Downregulation of Differentiation Specific Gene Expression by Oxidative Stress in ARPE-19 Cells

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Purpose. To investigate how the differentiation of ARPE-19 cells affects the relative expression of the *FGFR* genes in response to oxidative stress.

Methods. After differentiation in vitro, APRE-19 cells were treated with *t*-butyl hydroperoxide (tBH) or hydrogen peroxide (H_2O_2) to induce oxidative stress. Viability and reactive oxygen intermediate (ROI) production were measured using standard assays. The mRNA expression of *FGFR1*, *FGFR2*, cellular retinaldehyde-binding protein (*CRALBP*), *RPE65*, and heme oxygenase-1 (*HO-1*) were measured by Northern blot analysis as a function of treatment with tBH and H_2O_2.

Results. ARPE-19 cells were viable at all tBH concentrations tested but showed progressive loss of viability at concentrations greater than 300 μM H_2O_2. Differentiated ARPE-19 cells treated with tBH or H_2O_2 resulted in upregulation of the *HO-1* and *FGFR1* transcripts. The expression of RPE-differentiated specific genes, including *FGFR2*, *CRALBP*, and *RPE65* mRNAs, was downregulated with tBH or H_2O_2 treatment.

Conclusions. Oxidative stress in differentiated ARPE-19 cells alters the expression of *FGFR1*, *FGFR2*, *CRALBP*, and *RPE65* toward levels characteristic of the undifferentiated state. If similar changes take place in vivo, these events could alter the proliferative potential, viability, and even the function of the RPE. (*Invest Ophthalmol Vis Sci.* 2001;42:2706–2713)

The retinal pigment epithelium (RPE) is exposed to relatively high oxygen tensions of 70 to 90 mm Hg. As a result, these cells possess high levels of the enzymes required to detoxify reactive oxygen intermediates (ROIs).[1–3] To study the biochemistry and molecular biology of oxidative stress in RPE cells, several investigators have used in vitro cultures. Treatment of RPE cells with H_2O_2, for example, induces the expression of fibroblast growth factor (FGF)-2 and other trophic factors.[4] Metallothionein and HSP 70 expression and catalase activity are also induced by H_2O_2 in human RPE cells.[5,6] Treatment of RPE cells with *t*-butyl hydroperoxide (tBH) has recently been shown to lead to apoptosis.[7]

Many of the in vitro studies cited used RPE cultures, which were incompletely characterized with respect to cellular differentiation. It is important to consider the state of RPE differentiation in vitro because the overall oxidative stress response may be dramatically altered. Alternatively, oxidative stress may alter the differentiation state of the cultures. Our laboratory has recently introduced the ARPE-19 human RPE cell line and characterized the differentiated properties of these cells in a series of publications.[8,9] These properties include cuboidal morphology, functional polarity, and the expression of RPE-specific gene markers for differentiation, including *CRALBP*[10] and *RPE65*.[11,12] Our laboratory has also shown that the differentiation of ARPE-19 cells in vitro uncovers silencer activity in the *FGF-5* gene promoter.[13] Most recently, Alizadeh et al.[14] have shown that the differentiation of ARPE-19 cells alters the expression and alternative splicing of FGF receptor mRNAs. *FGFR2* is specifically upregulated by differentiation in vitro and is expressed in vivo.

We believe that changes in the expression of *FGFR2* may be significant for diseases in which RPE dedifferentiation occurs. Age-related macular degeneration (ARMD) and proliferative vitreoretinopathy are important examples. Not only do RPE cells lose characteristic features of differentiation, but several studies have also shown that aging cells in ARMD may be subject to increased oxidative stress.[2,15] In this study, we hypothesized that oxidative stress downregulates *FGFR2* gene expression as well as the expression of *CRALBP* and *RPE65* as markers of differentiation in ARPE-19 cells. To test this hypothesis, we examined the response of *FGFR1* and *FGFR2* gene expression to oxidative stress generated by tBH and H_2O_2 treatment.

Materials and Methods

Cell Culture

Routine experiments were performed with ARPE-19 cells, a nontransformed human diploid RPE cell line that displays many differentiated properties typical of RPE in vivo.[8] ARPE-19 cells were plated at high density (100,000 cells/cm^2) and maintained in culture for 3 days for undifferentiated cultures, or 3 months for differentiated cultures, at 37°C in 10% CO_2. All ARPE-19 cultures were fed weekly and maintained in Dulbecco's modified Eagle's medium (DMEM): nutrient mixture F12 with 15 mM HEPES buffer (DMEM/F12; BioWhittaker, Walkersville, MD) plus 10% fetal bovine serum (FBS; UBI, Lake Placid, NY), 0.348% additional sodium bicarbonate, 2 mM L-glutamine solution, and 0.1 mg/ml streptomycin (Gibco, Grand Island, NY). After 3 days or 3 months, cells were thoroughly washed with Hanks' balanced salt solution (HBSS) and were withdrawn from serum for 48 hours. Chemical treatments were performed in HBSS for 30 minutes.[16] After 30 minutes, the medium was removed and replaced with the original culture medium, and RNA was extracted after 4 hours. H_2O_2 (30% aqueous solution) and tBH (70% aqueous solution) were purchased from Fisher Scientific (Houston, TX) and Sigma (St. Louis, MO), respectively.

Fluorescent Detection of Intracellular ROIs

Sterile tissue-culture–treated 96-well plates (Corning Costar Corp., Cambridge, MA) were seeded with ARPE-19 cells at 100,000 cells/cm^2 and maintained in culture for 3 days or 3 months. After 3 days or 3

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months, the medium was removed, and cells were washed once with HBSS plus calcium and magnesium (Gibco) and loaded with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes, Eugene, OR) diluted in HBSS plus calcium and magnesium. After cells were incubated at 37°C for 30 minutes, the dye was removed and cells were washed once with HBSS. Various doses of tBH and H_2O_2 were added to the cells and incubated for 30 minutes, followed by washing. After 30 minutes, the intracellular ROI production was measured and quantified using a reader (HTS 7000 Bioassay; Perkin Elmer Corp., Norwalk, CT; excitation λ = 485 nm; emission λ = 535 nm.)

Measurement of Cell Viability

Cell viability was measured using a WST-assay (Roche Diagnostics, Indianapolis, IN). This assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases in viable cells. The amount of formazan dye produced is proportional to the number of metabolically active cells and was quantified by its absorbance at 485 nm with a multiwell spectrophotometer (Perkin Elmer Corp.). Sterile tissue-culture–treated 96-well plates were seeded with ARPE-19 cells at 100,000 cells/cm^2 and maintained in culture for 3 days or 3 months. After 3 days or 3 months, the medium was removed and reserved. ARPE-19 cells were treated with tBH or H_2O_2 in HBSS for 30 minutes at concentrations of 50 μM, 100 μM, 300 μM, 1 mM, and 3 mM. After 30 minutes the media were removed and replaced with the original culture medium for 3 hours. After 3 hours, the WST-1 reagent was added and the cells were incubated for 1 hour.

Northern Analysis

Total RNA was isolated from differentiated ARPE-19 cells, by using RNA extraction reagent per the manufacturer's instructions (Trizol; Gibco), and quantified by spectrophotometry. Total RNA (20 μg) was electrophoresed in formaldehyde-agarose gels and transferred to 0.45-μm membranes (Hybond-N; Amersham, Arlington Heights, IL) according to standard procedures.[17] After cross-linking, the blots were probed with ^{32}P-labeled cDNAs for *FGFR1* (American Type Culture Collection no. 1042862; [ATCC]), *FGFR2* (103 5480l), *RPE65*,[12] *CRALBP*,[10] and heme oxygenase-1 (*HO-1*), which was provided by Augustine M. K. Choi.[18] The blots were washed, and the signals were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) analysis after normalization with a 28S rRNA cDNA probe.

Statistical Analysis

Statistical significance at each time point comparing the control to the tBH-and H_2O_2-treated conditions was determined using the two-tailed Student's *t*-test. $P < 0.05$ was considered significant.

Results

The Time Course of *FGFR2*, *CRALBP*, and *RPE65* Gene Expression in Differentiating ARPE-19 Cells

ARPE-19 cells were seeded at a density of 100,000 cells/cm^2 on plastic and cultured from 1 to 90 days to evaluate the expression of *FGFR2*, *CRALBP*, and *RPE65* as a function of differentiation. Cultures were 100% confluent 24 hours after plating. *FGFR2* mRNA was first significantly upregulated at day 42 (P = 0.005) relative to control, and reached a maximum level at day 90, relative to all other points ($P < 0.05$) in differentiated ARPE-19 cells (Fig. 1A). *CRALBP* mRNA was first significantly upregulated at day 21 (P = 0.0025) relative to control, and reached a maximum value at day 90, relative to all other points ($P < 0.025$; Fig. 1B). *RPE65* mRNA was also upregulated as a function of RPE cell differentiation initially at day 28 (P = 0.05) and was unchanged thereafter by ANOVA (Fig. 1C). As a result of these observations, we chose 90 days as a standard period in culture for the studies involving differentiated ARPE-19 cells described in the following sections.

Effect of tBH and H_2O_2 Treatment on ARPE-19 Cell Viability

Before examining the effect of oxidative stress on gene expression, the viability of ARPE-19 cells after treatment with tBH or H_2O_2 was assessed using the WST-1 colorimetric assay for undifferentiated and differentiated cultures. The viability of undifferentiated ARPE-19 cells treated with tBH was greater than 90% of untreated control cells (Fig. 2A). There was no statistically significant change in viability after tBH treatment of undifferentiated cultures. In contrast, H_2O_2, showed a decrease in viability at concentrations greater than 300 μM. Figure 2B shows that the viability of differentiated ARPE-19 cells treated with tBH did not differ statistically from untreated control cultures. The viability of differentiated ARPE-19 cells after treatment with H_2O_2 was unchanged up to doses of 300 μM, but decreased to 55% at 1 mM and to 31% at 3 mM.

Fluorescent Detection of ROIs in ARPE-19 Cells

To quantify the level of cellular oxidative stress generated by tBH or H_2O_2, we used a fluorometric microplate assay to measure ROI.[19] For undifferentiated ARPE-19 cells, tBH treatment showed a steady dose-dependent increase, whereas H_2O_2 treatment showed a modest but significant increase in fluorescence (Fig. 3A). Whereas tBH treatment produced a dose-dependent increase in ROI production in differentiated ARPE-19 cells, H_2O_2 treatment did not result in ROI production (Fig. 3B).

Induction of *HO-1* mRNA Expression by Treatment with tBH and H_2O_2 in ARPE-19 Cells

Because *HO-1* mRNA expression is a general marker of oxidative stress in mammals, the expression of *HO-1* mRNA in ARPE-19 cells was used as a positive control for gene expression, due to oxidative stress after treatment with tBH or H_2O_2.[20,21] There was a dose-dependent increase in the mRNA expression of *HO-1* after a 30-minute treatment with tBH or H_2O_2 in both undifferentiated and differentiated cultures of ARPE-19 cells (Fig. 4). tBH treatment showed a marked dose-dependent increase in *HO-1* mRNA in undifferentiated ARPE-19 cells. A similar significant dose-dependent increase in *HO-1* expression was seen after undifferentiated cells were treated with H_2O_2 (Fig. 4A) up to 1 mM, after which *HO-1* expression declined slightly at 3 mM. tBH (3 mM) caused a 23-fold induction over basal levels of *HO-1* mRNA in differentiated ARPE-19 cells (Fig. 4B). H_2O_2 treatment also induced a 17-fold increase in *HO-1* mRNA expression at 1 mM, but *HO-1* expression decreased to seven times induction at 3 mM in differentiated cells.

Effect of tBH or H_2O_2 on *FGFR* Expression in ARPE-19 Cells

To assess the effect of oxidative stress on *FGFR1* and *FGFR2* mRNA expression, we treated undifferentiated and differentiated ARPE-19 cultures with various concentrations of tBH or H_2O_2. Undifferentiated ARPE-19 cultures treated with 3 mM tBH showed only a 1.4-fold increase at 3 mM (Fig. 5A). When treated with H_2O_2 (Fig. 5B), a more substantial increase in *FGFR1* expression was seen up to a fivefold increase at 3 mM. *FGFR2* was not expressed in the undifferentiated state (Fig. 1A).

Differentiated ARPE-19 cells treated with 3 mM tBH resulted in a 1.7-fold increase in *FGFR1* mRNA expression, whereas *FGFR2* mRNA expression declined twofold (Fig. 5C). The only

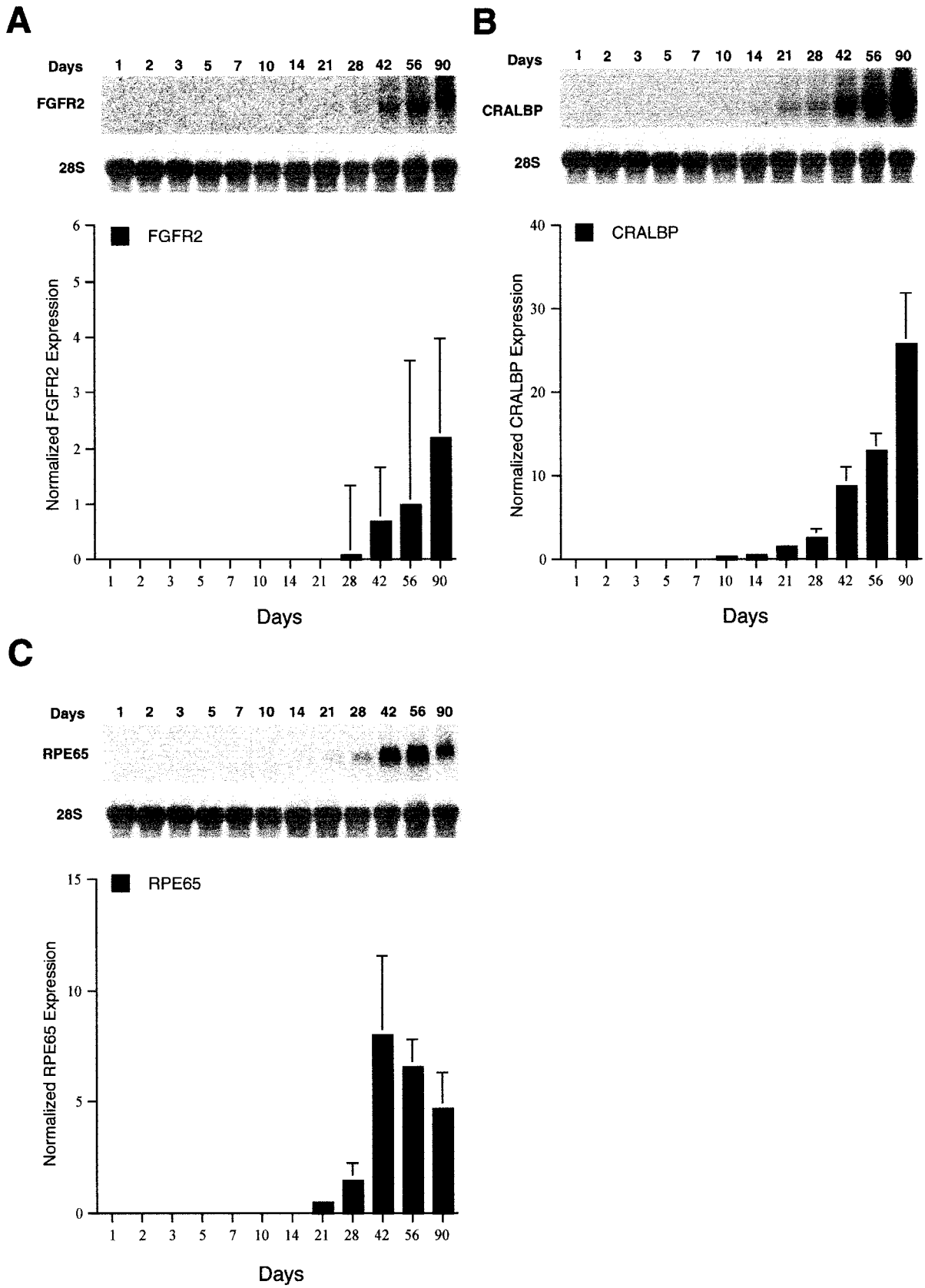

Figure 1. Northern blot analysis of RPE differentiation-specific genes (*FGFR2*, *CRALBP*, and *RPE65*) as a function of time. ARPE-19 cells were plated at 100,000 cells/cm^2 at various times. RNA was extracted from each culture. Northern blot analysis was performed, and blots were hybridized with ^{32}P-labeled *FGFR2* (**A**), *CRALBP* (**B**), and *RPE65* (**C**) cDNA. Northern blot analyses were normalized against 28S rRNA and quantified. The results are expressed as the average of two independent experiments and are shown as mean ± SE of two experiments, except when the SE is too small to be seen.

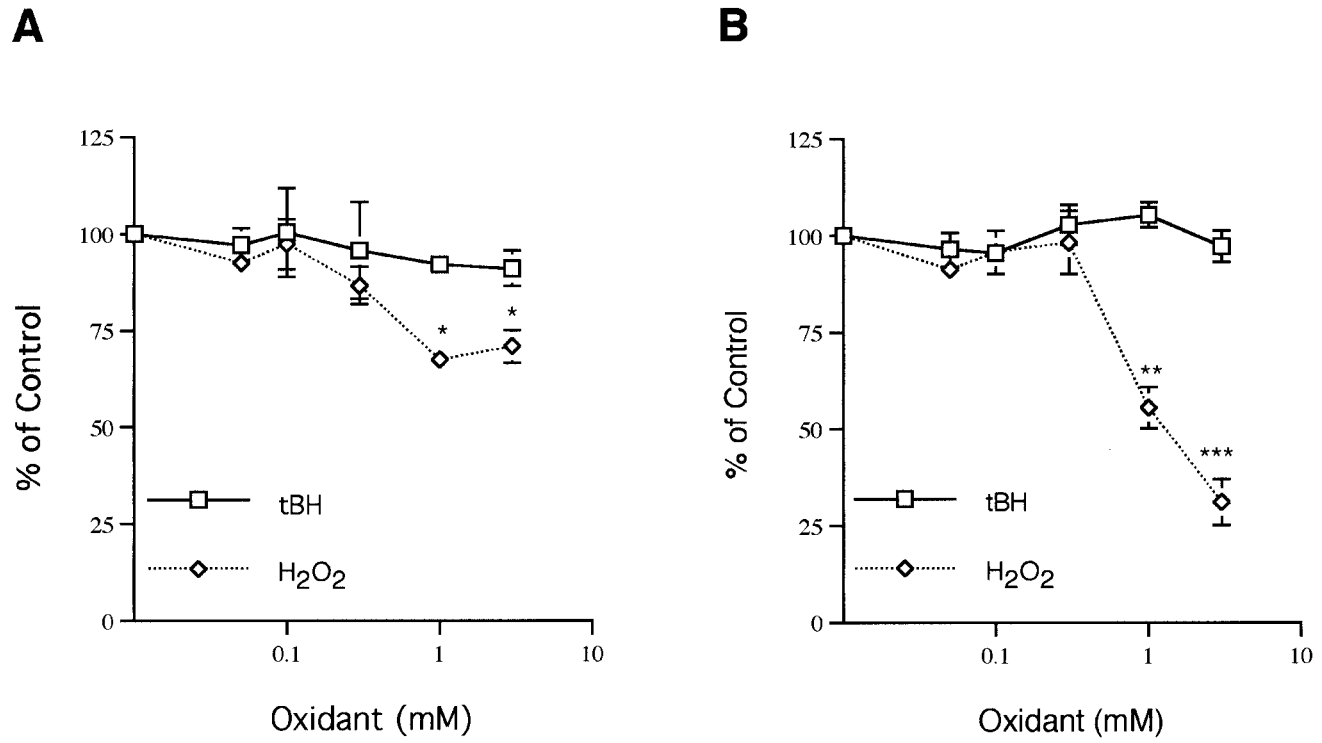

FIGURE 2. Viability of ARPE-19 cells as a function of tBH or H_2O_2. ARPE-19 cells were plated at 100,000 cells/cm^2 in 96-well plate and maintained in culture for 3 days or 3 months. The ARPE-19 undifferentiated (**A**) and differentiated (**B**) cells were treated with tBH or H_2O_2, and cell viability was measured by using the cell-proliferation reagent WST-1. Three independent experiments were performed in triplicate. Data are shown as mean ± SE of three experiments. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$; significant difference from control.

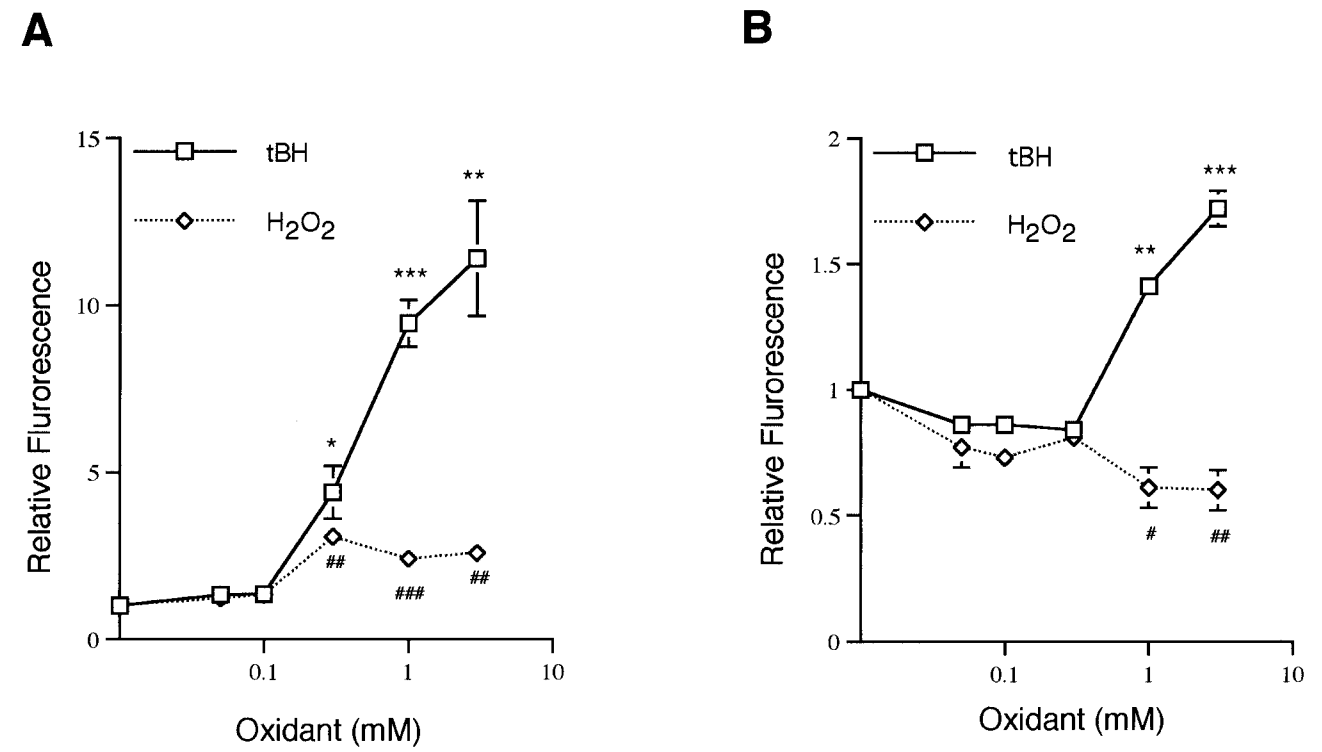

FIGURE 3. Fluorescent detection of intracellular ROIs. ARPE-19 cells were seeded in 96-well plates at 100,000 cells/cm^2 and maintained in culture for 3 days or 3 months. After 3 days or 3 months the medium was removed and cells were loaded with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). After incubation at 37°C for 30 minutes, the dye was removed and the cells were washed once with HBSS. Various doses of tBH or H_2O_2 were added to the undifferentiated (**A**) and differentiated (**B**) ARPE-19 cells and incubated for 30 minutes followed by washing. After 30 minutes, the intracellular ROI production was measured and quantified. Three independent experiments were performed in triplicate. Data are shown as mean ± SE of three experiments, except when the SE is too small to be seen. $^*P < 0.05$ (tBH), $^{**}P < 0.005$ (tBH), $^{***}P < 0.0005$ (tBH), $^{\#}P < 0.05$ (H_2O_2), $^{\#\#}P < 0.005$ (H_2O_2), $^{\#\#\#}P < 0.0005$ (H_2O_2); significant difference from control.

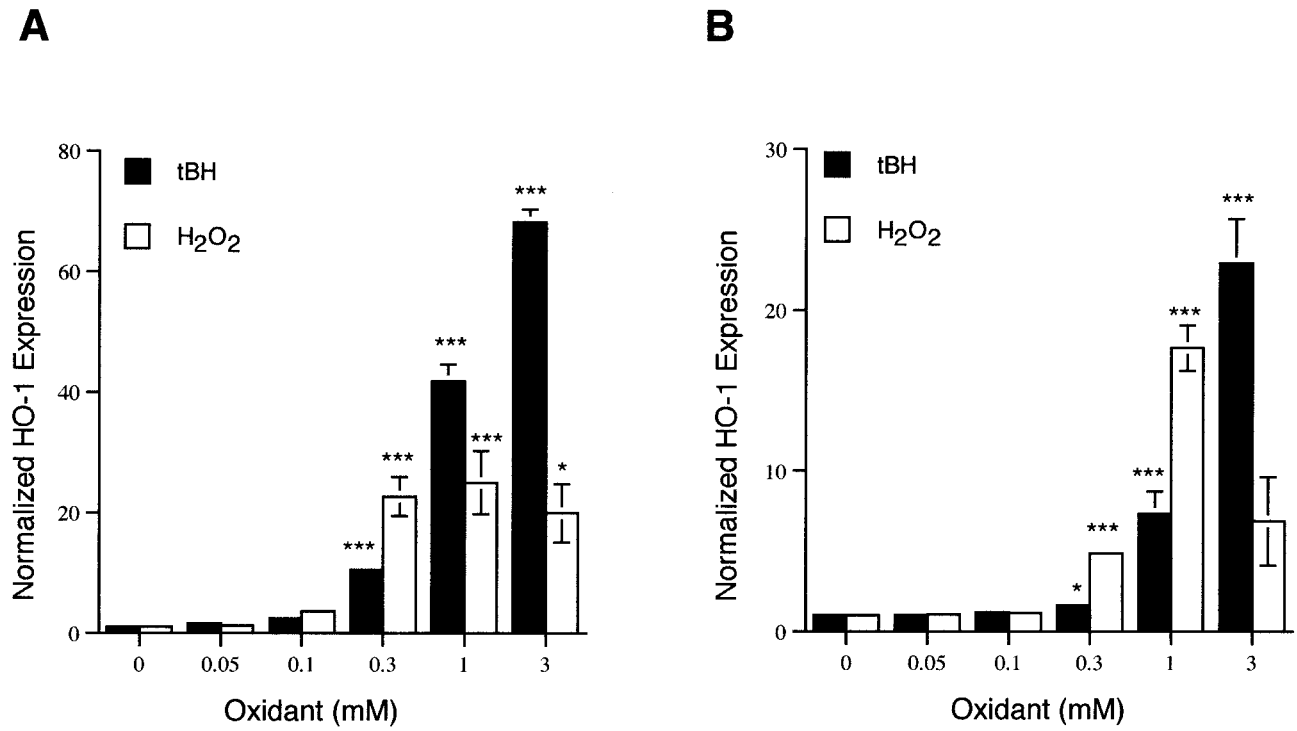

FIGURE 4. Expression of *HO-1* steady state mRNA by tBH or H_2O_2 treatment in ARPE-19 cells. ARPE-19 cells were plated at 100,000 cells/cm^2 in complete medium, maintained in culture for 3 days or 3 months, and then serum-starved for 48 hours. Chemical treatments such as tBH or H_2O_2 were performed in HBSS for 30 minutes at various concentrations (50 μM, 100 μM, 300 μM, 1 mM, and 3 mM). After 30 minutes the media were removed and replaced with the original culture medium, and the RNA was harvested after 4 hours for Northern blot analysis. The results are presented for undifferentiated (**A**) and differentiated (**B**) ARPE-19 cells relative to the untreated control at each indicated concentration. Three independent experiments were performed in duplicate. Data are shown as mean ± SE of three experiments, except when the SE is too small to be seen. $^*P < 0.05$, $^{***}P < 0.0005$; significant difference from control.

statistically significant changes in *FGFR1* and *FGFR2* gene expression occurred in differentiated cells treated with 3 mM H_2O_2 (data not shown). However, at this concentration, only 25% of cells were viable.

The expression of the RPE-specific markers *CRALBP* and *RPE65* was examined after treatment with tBH and H_2O_2. The expression of *CRALBP* and *RPE65* mRNA declined in a dose-dependent manner after tBH treatment in differentiated cultures (Fig. 6). Treatment of differentiated APRE-19 cells with 300 μM H_2O_2 resulted in no significant change in *CRALBP* and *RPE65* expression (data not shown).

DISCUSSION

The data presented in this study support the hypothesis that oxidative stress differentially alters the expression of *FGFR*s in differentiated ARPE-19 cells. *FGFR2* mRNA expression was downregulated, whereas *FGFR1* mRNA expression was upregulated, in ARE-19 cells as a function of treatment with tBH. The RPE-specific *CRALBP* and *RPE65* genes were also downregulated as a function of treatment with tBH. In contrast, H_2O_2 did not significantly alter *FGFR* expression in differentiated cells, although both tBH and H_2O_2 elevated *HO-1* gene expression, a general marker of oxidative stress.

Our laboratory has developed a fluorometric microplate assay for the detection of ROIs in ARPE-19 cells based on the original report by Rosenkranz et al.[19] H_2O_2 treatment generated far less fluorescence than tBH treatment, although positive results for *HO-1* expression were seen with both treatments. These results may be explained in several ways. The high catalase activity in RPE cells may react very efficiently with H_2O_2 to form water and molecular oxygen,[5,22,23] whereas tBH is not a substrate for catalase.[24,25] Alternatively, tBH and H_2O_2 may generate different cytosolic- or membrane-bound ROIs, which in turn, have different sensitivities of detection in our assay. Finally, technical issues related to the half-life of these chemical oxidants in solution may have complicated our results.

The oxidative stress response in differentiated cells also appeared to be quite different from the response seen in undifferentiated cells. For example, Franco et al.[26] compared the sensitivity of differentiated myotubes and undifferentiated myoblast cultures to oxidative injury. Their study indicates that antioxidant enzyme transcript and activity levels decrease with cellular differentiation, and therefore differentiated myotubes are more susceptible to oxidative injury. As animal cells lose their mitotic activity during differentiation, the level of glutathione (GSH) decreases. In contrast, the level of GSH increases during cellular proliferation when cells are in the mitotic phase.[27] Our own data on cell viability after H_2O_2 treatment in undifferentiated and differentiated cells parallel these observations.

Together, these findings allowed us to investigate how oxidative stress affects the expression of several genes that are transcribed only in the differentiated state of ARPE-19 cells. Our studies with H_2O_2 were complicated due to the loss of cell viability at concentrations above 300 μM, the concentration range in which all changes in gene expression were found. We measured cell viability and gene expression after 4 hours of treatment. Nonviable cells at 4 hours were most likely the result of necrosis due to H_2O_2 toxicity. Although apoptosis commonly results from oxidative stress, results in the literature demonstrate that early and late events occur in RPE cells in the range of 6 to 10 hours after

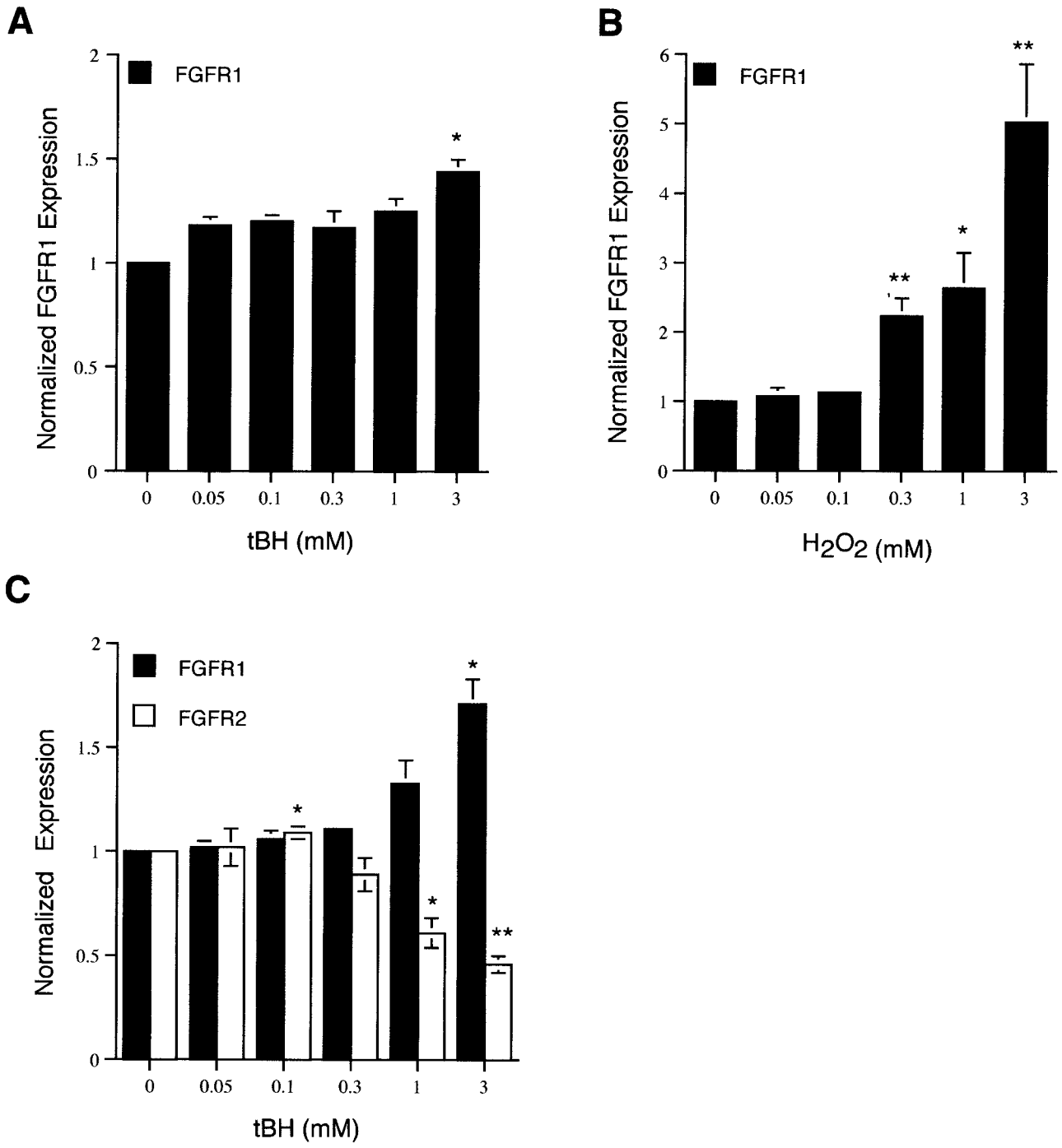

FIGURE 5. Northern blot analysis of *FGFR1* and *FGFR2* steady state mRNA levels in ARPE-19, as function of tBH or H_2O_2 treatment. Total RNA was isolated from ARPE-19 cells, and Northern blot analysis was performed. Blots were normalized against 28S rRNA and quantified. Normalized expression of *FGFR1* in undifferentiated cells is plotted as a function of tBH (**A**) or H_2O_2 (**B**) concentration. Normalized expression of *FGFR1* and *FGFR2* in differentiated cells are plotted as a function of tBH concentration (**C**). Three independent experiments were performed in duplicate. Data are shown as mean ± SE of three experiments, except when the SE is too small to be seen. $^*P < 0.05$, $^{**}P < 0.005$; significance from control.

tBH treatment.[7] This observation makes gene expression at 4 hours more likely to be the result of signal transduction directly related to oxidative stress. As a set, however, *FGFR2*, *CRALBP*, and *RPE65* were downregulated by oxidative stress generated by tBH above 1 mM in cells that were viable. This observation suggests, but does not prove, a more general relationship between oxidative stress and the differentiation status of RPE cells.

Growing sets of studies have documented differentiation-specific gene expression in ARPE-19 cells.[8,14] Based on these previously published data, we elected to study only the ARPE-19 cell line. We have shown that differentiation of ARPE-19 cells leads to a dramatic upregulation of *FGFR2* expression, whereas proliferating cells express *FGFR1*.[14] The differentiated state of ARPE-19 cells is similar, but not identical with the FGFR expression pattern of the RPE in vivo. Our

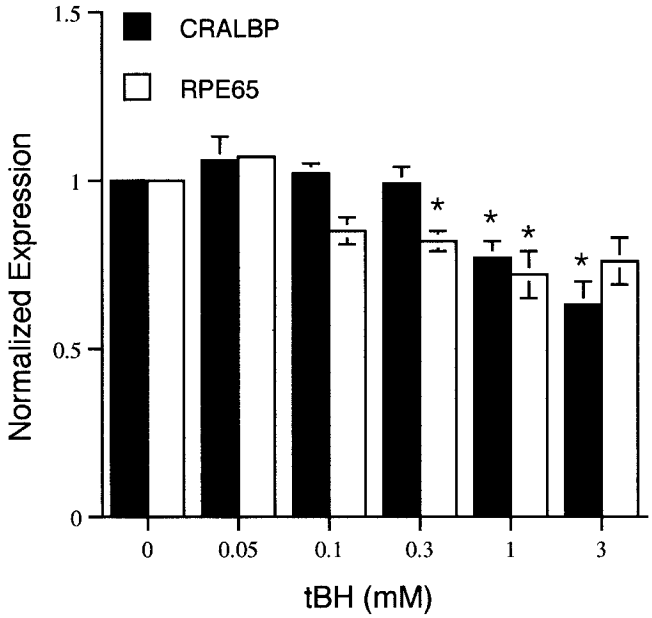

FIGURE 6. Northern blot analysis of *CRALBP* and *RPE65* steady state mRNA levels in differentiated ARPE-19, as function of tBH treatment. Total RNA was isolated from differentiated ARPE-19 cells after treatment with tBH and Northern blot analysis was performed. Steady state mRNA levels for *CRALBP* and *RPE65* (differentiated cells) are plotted as a function of tBH concentration. Blots were normalized against 28S rRNA and quantified. Three independent experiments were performed in duplicate. Data are shown as mean ± SE of three experiments, except when the SE is too small to be seen. $^*P < 0.05$; significance from control.

previous study showed that *FGFR3* is expressed in vivo, but not in differentiated ARPE-19 cells.

We believe that the downregulation of differentiation-specific gene expression may be significant in diseases in which abnormal cell differentiation occurs. Oxidative stress to RPE cells may be directly involved in the pathogenesis of ARMD.[2] It has been shown that the antioxidant capacity of the RPE decreases with age. Cohen et al.[28] demonstrated that GSH levels, glutathione reductase activity, and peroxidase activity decreases with age or in patients with ARMD. Liles et al.[22] reported decreased catalase activity with age and ARMD in both macular and peripheral RPE. Furthermore, Frank et al.[29] found decreased HO-1 immunoreactivity in the RPE of aged and ARMD specimens. It is therefore possible that enhanced oxidative stress in aging RPE cells differentially regulates *FGFR* and other differentiation-specific gene expression in vivo.

The functional consequence of these alterations may be loss of differentiated properties in the RPE, which in turn could contribute to the pathogenesis of diseases such as ARMD. FGFR2 for example, is a specific receptor for FGF9,[30,31] the expression of which we have recently demonstrated in human RPE and photoreceptors.[32] Loss of proteins involved in the vitamin A cycle such as RPE65 and CRALBP could also have direct functional and viability consequences on photoreceptors. The relationships among oxidative stress, gene expression, cell differentiation, and disease should be fruitful areas for future investigation.

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