of the markers RPE-65 and CRALBP. Steady state levels of Hsp27 and Hsp70 also increased. An increase in the constitutive levels of these stress proteins may represent a generalized increase in the ability of better-differentiated RPE cells to resist insult. The increase in steady state levels of Hsp27 with differentiation, which was also recently demonstrated in RPE by another group, could be protective through Hsp27's ability to increase intracellular glutathione, which in turn can play a protective role in oxidative stress of the RPE. It has also been suggested that Hsp70 may increase during differentiation of U-937 cells and protect against cytotoxic effects. Transfection of RPE cells with the small heat shock protein (Hsp) α-crystallin has shown that an increase in the expression of this chaperone confers resistance to apoptosis by hydrogen-peroxide-mediated injury. Alternatively, there may be a more specific role of these proteins, such as in the formation and maintenance of junctional complexes in a manner analogous to that in which Hsp25 binds vinculin and α-actinin and

![Figure 5](image-url)  
*Figure 5.* Effect of oxidative stress on proteins of tight and adherens junctions in 5-week ARPE-19 cultures. Cells were treated with 0.5 mM (B, E, H, K, N) or 2.0 mM (C, F, I, L, O) hydrogen peroxide for 24 hours before immunofluorescent detection of actin (A–C), ZO-1 (D–F), occludin (G–I), N-cadherin (J–L), and β-catenin (M–O). *Arrows:* areas of loss of localization to the periphery. (★) Increased cytoplasmic localization. Scale bar, 10 μm.

![Figure 6](image-url)  
*Figure 6.* Western blot of β-catenin in cytosolic (C) or membrane (M) fractions of ARPE-19 cells maintained in culture for 5 weeks. Cells treated for 24 hours with 0.5 mM or 2.0 mM hydrogen peroxide were compared with the untreated control. The band intensities were quantified and the results expressed as the means of four experiments ± SEM, normalized to the total β-catenin in each case. The 2.0 mM hydrogen peroxide treatment demonstrated a shift in β-catenin from the membrane to the cytosolic fraction (*P* < 0.05 compared with control). Close examination also reveals a change in banding which may relate to a shift in phosphorylation status.
potentially facilitates the maintenance of focal adhesion plaques.26

Changes in expression of Hsp27 and Hsp70 from steady state levels in response to stress were examined at the two culture time points. Upregulation of Hsp27 expression was seen in response to chronic peroxide treatment in the undifferentiated cells, but not in differentiated cells. This appears to be related to the vulnerability of these cells to hydrogen peroxide, yet heat shock stimulated an upregulation of both Hsp27 and Hsp70 at each time point under which treated cells remained viable. Comparing cell death and the Hsp27 response to that of HO-1, all undifferentiated cells contained HO-1 after treatment with hydrogen peroxide, but only a few cells showed HO-1 immunostaining with oxidative stress at the 5-week culture time point. Those few cells showing HO-1 immunostaining after 5 weeks in culture were generally slightly above the main cell layer and therefore may have been cells that would have been lost in the normal course of events. This seems to indicate a fundamental difference in the processes in which Hsp27 and HO-1 are involved in the response to oxidative stress, the details of which remain to be elucidated in RPE.

At both culture times, upregulation of Hsp27 and Hsp70 occurred in response to heat shock, and this was concurrent with a change in the cellular localization of these proteins that varied, depending on differentiation. On heat shock in undifferentiated cells, Hsp70, and to a lesser extent Hsp27, was detected in the nuclei of cells. In differentiated cells subjected to heat shock, Hsp70 was diffuse through the nucleus, and Hsp27 was not observed in the nucleus, indicating a differential response. An early demonstration of Hsp70 in nuclear extracts after heat shock was observed in Chironomus tentans salivary gland cells.29 Subsequently, a variety of immunologic studies have localized Hsp70 to the nucleus, specifically to the nucleolus, after heat shock. The formation of insoluble protein foci in the nucleoli in association with Hsp70 under stress conditions is reversible and is thought to function to prevent random aggregation and damage in the nucleus.30 Hsp27 has also been detected in the nucleus of heat-treated murine fibrosarcoma cells.31 It therefore seems that nucleolar localization of Hsps occurs as a response to heat shock, whereas the different localization in the differentiated cells reflects a different response, perhaps because of higher constitutive levels or because other regions of the cell have a greater need for the thermoprotective effect of Hsps in these more differentiated cells.

The RPE monolayer along with the vascular endothelia forms the BRB that strictly controls the nonspecific passage of circulating proteins to the retina. During chick embryonic development of the RPE, there is a decrease in monolayer permeability that correlates with maturation of apical junctions,32 and with ZO-1 and occludin localizing to the RPE borders.14 These observations are in agreement with our immunofluorescence results that show that increased junctional proteins at the periphery of differentiated cells correlates with reduced transepithelial flux compared with that in the 1 week cultures, despite the formation of a confluent monolayer by this time. Oxidative stress resulted in a dose-dependent increase in flux in the 5-week cultures, with increasing peroxide concentration. We suggest that this could correspond with a progressive loss of junctional integrity, which started at lower concentrations as observed by immunofluorescence, and reached a significant breach at concentrations above 1.0 mM, whereas the rapid change observed at 1 week above the 0.5-mM level correlated with cell death and complete breach of the monolayer.

The shift of β-catenin from the membrane to the cytosolic fraction is evidence that there is a tendency for this molecule to move from its membrane-sequestered state with N-cadherin to the cytosolic pool after oxidative stress. The phosphorylation status of β-catenin determines cell adhesive strength, with tyrosine phosphorylation promoting disassembly of junctional proteins from the cytoskeleton,33 as well as initiating nuclear signaling functions.34 A slight shift in the banding pattern in our Western blot of cytosolic and membrane fractions may reflect a number of phosphorylation states in this instance. Further investigation is needed to confirm a shift in signaling activity.

It has been shown that disruption of the BRB can cause extravascular albumin leakage and may be linked to macular edema.35 We have observed that chronic exposure to hydrogen peroxide may disrupt the localization of junctional proteins and increases flux across the RPE monolayer and therefore suggest that such exposure may also result in the breakdown of the posterior BRB and exposure of the retina to proteins such as serum albumin. In addition, the BRB breakdown may allow passage of immune cells into the retina, and subsequent release of inflammatory factors. This agrees with an
integrated hypothesis for drusen production in AMD, in which injured RPE release chemoattractants for monocytes causing incorporation of immune complexes into the drusen.\textsuperscript{36} Facilitation of such factors across the BRB would encourage neovascularization, which occurs in both AMD and diabetic retinopathy. Inflammation is well established as being among the principal etiological factors in the development of choroidal neovascularization.\textsuperscript{57,38}

The effect of oxidative stress on adherens junction proteins in RPE may also relate to the pathogenesis of retinal diseases. Expression of adherens junction proteins including N-cadherin and β-catenin are needed to maintain an epithelial phenotype,\textsuperscript{15} with stability of the adhesions increasing after confluence.\textsuperscript{39} Peroxide treatment has been used to induce cadherin internalization in bovine pulmonary artery endothelial cells, with the suggestion that this is an early event in the process of cell retraction before disruption of tight junctions,\textsuperscript{11} contributing further to BRB disruption. In addition, our results suggest that oxidative stress may increase cytoplasmic β-catenin in RPE cells. This may increase the free pool available to the signal transduction pathways in which the molecule is known to participate,\textsuperscript{40,31} and may facilitate processes that allow migration of RPE cells.\textsuperscript{15}

The precise pathways leading to these responses in the injury of RPE remain to be elucidated, but may indicate potential targets for therapeutic intervention in the pathogenesis of diseases such as AMD and diabetic retinopathy. Our results show that ARPE-19 cells have a differential response to stress on acquisition of a more differentiated phenotype, and this may be related to increased levels of Hsps and changing demands of cellular physiology. It may also be more physiologically relevant to look at lower doses of oxidant over longer periods to extrapolate to the in vivo situation and the true response to oxidative stress.

Acknowledgments

The authors thank Peter Munro for assistance with confocal microscopy and Karl Matter and Maria Balda for tight junction protein and flux advice.

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Disruption of RPE Junctions by Oxidative Stress 683


