The use of hyperoxia to induce chronic mild oxidative stress in RPE cells in vitro

Shigeru Honda,1 Leonard M. Hjelmeland,1,2 James T. Handa1

Departments of Ophthalmology and Molecular and Cellular Biology, University of California, Davis, CA

Purpose: To establish a model of mild and chronic oxidative stress using hyperoxia for retinal pigment epithelial (RPE) cells in vitro.

Methods: RPE340 cells and WI38 lung fibroblasts were grown in normal oxygen (20% O2) and hyperoxia (40% O2 or 60% O2). After cell viability was examined, the levels of reactive oxygen intermediates (ROI) by flow cytometry and heme oxygenase-1 (HO-1) mRNA by northern analysis were measured as markers of oxidative stress in both cell types. Proliferative ability and gene expression pattern of growth factors were studied to demonstrate the phenotypic changes induced by mild oxidative stress upon these cells.

Results: While decreased by 60% O2, 40% O2 did not affect viability in both cell types, ROI production and HO-1 mRNA expression were elevated in hyperoxia compared to controls, but were inhibited with the antioxidant dehydro-ascorbic acid (DHA). The proliferation of cells by hyperoxia was inhibited in both cell types. The expression of growth factors induced by hyperoxia was cell type dependent. Fibroblast growth factor-2 mRNA was unchanged in RPE cells, but was increased in fibroblasts. Transforming growth factor-β2 was decreased in RPE cells, but unchanged in fibroblasts. Vascular endothelial growth factor was downregulated in RPE cells, while upregulated in fibroblasts. Connective tissue growth factor was decreased in RPE cells, but was unchanged in fibroblasts.

Conclusions: The results demonstrate that hyperoxia induces mild oxidative stress which alters the phenotype of cells in a cell type specific manner.

The retinal pigment epithelial (RPE) cell is exposed to chronic oxidative stress in part from exposure to high partial pressures of oxygen and in part, from its high level of oxygen metabolism which generates large amounts of reactive oxygen intermediates (ROI) [1,2]. Several in vitro models have been developed to study the effects of oxidative stress on RPE cells, but most of these utilize the acute, high level administration of a chemical oxidant [3,4]. While these chemical oxidants induce a specific cellular oxidative stress that is useful for studying cellular responses to individual ROIs, the applicability of these acute chemical effects to the in vivo condition is open to question.

An alternative and perhaps more applicable approach to studying the effects of oxidative stress on RPE cells would apply chronic and lower levels of oxidative stress than the acute levels resulting from chemical oxidants. Recently, von Zglinicki et al. used chronic hyperoxia treatment to induce long term oxidative stress on lung fibroblasts [5]. The purpose of our study was to develop and characterize a model using chronic hyperoxia treatment of the RPE cell that induces mild oxidative stress without causing cell death. Our laboratory and others have studied in depth, the characteristics of the one cell line, designated RPE340 [6-9]. In this study, we have utilized RPE340 cells, and compared their response to WI38 lung fibroblasts, a cell line that has been utilized in oxidative stress studies, for comparison.

METHODS

Cell culture: The RPE340 cell line from one globe of a 1-year-old trauma victim was propagated as previously described [6]. While not an established cell line available for purchase, the growth characteristics and mRNA phenotype of this cell line have been characterized in a number of publications [6-9]. Cultures were maintained in Dulbecco’s modified Eagle medium/Nutrient mixture F12 with 15 mM HEPES buffer (DMEM/F12; Biowhittaker, Walkersville, MD) and 10% fetal bovine serum (FBS; UBI Upstate, Lake Placid, NY), 0.348% additional sodium bicarbonate, 2 mM L-glutamine solution (GIBCO, Grand Island, NY) at 20% oxygen and 40% oxygen conditioned in 10% CO2 at 37 °C. The pO2 in the medium was measured with a YSI 5331 oxygen probe (YSI Incorporated, Yellow Springs, OH) and found to be 162.9±4.6 mm Hg when placed in 20% oxygen and 296.9±2.9 mm Hg when in 40% oxygen. WI38 cells (ATCC, Manassas, VA) were cultured in Eagle’s minimum essential medium with Earle’s balanced salt solution, 2 mM L-glutamine (EMEM), 1 mM sodium pyruvate (GIBCO) and 10% FBS at 20% oxygen and 40% oxygen conditioned in 5% CO2 at 37 °C. This culture medium was selected because it is considered the optimum growth medium for WI38 cells (personal communication, ATCC). In addition, since EMEM has been used in published studies of WI38 cell response to hyperoxia, we selected this medium to allow for comparison of our results with those in...
the literature. For experiments, cells were grown in 75 cm² flasks at an initial seeding density of 10,000/cm². For proliferation assays, cells were passaged before reaching confluence to avoid contact inhibition. At each passage, cell number was counted using a Coulter Counter Z1 (Coulter, Miami, FL) and population doubling level (PDL) was determined as current PDL = last PDL + log₂ (collected cell number/seeded cell number).

Cell viability: Viability was quantified using the WST-1 assay according to the manufacturer’s recommendation (Boehringer Mannheim, Mannheim, Germany). This assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases in viable cells. Cells were seeded in 96 well plates at 20,000 cells/cm² in DMEM/F-12 (RPE340 cells) and EMEM (WI38 cells) including 10% FBS and grown for 2 days in 20% oxygen before placing half of the cultures in 40% or 60% oxygen for 48 h. WST-1 reagent was added for two h and the absorbance was measured at 485 nm using a HTS 7000 Bioassay reader (Perkin Elmer Corporation, Norwalk, CT). Three independent experiments were conducted.

Flow cytometry: Cells were plated at 10,000 cells/cm² in DMEM/F12 plus 10% FBS (RPE340 cells) and EMEM plus 10% FBS (WI38 cells) and grown for three days in 20% oxygen. At 80-90% confluence, half of the flasks were transferred to hyperoxia (40% oxygen) with or without 150 μM dehydroascorbic acid (DHA; Sigma, St. Louis, MO) as an antioxidant for the duration of the experiments. Cells were rinsed twice with Hanks’ balanced salt solution (HBSS) and treated with 10 μM of 2',7'-dichlorofluorescein diacetate (H2DCFDA; Molecular Probes, Eugene, OR) for 30 min. H2DCFDA is a nonfluorescent compound that readily crosses cell membranes. It is hydrolyzed to 2',7'-dichlorofluorescein intracellularly, and becomes fluorescent when it is oxidized by reactive oxygen species. Cells were then trypsinized and resuspended in 1% paraformaldehyde/HBSS and fluorescence was measured with a FACscan (Becton Dickinson, San Jose, CA) [10,11]. Three independent experiments were conducted.

RNA extraction and northern analysis: Total RNA was extracted using TRIZOL reagent (GIBCO) according to the manufacturer’s recommendations. Fifteen-microgram aliquots of each sample were electrophoresed in 1% agarose gels, transferred to nylon membranes, and prehybridized for 3 h at 42 °C in 50% formamide. 5X Denhardt’s solution, 5X SSC, 100 ng/ml salmon sperm DNA and 0.1% SDS. The membranes were hybridized for 18 h at 42 °C with 25 ng of the 32P labeled cDNA probe. Blots were washed in 0.1% SDS/0.1X SSC three times at room temperature, then once at 50 °C before being subjected to phosphorimage analysis (Molecular Dynamics, Sunnyvale, CA). The blots were stripped and sequentially hybridized with the following cDNA probes: heme oxygenase-1 (HO-1), a generous gift from Dr. A. Choi [12], fibroblast growth factor-2 (FGF-2) [13], transforming growth factor-β2 (TGF-β2) [14], vascular endothelial growth factor (VEGF), a gift from Dr. A. Singh (Genentech, Inc., South San Francisco, CA), connective tissue growth factor (CTGF) from Dr. G. Grotendorst [15], and 28S rRNA [16]. Hybridization signals were normalized against 28S rRNA. Three independent experiments were conducted for each growth factor.

Statistical analysis: Since the data were normally distributed, statistical significance at each time point comparing control to the hyperoxia treated condition was determined using the two-tailed Student’s t-test. p<0.05 was considered significant.

RESULTS

Cell viability: To determine a concentration of O₂ that would induce chronic oxidative stress to cells without affecting viability, RPE340 and WI38 cells were grown in various concentrations of O₂. When grown in 60% O₂, RPE340 cells showed 77±14% (mean±SEM) viability compared to growth in 20% O₂ (p<0.05) while WI38 cells were 71±13% viable when compared to control (p<0.01; Figure 1). In contrast, RPE340 cells cultured in 40% O₂ for 48 h showed no change in viability (97±7%) compared with growth in 20% O₂ (p=0.7), while WI38 cells showed 92±9% viability under 40% O₂ against control (p=0.6). Thus, the remaining experiments were conducted in 40% O₂.

Reactive oxygen intermediate (ROI) production: ROI production in RPE340 cells became elevated soon after exposure to 40% O₂ (Figure 2A). Within 8 h of incubation, ROI production reached 145% of control (p<0.01), which was maintained throughout the 48 h experimental period. The antioxidant DHA (150 μM) inhibited the hyperoxia induced ROI production after 24 h of incubation (p<0.05). WI38 cells treated with 40% O₂ also showed a rapid increase in ROI production (Figure 2B). This increase in ROI production plateaued at 169% of control (p<0.01) after 24 h of incubation. As with RPE cells, 150 μM DHA inhibited ROI production after 24 h (p<0.05). DHA also tended to decrease the ROI production in both cell types when

![Figure 1. Viability of cells after hyperoxic treatment.](http://www.molvis.org/molvis/v7a10/)
grown in 20% O₂, but this change did not reach statistical significance. Further experiments with DHA revealed a dose dependent inhibition of ROI production in both cell lines grown in 40% O₂, reaching significance at ≥100 µM DHA in RPE 340 cells (p<0.0005 to p<0.05), and ≥10 µM DHA in WI38 cells (p<0.01 to p<0.05; Figure 3A,B). In both cell lines, 150

Figure 2. Measurement of intracellular ROI production. Flow cytometric measurement of ROI production in RPE340 cells (A) and WI38 cells (B) over time. Cells were preincubated at each time point in 20% O₂ (Control) and 40% O₂ with or without 150 µM DHA before adding H2DCFDA. Values are shown as mean±SE of three independent experiments. Data of each condition were compared at the same time point. Significance levels for comparison to controls: *p<0.05, **p<0.01. Significance levels for comparison to hyperoxia treated cells without DHA: #p<0.05, ##p<0.01.

Figure 3. The antioxidant effect of DHA. Dose dependent effect of DHA on ROI production in RPE340 (A) and WI38 cells (B) grown in hyperoxia. Cells were preincubated in 20% O₂ (Control), and 40% O₂ with the indicated DHA dose for 24 h before adding H2DCFDA. Values are shown as mean±SE of three independent experiments. Significance levels for comparison to controls: ***p<0.001. Significance levels for comparison to hyperoxia treated cells without DHA: #p<0.05, ##p<0.01, ###p<0.005, ####p<0.0005.
µM DHA completely inhibited the ROI production induced by hyperoxia.

**HO-1 mRNA expression is induced by hyperoxia:** As an alternative measure of the oxidative stress induced by cells grown in hyperoxia, the induction of HO-1 mRNA, a known marker of oxidative stress, was assessed by northern analysis [17]. Cells grown in 40% O₂ showed a modest induction of HO-1 mRNA steady state expression. In general, HO-1 mRNA expression was higher in WI38 than RPE 340 cells (Figure 4A,B). HO-1 mRNA expression gradually decreased in both cell lines with incubation time under both hyperoxic and control conditions, an effect that could be due to increasing cell density during the experimental period [18] (Figure 4A,B) or from the antioxidative effect of HO-1 [19]. We therefore took the ratio of HO-1 mRNA expression from cells grown in 40% to 20% O₂ at each time point to assess the effect of hyperoxia on HO-1 expression. This ratio of HO-1 mRNA expression increased gradually with time in both cell lines (Figure 4C,D). In RPE340 cells grown in 40% O₂, HO-1 mRNA expression increased 146±10% at 24 h (p<0.001) and 187±22% at 96 h (p<0.005) compared to control conditions. Likewise, WI38 cells grown in 40% O₂ increased HO-1 expression to 185±14% by 24 h (p<0.005), and remained at this level for the duration of the 96 h (p<0.01). Incubation of both cell types with 150 µM DHA, after a short period of enhanced HO-1 induction, inhibited HO-1 mRNA expression. In RPE340 cells, inhibition of HO-1 mRNA induction by hyperoxia treatment reached significance after a 48 h incubation with 150 µM DHA (p<0.05) while in WI38 cells, DHA inhibited HO-1 mRNA induction by hyperoxia after 96 h (p<0.05).

**Effect of hyperoxia treatment on cell proliferation:** Cell proliferation was used to assess a functional effect of hyperoxic treatment on both cell types. RPE340 cells cultured under normal oxygen proliferated with a constant rate (0.46 PDL/day) until about PDL50, and gradually ceased proliferation by PDL57 (Figure 5A). Hyperoxia inhibited the proliferation of RPE340 cells and shortened their life span to PDL47. WI38 cells grown in 20% O₂ had constant proliferation (0.34 PDL/day) until approximately PDL37, and ceased proliferating by PDL41 (Figure 5B). In contrast, hyperoxia rapidly inhibited

---

**Figure 4. Northern analysis of HO-1 expression.** Northern analysis of HO-1 mRNA expression by RPE340 cells (A) and WI38 cells (B). Cells were cultured in 20% O₂ (Control), 40% O₂, and 40% O₂ with DHA (150µM). Signals of HO-1 were normalized against that of 28S rRNA. The ratio of hyperoxia treated: controls at each time point was determined for RPE340 cells (C) and WI38 cells (D) from three independent experiments. Values are shown as mean±SE. Data of each condition were compared at the same time point. Significance levels for comparison to controls: *p<0.05, **p<0.01, ***p<0.005, ****p<0.001. Significance levels for comparison to hyperoxia treated cells without DHA: #p<0.05.
proliferation and shortened the life span of WI38 cells within three PDLs.

Alteration of gene expression: Our laboratory has been interested in a set of growth factors that can alter the phenotype of the RPE-Bruch’s membrane-choriocapillaris complex after exposure to oxidative stress. Our initial work treated RPE cells with acute exposure to chemical oxidants [20]. Using this set of genes, we found modest alterations in gene expression that were cell type dependent (Figure 6). FGF-2 mRNA expression in RPE340 cells showed no change after 24 h of incubation, but was upregulated in WI38 cells (126±12%, p<0.01) treated with hyperoxia (Table 1). TGF-β2 mRNA expression was downregulated in RPE340 cells (86±9%, p<0.05), but remained unchanged in WI38 cells grown in hyperoxia (99±11%, p =0.85). VEGF was downregulated in RPE340 with hyperoxia (80±5%, p<0.05), but was upregulated in WI38 cells with hyperoxia (144±18%, p<0.05). CTGF was downregulated with hyperoxia (70±8%, p<0.05) in RPE340 cells, but not in WI38 cells (92±13%, p = 0.31).

DISCUSSION
We demonstrate here that 40% hyperoxia treatment caused detectable oxidative stress in RPE340 and WI38 cells, as measured by ROI production and induction of HO-1 mRNA, without affecting cell viability. ROI production was induced soon after hyperoxic treatment and became significant within 8 h, an effect that was ameliorated by the antioxidant DHA. To test our model, we also assessed functional consequences of hyperoxia on RPE340 and WI38 fibroblasts, two cell lines which have been characterized in detail [5-9,21,22]. Hyperoxia inhibited cell proliferation and shortened their life span in vitro, an effect that was dramatic in WI38 fibroblasts. Hyperoxia also induced a mild, cell specific alteration in the expression of several growth factors.

Many oxidative stress models have been previously established which use a chemical oxidant like hydrogen peroxide to induce oxidative stress [23]. Chemical oxidants generate significant intracellular ROIs, but most are degraded enzymatically, and consequently, are short-lived in the cell [24]. The dose of oxidant necessary for typical experiments induces peak, temporary effects that might not be relevant to the in vivo condition. It is likely that oxidative stress in vivo is chronic and below levels that cause necrosis or apoptosis. We therefore wanted to establish a model of low grade, chronic oxidative stress in vitro that more closely simulates the situation in vivo. In general, the degree of oxidative stress induced by hyperoxia is lower than that generated by chemical oxidants even when a maximum oxygen tension (95%) is applied [18]. However, chronic exposure to high levels of hyperoxia can induce cytotoxicity, depending on the cell type [24-26]. We sought to use the highest level of oxygen that induced oxidative stress, as measured by intracellular ROI production and induction of HO-1 mRNA, without causing a loss of viability. We chose to use 40% O2 for our studies because it generated modest levels of ROI production and induced HO-1 mRNA expression without inducing cytotoxicity as was found at

Figure 5. The effect of hyperoxia on cell proliferation. Cumulative growth curve of RPE340 cells (A) and WI38 cells (B) cultured in 20% O2 (Control) and 40% O2. Cells were grown in 75 cm2 flasks at an initial seeding density of 10,000/cm2, and were split to 1:2.5 before reaching confluence to avoid contact inhibition.
higher O₂ (60% O₂) in both RPE340 and WI38 cells. While the intention of our experiments was to document oxidative stress to cells with hyperoxia, we did not perform a detailed analysis of which specific oxidant species were generated. Further studies identifying the specific ROIs could provide insight into oxidative stress mediated changes to RPE cells.

The induction of ROI was consistently lower in RPE340 than WI38 cells with hyperoxic treatment. We presume that this effect can be explained in part, by the considerable antioxidant capability of the RPE compared to WI38 cells, since RPE cells have abundant glutathione stores and high catalase activity that can reduce oxidative stress [27,28]. Alternatively, differences in the culture media used could have influenced the effect of oxidative stress or the response by the cells. However, our preliminary experiments showed no significant difference in ROI production of RPE340 cells between the two medium types (data not shown). Thus, the influence of medium type may be negligible.

DHA, an effective ROI scavenger [29,30], inhibited ROI production induced by hyperoxia (40% O₂) in a dose dependent manner. Interestingly, high doses of DHA reduced the ROI level of cells grown in 40% O₂ below that of control cells grown in 20% O₂. We furthermore, observed a reduction in ROI production after cells grown in 20% O₂ were treated with DHA. Our flow cytometric analysis of DHA treatment on cells grown in 20% oxygen, though not statistically significant, suggests that even 20% O₂ produces some ROI in RPE340 and WI38 cells. It is interesting that DHA showed temporal enhancement of HO-1 mRNA expression in the early period of hyperoxia exposure. DHA is rapidly incorporated by the cell and reduced to ascorbic acid via nonenzymatic reaction with glutathione [31]. Since ascorbic acid acts not only an antioxidant but also as a strong reductant [32], this temporal increase of HO-1 mRNA may be related to the reduction of thiol residues in some regulatory proteins (i.e., transcription factors) [18]. N-acetylcysteine, a reducing reagent and a precursor of glutathione, was also reported to enhance the expression of HO-1 mRNA in hepatoma cells cultured in 95% O₂ for 6 h [18].

One phenotypic alteration of oxidative stress is the inhibition of cell proliferation without inducing cell death [5,24,33-36]. This effect is probably due to the activation of cell cycle regulatory genes such as p53 and p21 that respond to oxidative stress induced DNA damage [35,37-39]. We showed that hyperoxia had a lower antiproliferative effect in RPE340 than WI38 cells, an effect that correlates with lower oxidative stress levels in RPE340 than WI38 cells when grown in 40% O₂. The stimulation of cell growth with an antioxidant and low oxygen tension has been reported in various cell types [36,40-42]. Akeo et al reported that 20% oxygen inhibited the prolif-

### Table 1. The percentage change in gene expression for selected growth factors

<table>
<thead>
<tr>
<th></th>
<th>RPE340</th>
<th>p</th>
<th>WI38</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-1</td>
<td>145±16</td>
<td>&lt;0.0005</td>
<td>193±33</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>FGF-2</td>
<td>90±10</td>
<td>0.12</td>
<td>126±12</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>86±9</td>
<td>&lt;0.05</td>
<td>99±11</td>
<td>0.85</td>
</tr>
<tr>
<td>VEGF</td>
<td>80±5</td>
<td>&lt;0.05</td>
<td>144±18</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CTGF</td>
<td>70±8</td>
<td>&lt;0.05</td>
<td>92±13</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Percentage change of the signal from 40% O₂ to 20% O₂ determined for each blot. Values are shown as mean ± SE from three independent experiments.

Figure 6. The effect of hyperoxia on gene expression. The change in gene expression by RPE340 (A) and WI38 cells (B) grown in hyperoxia. RPE340 cells and WI38 cells were grown in 20% O₂ (C; control) and 40% O₂ (H; hyperoxia) for 24 h, followed by RNA extraction and northern blot analysis. Signals were normalized to 28S rRNA.
eration of porcine RPE cells, and that antioxidant enzymes added to the medium stimulated proliferation [43]. The partial pressure of O₂ in the culture medium under ambient oxygen (20%) was 162.9 ± 4.6 mm Hg in this study similar to published data (130–160 mm Hg) [36,40,44] while that in the RPE layer is 70–90 mm Hg in vivo [1,45], making standard room air (20%) incubation, compared to the environment of the RPE in vivo, a relative oxidative stress environment.

We chose to sample the expression of growth factors known to be important in the maintenance of the RPE-Bruch’s membrane-choroidal complex. The response of these growth factors has been studied in RPE cells treated with chemical oxidants [20,46]. In comparison, we found modest changes in gene expression. Changes in age-related disease such as age-related macular degeneration (AMD) occur over a long duration. We hypothesize that chronic, modest changes in gene expression approximates the response to the exposure of the high partial pressure oxygen that occurs in vivo. The pattern of gene expression induced by hyperoxia depends on the cell type, presumably because the basic expression and the importance of each gene varies among cell types. While we found no change in FGF-2 expression in RPE340 cells after hyperoxic treatment, Khalili et al reported that FGF-2 mRNA was upregulated in human RPE cells grown in hypoxia [47]. Hackett et al reported that FGF-2 mRNA was upregulated in human RPE cells with 100 µM H₂O₂ [48]. These findings may represent a characteristic difference between hyperoxia and chemical oxidant treatment. In contrast, hyperoxia increased FGF-2 mRNA expression in WI38 cells. Care must be taken in interpreting these results since they represent the expression changes in one cell line which may not be applicable to RPE or lung fibroblast cells in general, or to these cells in vivo. Furthermore, since different culture media were used to optimize the growth conditions for each cell type, a direct comparison of gene expression changes may not be valid. However, the gene expression changes found in each cell type do illustrate a phenotypic alteration induced by hyperoxia treatment in each of these cell lines.

It is likely that chronic oxidative stress to the RPE plays some role in the pathogenesis of AMD [1] due to the high oxidative stress environment from the high partial pressure of oxygen, the high metabolic rate of the RPE, chronic light exposure, accumulation of lipofuscin, and the formation of advanced glycation endproducts in Bruch’s membrane [1,49]. The accumulated oxidative stress could alter the phenotype of the RPE during aging and AMD [2,50]. Therefore, it is useful to establish a chronic oxidative stress model of RPE cells to investigate the mechanism of how oxidative stress changes the function of the RPE. Further studies may be directed at more fully characterizing the RPE cell phenotype induced by chronic hyperoxia.

ACKNOWLEDGEMENTS
This work was supported by National Institutes of Health grants NIH/EY00344 (JTH), NIH/EY06473 (LMH), UCD Health System Awards (JTH), Manpower Award (JTH), an unrestricted grant from Research to Prevent Blindness (Department of Ophthalmology, University of California, Davis), and the Nippon Eye Bank Association.

REFERENCES
18. Takahashi S, Takahashi Y, Yoshimi T, Miura T. Oxygen tension...


