Repetitive Nonlethal Oxidant Injury to Retinal Pigment Epithelium Decreased Extracellular Matrix Turnover In Vitro and Induced Sub-RPE Deposits In Vivo

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Purpose. To determine the impact of repetitive nonlethal oxidant injury with hydroquinone (HQ) on regulation of cell membrane blebbing and molecules, which are essential in extracellular matrix turnover (ECM) maintenance, especially matrix metalloproteinase (MMP)-2, tissue inhibitor of MMP (TIMP)-2, and type IV collagen in cultured RPE. In addition, to determine whether chronic oral HQ causes induction of sub-RPE deposit formation in a mouse model.

Methods. An ARPE-19 cell line stably expressing membrane-targeted green fluorescent protein (GFP) was challenged by exposure to HQ (100 μM). Repetitive acute (6 hours every 3 days for 4 weeks) or transient (6 hours followed by a recovery phase, every 5 days for 6 weeks) exposure to HQ were evaluated. An MTS assay, cell counts, and bromodeoxyuridine (BrdU) incorporation were used to detect cell viability and proliferation. Supernatants and cell homogenates were collected to assess MMP-2 and TIMP-2 activity by zymography and reverse zymography, proteins by Western blot, and type IV collagen accumulation by ELISA and immunostaining. Expression of MMP-2 and type IV collagen was examined by real-time RT-PCR on total RNA. Sixteen-month-old C57BL/6 female mice were fed a regular fat diet, with or without HQ (0.8%) in the drinking water, for 4 months. The eyes were removed for transmission electron microscopy of the retina and choroid after treatment. Semiquantitative grading of deposit severity was performed.

Results. In vitro, high doses of HQ (400–250 μM) killed a significant fraction of RPE cells (~60% of control). Low doses (50–100 μM) were nonlethal but induced significant blebbing. Both nonlethal repetitive acute and transient exposure to HQ were associated with diminished MMP-2 activity and increased collagen type IV accumulation. In vivo, mice exposed to oral HQ demonstrated moderately thick basal laminar deposits and a variable degree of deposits within Bruch's membrane (BrM). These homogeneous sub-RPE deposits accumulated in the eyes, consistent with early laminar deposits.

Conclusions. In cultured RPE, nonlethal injury with HQ upregulated nonlethal blebbing and decreased ECM turnover. Similarly, in vivo exposure to oral HQ induced nonlethal bleb injury and sub-RPE deposits. These data support the hypothesis that HQ may regulate blebbing and molecules that influence ECM turnover. This study suggests that HQ may be another type of oxidant that causes injury to the RPE and may explain the association between environmental oxidants and early AMD. (*Invest Ophthalmol Vis Sci.* 2006;47:4098–4112) DOI: 10.1167/iovs.05-1230

Age-related macular degeneration (AMD) is the most common cause of blindness in the elderly in developed countries.[1] It has been suggested that both the incidence and severity of this disease have increased among populations exposed to Western lifestyle and urban environments.[1–3] However, the pathogenic mechanisms whereby environmental factors contribute to the induction of AMD remain elusive. There is growing evidence that cumulative oxidant injury to the retinal pigment epithelium (RPE) plays an important role in development of AMD. Thus, toxic substances associated with lifestyle such as oxidative stressors may directly contribute to the development of AMD.[1–3]

Most studies on early development of AMD have focused on oxidative injury affecting the RPE. This type of oxidative insult induces a set of profound physiological responses in RPE consisting of cell membrane blebbing, which is compatible with the continued survival of the cell but leads to dysfunction in the tissue or organ without initiation of cell death.[4] In particular, the oxidant-mediated death of RPE, a very late stage of dry AMD, has been largely addressed by the literature.[5–10] However, our goal was to determine, at earlier stages of dry AMD, whether oxidant injury can dysregulate the degradation of the extracellular matrix (ECM) that accumulates between the basal lamina of the RPE and the inner of Bruch's membrane (BrM). This process leads to accumulation of ECM deposits, a hallmark of early AMD that develops decades before the RPE actually dies. Thus, nonlethal cellular responses to RPE oxidant injury may contribute to early AMD.

The concept of nonlethal cell membrane blebbing as a possible pathogenic mechanism in drusen formation was introduced 25 years ago,[11–15] suggesting the role for blebs in the sub-RPE deposit accumulation and progression to drusen in vivo. We have recently demonstrated that exposure for a short time to nonlethal oxidant injury to the RPE cells induces nonlethal cell membrane blebbing,[16] a process that we propose is related to deposit formation in AMD later on.

Another injury response relevant to AMD is imbalanced ECM turnover. It has been shown that relatively small dysregulation in the relative production of ECM proteins like matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), and collagen IV[17,18] may lead to net changes in the ECM, including thickening and deposit formation.[19,20] Accordingly, dysregulated turnover of ECM is a major mechanism of disease pathogenesis in many tissue sites, including renal disease, atherosclerosis, lung disease, and others.[18–21] Unfor-

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tunately, minimal information is available concerning normal turnover in healthy BrM or imbalanced turnover in AMD.

Our group has recently demonstrated that sustained, a brief, nonlethal oxidant injury to the RPE induces a wide range of changes in gene expression, especially for those genes involved in regulation of extracellular matrix,[22] and decreases MMP-2 activity without modification in collagen type IV accumulation.[16] Therefore, the regulation of collagen synthesis and secretion by brief exposure to oxidants has been well documented in nonocular and ocular tissues.[23–26] However, minimal data are available regarding the role of prolonged nonlethal oxidant injury to the RPE in the regulation of ECM turnover.

To our knowledge, there is little information comparing differences in cellular responses in the setting of repetitive transient exposure to oxidant injury, like cigarette smoking, intense sunlight or the more sustained exposure to oxidants that may occur in diseases associated with circulating plasma oxidants. Accordingly, in the present study, the effect of repetitive, prolonged nonlethal oxidant injury in regulating cell membrane blebbing and molecules relevant to ECM accumulation (i.e., MMP-2, TIMP-2, and collagen type IV) was examined. We compared repetitive acute with repetitive transient exposure to oxidant stimuli and extended the in vitro observations to a more physiological environment, using the mouse model of dry AMD published by our laboratory.[27–30] We provided an alternative source of oxidant stimulus by replacing exposure to blue light with exposure to HQ in drinking water, and evaluated the impact of this compound on the development of sub-RPE deposits. In vitro, we found that both repetitive acute and transient oxidant injuries caused nonlethal blebbing, decreased release of active MMP-2 and increased collagen type IV accumulation. In addition, oxidant injury did not affect TIMP-2 activity and protein expression. None of these effects recovered to baseline after cessation of both the acute and the final transient oxidant exposure. In vivo, we observed accumulation of moderately dense homogeneous and granular material between the RPE and its basement membrane and occasional blebs. BrM was thickened with coated vesicles and membranous profiles, and basal laminar deposits (BLDs) with banded structures were often present. These results suggest that repetitive treatment with nonlethal oxidative stimulus favors accumulation of the sub-RPE deposits. These observations may be of relevance to AMD.

Materials and Methods

Cell Culture Conditions

ARPE-19 cells stably expressing green fluorescent protein (GFP) targeted to the inner leaflet of the plasma membrane (GFP-ARPE-19) were generated as described.[31] ARPE-19 cells were split and then plated at subconfluent density onto T-75 (75 cm^2) flasks and grown to confluence in maintenance medium (Dulbecco's modified Eagle's medium-Ham's F12 (DMEM)/F12; 1:1 vol/vol) supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine, 100 μg/mL penicillin-streptomycin, and 0.348% Na_2HCO_3 in a 10% CO_2 humidified air incubator at 37°C. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). The cells were then subcultured, propagated, and maintained in the same medium. For experiments, cells were seeded at subconfluent density in plates coated with 0.5 mg/mL collagen IV (Sigma-Aldrich, St. Louis, MO) and 0.5 mg/mL laminin (Invitrogen) mixed 1:1 vol/vol and grown to confluence.

Cell Viability Assay

Confluent GFP-ARPE-19 cells were split and plated onto 96-well plates coated with collagen IV/laminin at a density of 10,000 cells per well. After 4 days, they were rendered confluent. At the time of confluence, the cells were prepared for the experiment by changing the maintenance medium to the assay medium (i.e., maintenance medium without phenol red) for 2 days. This medium was then replaced for 1 day with assay medium that was supplemented with 1% FBS instead of 10%. Subsequently, the medium was changed to the assay medium supplemented with 0.1% FBS. At this time, the cells were treated with different concentrations of hydroquinone (HQ: Sigma-Aldrich, St. Louis, MO) for 6 hours or 100 μM HQ for different duration. The number of surviving cells was measured by cell counting (Coulter ZI cell counter; Beckman Coulter, Hialeah, FL), and by MTS (a tetrazolium salt) assay (Cell Titer 96 Aqueous One Solution kit; Promega, Madison, WI) after a 24-hour recovery period.

RPE Membrane Blebbing

Confluent GFP-ARPE-19 cells were split and plated onto six-well plates coated with their matrix environment, collagen IV/laminin at a density of 200,000 cells per well. At the time of confluence, the cells were prepared for the experiment for 3 days, as described previously, and then incubated with or without 100 μM HQ in assay medium supplemented with 0.1% FBS for 6 hours. After exposure to HQ, cells were examined under a fluorescence microscope (Axiophot; Carl Zeiss Meditec, Inc., Oberkochen, Germany).

HQ Injury

Cells were plated at subconfluent density (2×10^5 cells) onto six-well plates coated with collagen IV/laminin. At the time of confluence, cells were prepared for the experiment as described in previous sections. At this time, 100 μM HQ was added for 6 hours every 3 days for 4 weeks and/or for 6 hours (acute transient injury phase) followed by reassessment during the subsequent 6 to 72 hours (recovery phase) every 5 days for 6 weeks. Culture medium was withdrawn, and cells were washed two times with phosphate-buffered saline (PBS). After that, fresh assay medium supplemented with 0.1% FBS was added for 24 hours. Cells were harvested for protein and/or RNA assessment and for quantification of collagen type IV accumulation. Supernatants were also collected to measure MMP-2 and TIMP-2 protein expression and activity. Protein was quantified in all samples by the Bio-Rad method (Hercules, CA).

RPE Cell Proliferation Assay

Proliferation was determined by three different methods: MTS assay, cell count (as described previously), and quantitative cellular enzyme immunoassay (Biotrak; GE Healthcare, UK), using mAbs directed against bromodeoxyuridine (BrdU).

Cells were seeded in 96-well collagen-laminin–coated plates and treated with HQ as described in the HQ injury section. After that, the cells were labeled with 10 μM BrdU (100 μL/well) and incubated for 4 hours at 37°C. The cells were fixed, and genomic DNA was denatured by adding 200 μL/well of blocking reagent (1:10) for 30 minutes at room temperature. Peroxidase-labeled anti-BrdU antibody (1:100) was added (100 μL/well) and incubated for 90 minutes at room temperature. After the cells were washed three times, TMB (3, 3′5,5′-tetramethylbenzydine) substrate solution was added (100 μL/well) and incubated for 15 minutes at room temperature to elicit color. Optical density was measured using a microplate reader set at an absorbance wavelength of 450 nm. Absorbance values correlated directly to the amount of DNA synthesis and thereby to the number of proliferating cells in culture.

MMP-2 Activity and Protein Expression

Culture medium was collected 24 hours after treatment and then centrifuged at 13,000*g* for 30 minutes at 4°C. Insoluble material was removed, and the supernatant collected. Protein quantification was determined as described earlier, and MMP-2 activity and protein expression were assessed by zymography and Western blot, respectively, as described previously.[16] Gels were analyzed by densitometry using ImageJ densitometry program (ver. 1.17; available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD), to determine relative MMP-2 activity. Each zymography assay was repeated

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at least three times. Inhibition of gelatinase activity was assayed by incubating gels with 1 mM EDTA, a specific metalloproteinase inhibitor (data not shown).

Degradation of Active MMP-2 Induced by HQ

We evaluated whether degradation of active MMP-2 induced by an oxidant might have caused decreased release of active MMP-2 into the supernatant. For this study, an equal amount of active MMP-2 was placed on top of cell culture inserts (BD Biosciences, Bedford, MA) and 100 μM HQ was added for 6 and 24 hours. Then, medium was centrifuged at 15,000*g* for 30 minutes at 4°C, the supernatant collected and protein content determined by BCA assay. MMP-2 activity was assessed by gelatin zymography, using 10 μg of protein from each sample.

Reverse Zymography

Culture medium was collected and centrifuged to remove cellular debris. Protein quantification was determined by BCA assay. Then, samples were diluted as needed in Laemmli's buffer and combined with an equal volume of Tris-glycine SDS (Novex, San Diego, CA).

For the sample buffer (Invitrogen Corp.), 10 μg of protein extracts from each experimental condition were loaded. TIMP-1 standard was loaded on each gel (EMD Biosciences, Inc., San Diego, CA). Protein samples were electrophoresed at 75 V for 2.5 hours on a standard separating gel composed of 2.25 mg/mL porcine gelatin, 0.25 M Tris-HCl (pH 8.8), 0.125% SDS, 1 μL/mL TEMED, 0.4 mg/mL ammonium persulfate, 15% acrylamide and 0.4% bisacrylamide, and 100 ng/mL proenzyme MMP-2 (EMD Biosciences, Inc.). A 4% stacking gel was used. After electrophoresis, gels were incubated in 1× zymogram renaturing buffer (Novex) with gentle agitation for 3 hours at room temperature, replacing the solution every hour. Then, the renaturing buffer was replaced with 100 mL 1× developing buffer (Novex). Gels were then incubated at 37°C overnight. Each gel was stained with 0.5% Coomassie Blue G250 in 30% methanol/10% acetic acid for 4 hours followed by four washes of destaining solution of 30% methanol/10% acetic acid for 1, 15, 30, and 60 minutes. Gels were further destained in 1% TritonX-100 solution for 1 hour and stored in distilled water until densitometry was performed. Gels were analyzed by densitometry using ImageJ 1.17 software as described earlier.

TIMP-2 Expression by Western Blot Analysis

Culture medium was collected after due treatment, and then centrifuged at 13,000*g* for 30 minutes at 4°C. Insoluble material was removed and the supernatant collected. Protein quantification was determined by BCA protein assay. Ten micrograms of protein extracts from each experimental condition were denatured with SDS sample buffer followed by 5 minutes of boiling and then were resolved by 10% to 12% polyacrylamide gel (Novex). Proteins were transferred in 1× transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol [pH ~ 8.4]) into a 0.45-μM polyvinyl difluoride membrane (Immobilon-P; Millipore Corp., Billerica, MA), using a transfer cell (mini-PROTEAN II; Bio-Rad Laboratories, Inc., Hercules, CA) set at a constant voltage of 120 mV for 2 hours. Membranes were then blocked in a 5% nonfat dry milk TBS-T solution for at least 1 hour at room temperature. Incubation with the primary antibody (polyclonal anti-TIMP-2 antibody, 1 μg/mL; Chemicon International, Temecula, CA) proceeded overnight at 4°C. Membranes were washed four times with TBS-T, incubated with horseradish peroxidase-linked donkey anti-rabbit antibody (1:1000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 hours at room temperature and then washed four times with TBS-T. Immunoreactive bands were detected as described previously. Relative TIMP-2 band intensities were determined by the ImageJ 1.17 densitometry program.

MMP-2 mRNA Levels

Total RNA was extracted and reverse-transcription was performed as previously described.[16] Every sample was normalized to the 18S transcript content. The primer probe mixture was purchased from Applied Biosystems (ABI; Foster City, CA) and used as specified by the manufacturer. The standard curves for MMP-2, and 18S were generated with serially diluted solutions (0.001–100 ng) of mRNA from cultured RPE cells. PCR assays were conducted in duplicate for each sample. Data are expressed as a percentage of untreated cells and represent the mean ± SEM of four independent experiments run in triplicate.

Assessment of Collagen Type IV

Cell layers were collected after 24 hours of incubation, and an ELISA assay was performed as described.[16] Concentrations of collagen type IV standards were 0 to 3 ng/well. Three independent experiments were performed in duplicate. Final values were expressed as nanograms per 10^5 cells and results as a percentage of control (untreated cells).

Immunostaining for Collagen Type IV

Changes in the cellular production of collagen type IV was also determined by immunostaining as in other studies.[32,33] Confluent cells were split and plated at subconfluent density (1×10^5) onto 24-well plates containing thin inserts (1.0 μm) with collagen type IV/laminin. At the time of confluence, cells were treated with 100 μM HQ, as described in the HQ injury section. Twenty-four hours after the last injury, cells were washed two times with PBS, fixed with 2% paraformaldehyde for 10 minutes at room temperature, and permeabilized with 1% Triton X-100. After blocking with 5% BSA, they were incubated with antibody against human collagen IV diluted 1:200 (Biodesign International, Saco, ME) overnight at 4°C, followed by application of an Alexa Fluor goat anti-rabbit IgG diluted 1:500 (Invitrogen, Inc., Eugene, OR) for 2 hours at room temperature. Inserts were mounted and images examined under an fluorescence microscope (Axiophot; Carl Zeiss Meditec, Inc., Oberkochen, Germany). Collagen intensity was measured from 20× magnification digital images (Photoshop 6.0; Adobe Systems, Inc., Mountain View, CA) as described in other studies.[32,33] Results are expressed as a percentage of the control (untreated cells).

Type IV Collagen mRNA Levels

mRNA for collagen α1 (IV) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time polymerase chain reaction (PCR; Prism 7700; ABI). The forward and reverse primers (Invitrogen-Gibco BRL) and probes (*Taq*Man; Perkin-Elmer Biosystems, Wellesley MA) for each molecule were 5′-ACTCTTTTGTGATGCACACCA-3′, 5′-AAGCTGTAAGCGTTTGCGTA-3′ and 5′-AATGGCGCACTTCTAAACTCCTCCAGGCAGG-3′ [collagen α1(IV)]; 5′-TTCCAGGAGCGAGATCCCT-3′, 5′-CACCCATGACGAACATGGG-3′, and 5′-CCCAGCCTTCTCCATGGTGGTGAA-3′ (GAPDH). The probe sequence for each transcript was chosen over an exon–intron junction to prevent amplification of genomic DNA. The 5′-end of the collagen probe was labeled with the reporter dye tetrachloro-6-carboxyfluorescein (TET), and the 5′-end of the GAPDH probe was labeled with 6-carboxyfluorescein (6-FAM). The 3′-ends of all probes were labeled with the quencher dye 6-carboxyfluorescein (TAMRA). The PCR reaction contained 100 nM (GAPDH) or 80 nM (collagen) probe, 300 nm/L both primers master mix (*Taq*Man Universal PCR Master Mix; Perkin-Elmer), including 300 μm/L dNTP, 2.5 mM $MgCl_2$, 0.5 U of AmpErase UNG, and 1.25 U of DNA polymerase (Ampli-*Taq* Gold). Reactions were performed in optical 96-well reaction plates covered with optical caps (Perkin-Elmer). Amplification cycles were 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. Signals for collagen in each sample were standardized against the GAPDH mRNA signal. All measurements were performed in duplicate. As standards, each PCR was run on a fivefold dilution range of 2 pg of plasmid containing the appropriate template.

Mice

Sixteen-month-old, female C57BL/6 mice (National Institute of Aging, Bethesda, MD) were used in the study. All experiments were conduced according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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To evaluate the effect of HQ on sub-RPE deposit formation, two groups of mice were studied: mice on a regular fat diet (Diet 5001; PMI Nutrition International Test Diet, Richmond, IN) without HQ for 4 months ($n = 6$, group 1) and mice on regular fat diet with 0.8% HQ in drinking water for 4 months ($n = 6$, group 2). Mice had free access to food and water, were housed in plastic cages, and were kept on a 12-hour light–dark cycle. After treatment, eyes were removed for transmission electron microscopy of the retina and choroid.

Serum Levels of HQ

Blood samples (850 μL) were removed by cardiac puncture and serum was obtained for determination of HQ concentration, using gas chromatography (National Medical Services, Willow Grove, PA).

Transmission Electron Microscopy

After enucleation, eyes were fixed in 3% glutaraldehyde and 2% paraformaldehyde in PBS overnight. Lens was removed, and the posterior segment (retina, choroids and sclera) was quadrisected to contain the perioptic nerve portion at the apex and the ciliary body at the base. The superotemporal quadrant of the retina, choroids, and sclera was submitted for electron microscopic sectioning. The tissue was fixed in 1% osmium tetroxide for 1 hour, rinsed in PBS, dehydrated in ethanol and then embedded in Spur's resin. Thick (0.5–1.0 μm) and ultrathin sections (0.1 μm) were cut on a microtome (Porter Blum MT-2; Sorvall, Newtown, CT), stained with 4% uranyl acetate and lead citrate. Then, the sections were examined with a transmission electron microscope (CX-100; JEOL, Tokyo, Japan).

Semiquantitative Grading System

For each animal, a single cross section was examined, and low-power transmission electron micrographs (i.e., magnification, ×7200) were made of the entire section, from the perioptic to the ciliary body portion (approximately 10 micrographs). Then, one representative high-power micrograph (i.e., magnification, ×25,000) was made from each low-power section by an individual unaware of the experimental conditions and used for semiquantitative scoring. The high-power micrographs were graded by two independent examiners for the presence and severity of BLD. A severity score of 0 to 15 points was determined for each section by summation of the median scores of all the micrographs from a section on each of four different categories of abnormalities (from 0–3 points for each): continuity of BLD (score: 0, no BLD16 months old; 1, occasional BLD16 months old i.e., focal nodule; 2, BLD16 months old, extending under fewer than two RPE cells; and 3, BLD16 months old extending under two or more RPE cells); maximum thickness of BLD (score: 0, no BLD; 1, flat BLD16 months old; 2, deposits thickness <25% of RPE cell cross-sectional thickness; and 3, deposit thickness ≥25% of RPE cell cross-sectional thickness); nature of deposit content (score: 0, no BLD16 months old; 1, homogeneous BLD16 months old; 2, any banded structures within BLD16 months old; and 3, three or more banded structures within BLD16 months old); presence of BrM abnormalities (score: 0, no abnormalities; 1, collagenous thickening, no deposit; 2, thickening with circular profiles or nonspecific debris; and 3, presence of basal linear deposits represented as banded structures, granular material or membranous debris); and assessment of other choriocapillaris endothelial damage or invasion (score 0, no alterations; 1, loss of fenestrations; 2, loss of fenestrations and thickening; and 3, choriocapillaris invasion into BrM). BrM thickness was also directly measured in three different standardized locations in each image and then averaged to provide a mean score for that micrograph. The mean of 10 micrographs was used to assign and "average" BrM thickness for an individual specimen.

Groups were compared by determining the mean and standard deviations. A *t*-test was used for statistical analysis of differences. In addition, the frequency of BLD was determined using two different criteria. "Any BLD" was defined as the presence of any discrete focal nodule of homogeneous material of intermediate electron density between the RPE cell membrane and BrM in at least one micrograph within a section. "Moderate BLD" was defined as the presence, in at least three micrographs, of the following: continuous BLD extending under two or more cells, deposit thickness equal to or greater than 25% of RPE cell cross-sectional thickness, or the presence of any banded structures within the BLD. Differences in the relative frequency were tested using χ^2 test (χ^2).

Statistical Analyses

All experiments were performed three to four times on cultured cells, with reproducible results. Data are expressed as a percentage of control. Results are the mean ± SEM of three to four independent experiments, performed in duplicate or triplicate (as indicated). One-way ANOVA and the Dunnett multiple comparison post-hoc tests were performed.

Results

Lethal and Nonlethal Injury after Oxidant Exposure

To determine the appropriate concentration and duration of exposure to HQ, concentration and time course of HQ exposure followed by determination of viability were performed in GFP-ARPE-19 cells. The number of surviving cells was measured by cell count 24 hours after oxidant injury with various concentrations of HQ applied for 6 hours. We found that high-dose HQ (400–250 μM) killed a significant fraction of RPE cells (~60% of control). However, concentrations of 100 μM HQ or less were nonlethal within 24 hours (Fig. 1A). Comparable results were observed for cell viability measured by MTS and BrdU assays (data not shown). Thus, exposure to 100 μM HQ or less for 6 hours was nonlethal.

Next, to determine the time course, we challenged the GFP-ARPE-19 cells with 100 μM HQ at different times. Cell counts were performed 24 hours after initiating oxidant exposure for 6, 8, 10, 12, 16, 18, 24, 48, and 72 hours. As shown in Figure 1B, exposure to 100 μM HQ resulted in RPE cell death only when present for more than 18 hours in the incubation medium, confirming previous reports of the relatively slow time course of HQ entrance and intracellular generation of oxidative reactants.[34,35] Based on these results, we focused our attention on the nonlethal concentration of 100 μM HQ and 6 hours of incubation time for the planned experiments in this study.

Induction of Blebbing in GFP-ARPE-19 Cells by Nonlethal and Lethal Oxidant Injury

RPE cells are able to tolerate oxidative stress without initiation of cell death.[4] These cells exhibit a distinct set of physiological responses, including cell membrane blebbing, when subjected to a nonlethal oxidative injury.[4]

The GFP-RPE-19 cells were used to evaluate membrane blebbing after exposure to nonlethal and lethal oxidant injuries. Figure 1D shows the extensive blebbing that occurs in GFP-RPE-19 cells after exposure to nonlethal concentrations of HQ for 6 hours. At 100 μM HQ, small blebs were apparent on the surface of the plasma membrane (Fig. 1D). At HQ concentration greater than 250 μM, many cells showed large protruding cell membrane blebs, as well as morphologic characteristics of cell death, such as cell shrinkage or detachment (data not shown).

Repetitive Oxidant Injury Did Not Induce Proliferation of RPE Cells

To discard a possible effect of HQ on RPE cell proliferation, cells were monitored by MTS assay, cell count, and BrdU incorporation, as described in the Materials and Methods section. Repetitive acute exposure to 100 μM HQ did not affect

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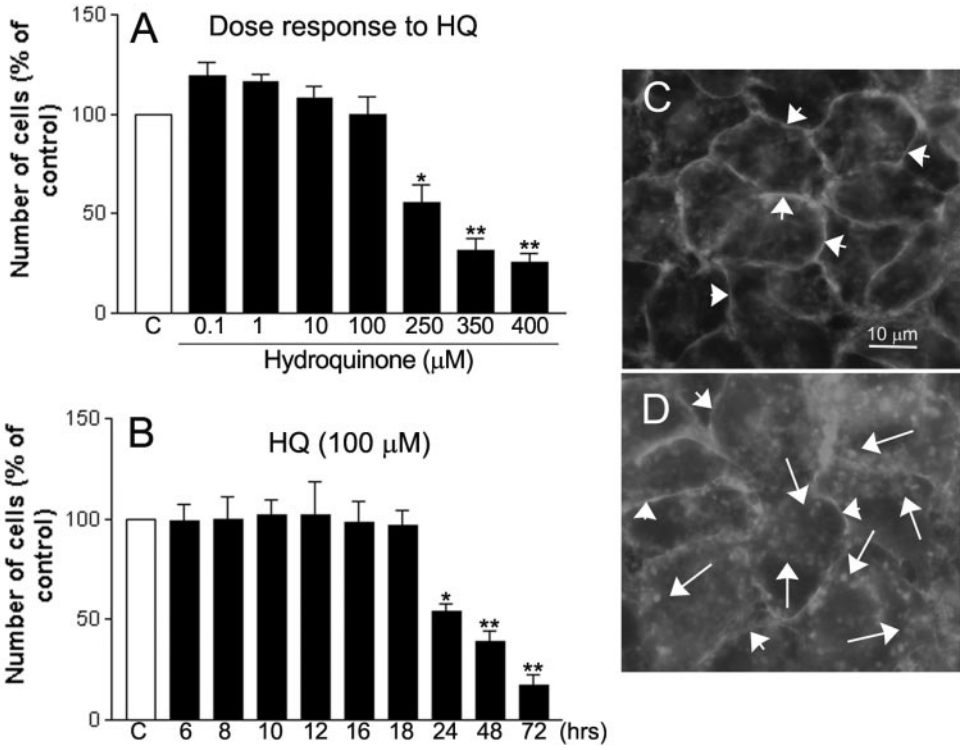

FIGURE 1. Concentration and time–response curves for cell count and induction of cellular changes in GFP-ARPE-19 cells. Confluent cells were exposed to various concentrations of HQ for 6 hours (**A**) or to 100 μM HQ for different times (**B**) and then washed with PBS and incubated in assay medium (0.1% FBS) for 24 hours. Data are expressed as the percentage of control and are shown as the mean ± SEM of results in three independent experiments run in triplicate. Statistical significance is indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$ for comparison to the control. (**C**, **D**) Fluorescent GFP-ARPE-19 derived blebs before and after exposure to oxidant-mediated injury with HQ. Cells were exposed to 100 μM HQ for 6 hours and observed immediately under a fluorescence microscope. (**C**) Control cells in which GFP is localized to the membrane (*arrows*). (**D**) The membrane blebs (*arrowheads*) and GFP were present in the membrane (*arrows*) after exposure to 100 μM HQ. Magnification, ×400.

the number of cells (Table 1). Therefore, there was no association with increased DNA synthesis (Table 1). Results observed for RPE cells that were repeatedly exposed to transient oxidant were of a magnitude similar to those observed in cells exposed to acute oxidant (Table 1). In summary, there was no significant change in RPE cell proliferation after treatment with 100

TABLE 1. Effect of Repetitive Acute and Transient HQ Oxidant Injury on the Number of Cells and Proliferation of ARPE-19 Cells

	1st Injury	2nd Injury	3rd Injury	4th Injury
Acute				
Number of cells % of control				
Control	100.0 ± 1.3	95.5 ± 10.8	96.6 ± 6.3	96.3 ± 2.8
6 hours	107.7 ± 11.4	98.1 ± 4.6	92.1 ± 5.7	93.7 ± 4.0
BrdU incorporation (% of control)				
Control	100.0 ± 1.6	96.5 ± 5.7	93.3 ± 6.2	90.6 ± 5.8
6 hours	103 ± 6.85	99.9 ± 10.3	87.6 ± 3.4	88.0 ± 7.4
Transient				
Number of cells % of control				
Control	100.0 ± 1.3	109.7 ± 6.2	113.6 ± 2.8	106.0 ± 8.8
6 hours	101.0 ± 5.9	115.6 ± 7.3	102.9 ± 5.3	116.0 ± 5.4
Control	111.0 ± 2.8	113.8 ± 6.1	114.0 ± 0.6	113.3 ± 3.2
24 hours	106.6 ± 7.64	104.6 ± 2.9	101.6 ± 2.9	119.0 ± 5.8
Control	106.1 ± 2.05	103.3 ± 3.8	102.1 ± 5.1	104.6 ± 3.8
48 hours	114.2 ± 2.8	109.3 ± 6.5	101.0 ± 4.4	107.0 ± 5.6
Control	116.6 ± 3.5	107.1 ± 2.6	105.6 ± 7.3	117.3 ± 7.0
72 hours	110.8 ± 7.3	112.3 ± 5.7	103.6 ± 8.0	110.0 ± 5.2
BrdU incorporation (% of control)				
Control	100.0 ± 0.2	108.6 ± 2.8	109.6 ± 5.0	114.6 ± 9.1
6 hours	103.4 ± 2.9	105.2 ± 5.7	114.0 ± 6.1	112.3 ± 6.7
Control	111.3 ± 3.8	109.3 ± 5.3	99.9 ± 2.1	116.4 ± 2.9
24 hours	112.8 ± 5.3	107.0 ± 5.9	105.9 ± 2.3	104.2 ± 8.2
Control	110.5 ± 5.8	116.0 ± 8.6	107.0 ± 5.4	110.7 ± 5.8
48 hours	113.2 ± 7.3	108.9 ± 8.9	105.6 ± 2.4	111.4 ± 3.7
Control	106.8 ± 5.6	115.6 ± 2.0	110.3 ± 8.4	119.6 ± 3.4
72 hours	109.7 ± 9.2	112.0 ± 5.1	106.0 ± 8.1	108.7 ± 4.4

Cell count after the repetitive acute and transient protocols. In the acute protocol, cells were exposed to 100 μM HQ for 6 hours every 3 days for 4 weeks. In the transient protocol cells were exposed to 100 μM HQ for 6 hours followed by reassessment during the subsequent 24, 48, and 72 hours every 5 days for 6 weeks. BrdU incorporation shows cell proliferation during the repetitive acute and transient HQ oxidant-injury, evaluated by BrdU incorporation. The results were expressed as the percentage of the control (first injury; untreated cells) and shown as means ± SEM of three independent experiments run in triplicate.

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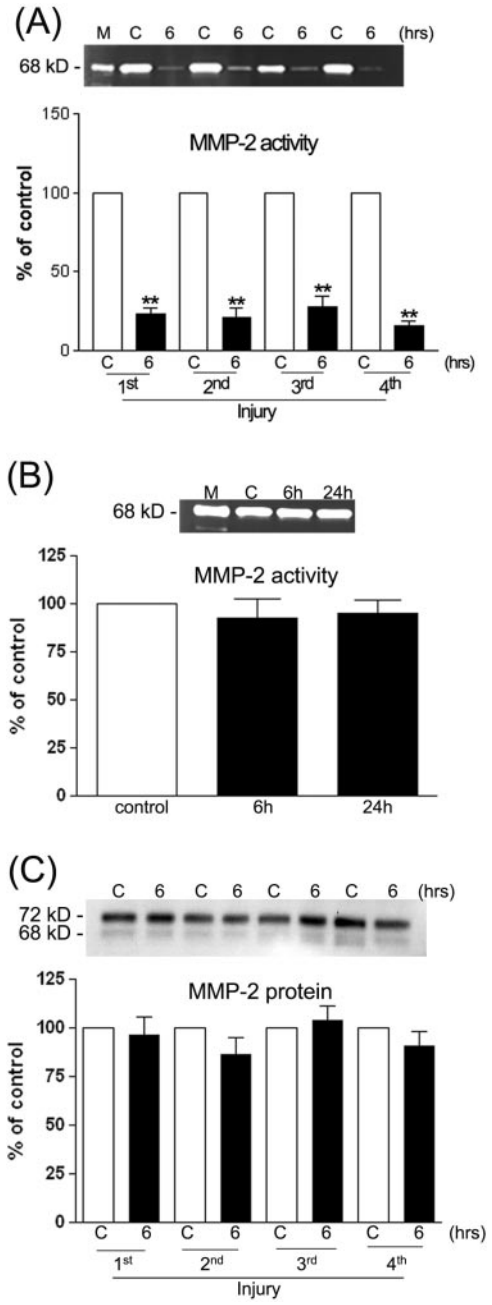

FIGURE 2. Repetitive acute exposure to HQ oxidant injury downregulated MMP-2 activity but did not modify MMP-2 protein. The number on the *left* of the zymograms (**A**, **B**, *top*) and Western Blot (**C**, *top*) represents the molecular mass. *Lane M*: molecular weight standard. *Lane C*: control. Graphs show average results of three independent experiments run in triplicate on cultured cells. (**A**) ARPE-19-derived MMP-2 protein activity evaluated by zymography in the presence of 100 μM HQ. Cells were exposed to HQ for 6 hours every 3 days for 4 weeks. $^{**}P < 0.01$, statistically significant difference) compared with the control. (**B**) Sustained HQ oxidant-mediated injury did not affect degradation of active MMP-2. An equal amount of active MMP-2 was treated with 100 μM HQ for 6 and 24 hours, respectively. (**C**) ARPE-19-derived MMP-2 protein expression evaluated by Western blot analysis. Data represent the relative amount of the 72-kDa form.

μM HQ in either repetitive acute or transient treatments, compared with untreated controls. It cannot be excluded that an adaptive response is a major antioxidant defense for RPE cells exposed to oxidizing microenvironments.

Effect of Repetitive Acute Oxidant Injury on MMP-2 Activity and Protein Expression in ARPE-19 Cells

Because of the importance of the extracellular matrix in RPE-related diseases, especially MMP-2, TIMP-2, and collagen type IV accumulation were examined after repetitive acute HQ injury to the ARPE-19 cells.

MMP-2 is secreted as a partially active proenzyme (72 kDa) which is sequentially processed to an intermediate and then to an active 68-kDa form.[36] Oxidant injury has been shown to regulate MMP-2 in nonocular tissues.[37–39] We have recently shown that brief, nonrepetitive sustained and transient oxidant injuries to the ARPE-19 cells increase the release of pro-MMP-2 but decrease the release of active MMP-2.[16] However, the regulation of MMP-2 activity by prolonged, repetitive nonlethal oxidant injury remains largely unknown. In the current stuy, we sought to determine whether repetitive acute oxidant injury has the ability to modulate MMP-2 activity and protein expression. We exposed RPE cells to HQ injury for 6 hours every 3 days for 4 weeks. By zymography, we found that repetitive acute oxidant-mediated injury downregulated MMP-activity released into the medium (Fig. 2A).

In the presence of 100 μM HQ for 6 hours, MMP-2 activity diminished by approximately 74% during the first and second injuries. MMP-2 activity was downregulated by approximately 70% during the third injury, and a further decrease in the activity of this MMP was observed after the last acute injury (5.8-fold decrease, $P < 0.01$).

To explain the decrease of active MMP-2 present in the supernatant during times at which one would expect the same or higher amounts of surface MMP-2 in the culture medium, we performed two types of analyses. First, we confirmed that the persistence of the oxidant within the culture medium did not degrade the enzymatic activity of exogenous MMP-2. As shown in Figure 2B, the presence of HQ did not cause a decline in the activity of exogenously added active MMP-2. Second, we used Western blot analysis to compare the impact of oxidant injury on the ratio of secreted pro-MMP-2 protein (72 kDa) to cleaved, active MMP-2 (68 kDa). We confirmed that there were no significant differences in pro-MMP-2 (72 kDa) and active (68 kDa) forms in the supernatant after oxidant injury (Fig. 2C). In contrast, the ratio of the cell-associated pro-MMP-2, latent to active protein was greater than 1.8 for untreated and treated RPE cells.

We also performed real-time RT-PCR on total RNA extracts, to determine the impact of oxidant injury on MMP-2 mRNA expression. Minimal modifications in the levels of mRNA were observed after repetitive acute oxidant injury. Only, a small (but statistically significant) increase of 44.9% was observed after exposure of RPE cells to the fourth injury (Table 2). In

TABLE 2. Regulation of MMP-2 mRNA by Repetitive Acute Oxidant-Mediated Injury in ARPE-19 Cells

		MMP-2 mRNA	
Injury	**Time**	**Amount (ng)**	**% Change**
1st	Control	98.6 ± 7.5	
	6 hours	99.13 ± 1.3	+0.54
2nd	Control	102.4 ± 18.3	
	6 hours	108.21 ± 9.7	+5.67
3rd	Control	112.7 ± 4.12	
	6 hours	114.9 ± 1.3	+1.95
4th	Control	119.12 ± 13.4	
	6 hours	172.7 ± 15.1	+44.9

MMP-2 mRNA expression was normalized to 18S transcript content. Data are expressed as a percentage of the control. Results are mean nanograms ± SEM of three independent experiments run in triplicate on cultured cells.

* Statistically significant at $P < 0.05$.

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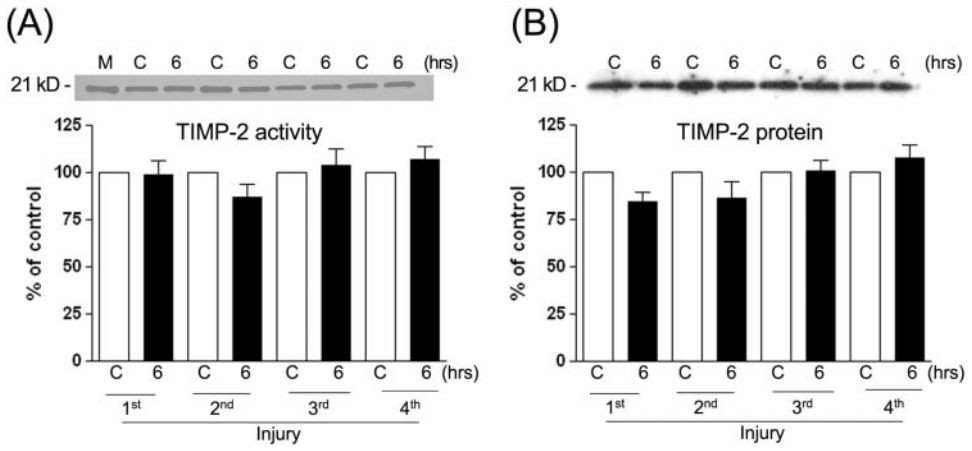

FIGURE 3. TIMP-2 activity and protein were not affected in ARPE-19 cells exposed to repetitive acute oxidant injury. *Top*: zymogram or Western blot from a representative experiment. The number on the *left* represents the protein's molecular mass. *Lane M*: molecular size standard. *Lane C*: control. *Bottom*: averages of results of three independent experiments run in triplicate on cultured cells. (**A**) TIMP-2 activity evaluated by reverse zymography. (**B**) TIMP-2 protein evaluated by Western blot.

summary, sustained oxidant injury greatly diminished MMP-2 activity but did not affect pro-MMP-2 protein in the supernatant. The large increase in the ratio of pro-MMP-2 to active MMP-2 suggests the loss of cell surface proteases responsible for cleavage and activation.

Effect of Repetitive Acute Oxidant Injury on TIMP-2 Activity and Protein Expression in ARPE-19 Cells

MMP-2 is secreted as a partially active proenzyme (72 kDa) that is subsequently processed into an active 68-kDa form, as men-

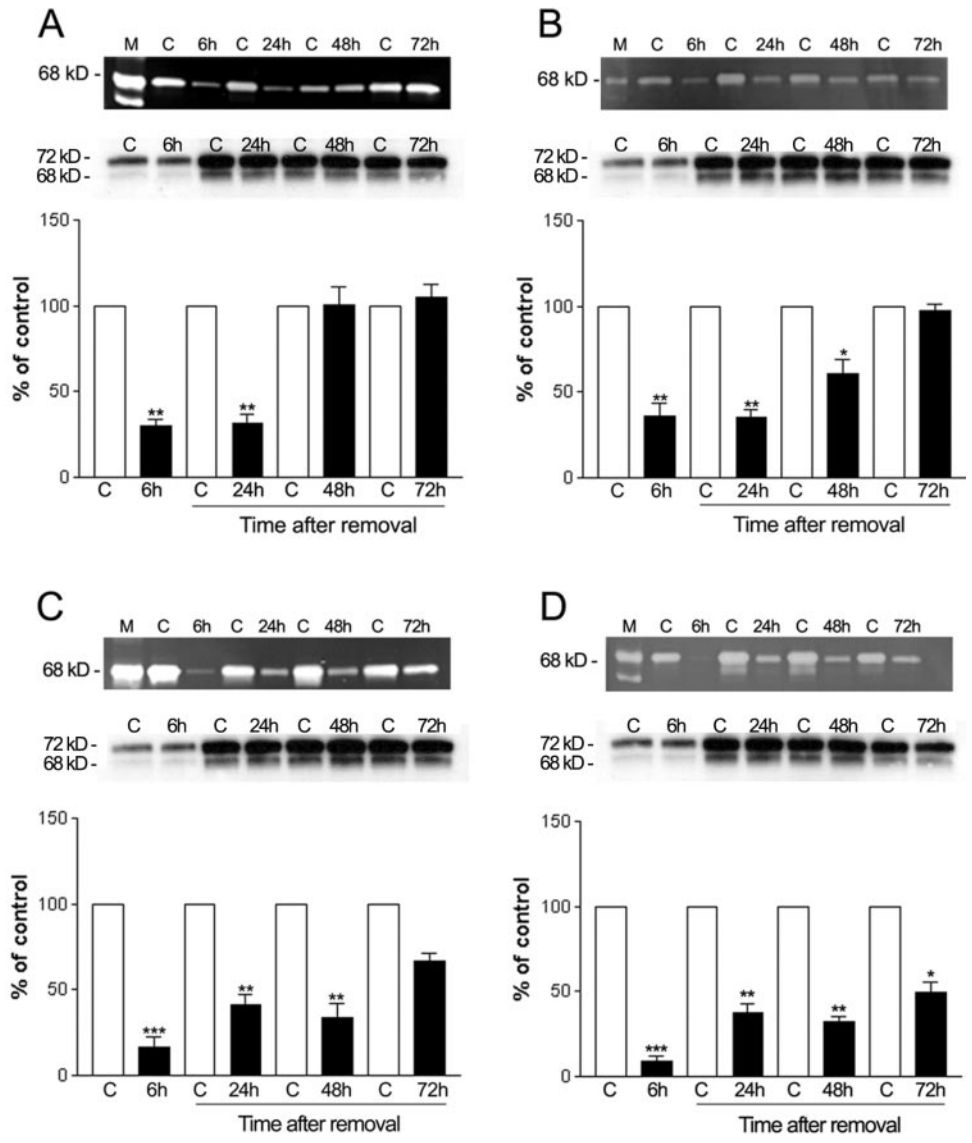

FIGURE 4. Repetitive transient exposure to oxidant injury was associated with diminished MMP-2 activity. Incubation of ARPE-19 cells with 100 μM HQ downregulated MMP-2 activity in the first (**A**), second (**B**), third (**C**), and fourth (**D**) injuries. *Top*: gelatin zymogram from a representative experiment. Number on the *left* represents protein molecular mass in kilodaltons. *Lane M*: molecular size standard. *Lane C*: control. *Middle*: Immunoblot from a representative experiment, showing no change in protein expression. *Bottom*: average of results of three independent zymograms run in triplicate on cultured cells. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$: statistically significant differences compared with the control.

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tioned. The regulation of MMP-2 activity is complex with possible loci of regulation being at the level of transcription, translation, or posttranslational processing by endogenous activators and inhibitors, including TIMP-2.

To evaluate potential posttranslational regulation of MMP-2 activity, the endogenous regulator TIMP-2 was studied using reverse zymography and Western blot analysis. As shown in Figure 3, there were no significant differences in TIMP-2 activity and protein expression in RPE cells that were repeatedly acutely exposed to the oxidant.

Regulation of MMP-2 by Repetitive Transient Oxidant Injury in ARPE-19 Cells

Because we believe that transient exposure to oxidant injury followed by a period of recovery probably represents conditions more physiologically relevant to oxidant-induced injury in vivo, experimental protocols using repetitive nonlethal transient oxidant injury were applied. We exposed RPE cells to HQ injury for 6 hours (acute transient oxidant injury phase), followed by replacement with maintenance medium (reassessment after removal of injury stimulus during the subsequent 6–72 hours) every 5 days for 6 weeks. By zymography, we found that the first transient exposure to oxidant injury was associated with diminished MMP-2 activity (~3.4-fold decrease, $P < 0.01$), 6 and 24 hours after removal of the oxidant. However, the MMP-2 activity released into the culture medium quickly recovered to control levels at 48 hours after injury (Fig. 4A).

We also observed a significant decrease in MMP-2 activity in the supernatant from 6 to 48 hours after acute injury during the second exposure of the cells to 100 μM HQ. An approximately 2.7-fold (267% ± 11.3%, $P < 0.01$) decrease in MMP-2 activity 6 and 24 hours after removal of the HQ, and an approximately 1.6-fold (163.61% ± 1.4%, $P < 0.01$) decrease in the activity of this protein 48 hours after injury was observed; both recovered to normal levels 72 hours after injury (Fig. 4B).

During the third transient oxidant injury, a further decrease in MMP-2 activity was observed 6 hours after acute oxidant injury (~5.6-fold decrease, $P < 0.001$), whereas 24 and 48 hours after removal of the oxidant, the decrease in MMP-2 activity was approximately 2.9-fold (287.31% ± 6.61%; $P < 0.01$) and 3.1-fold (311.21 ± 13.24%, $P < 0.01$) respectively (Fig. 4C). In contrast, after the third transient oxidant injury MMP-2 activity did not recover to normal levels up to 72 hours after injury.

Similar to the results observed in Figure 4C, during the fourth oxidant injury, there was a decrease by 80% in MMP-2 activity observed 6 hours after last acute oxidant injury. After 24 and 48 hours, the decrease was approximately 57% and 63%, respectively. Finally MMP-2 activity decreased by approximately 47% at 72 hours after injury (Fig. 4D). Thus, oxidative damage to the enzyme activity of released MMP-2 was unlikely to explain the observed decreased in MMP-2 activity. Regarding the effect of HQ oxidant injury on the ratio of secreted pro-MMP-2 protein to cleaved, active MMP-2, we found results similar to those observed for RPE cells that were repeatedly exposed to acute oxidant.

Minimal changes in MMP-2 mRNA expression were observed after repetitive transient injury (Table 3), although a small but significant increase in mRNA expression was observed (47.6% and 49.62%) 48 and 72 hours, respectively, after the last transient injury with HQ.

In summary, repetitive, prolonged transient nonlethal oxidant injury in RPE cells caused a decrease in MMP-2 activity, which did not recover to baseline levels after the last repetitive acute injury. These events resulted in similar changes of released pro-MMP-2 protein.

TABLE 3. Regulation of MMP-2 mRNA by Repetitive Transient Exposure to Oxidant Injury in Cultured ARPE-19 Cells

Times	MMP-2 mRNA	
	Amount (ng)	% Change
1st Injury		
Control 6 hours	123.6 ± 1.2	
6 hours	125.3 ± 0.8	+1.37
Control 24 hours	128.2 ± 3.7	
24 hours	131.04 ± 1.4	+1.02
Control 48 hours	129.8 ± 6.3	
48 hours	136.2 ± 9.7	+4.93
Control 72 hours	143.1 ± 2.1	
72 hours	156.7 ± 4.0	+9.5
2nd Injury		
Control 6 hours	131.4 ± 5.7	
6 hours	135.3 ± 3.1	+2.96
Control 24 hours	147.3 ± 2.8	
24 hours	151.4 ± 3.2	+2.78
Control 48 hours	149.16 ± 7.9	
48 hours	152.5 ± 1.3	+2.23
Control 72 hours	147.4 ± 7.0	
72 hours	162.7 ± 4.8	+10.37
3rd Injury		
Control 6 hours	127.38 ± 7.3	
6 hours	138.9 ± 4.1	+9.04
Control 24 hours	134.6 ± 9.2	
24 hours	141.12 ± 6.2	+11.56
Control 48 hours	148.2 ± 10.2	
48 hours	153.5 ± 11.6	+3.57
Control 72 hours	150.8 ± 9.7	
72 hours	162.16 ± 8.3	+7.53
4th Injury		
Control 6 hours	117.8 ± 10.6	
6 hours	125.7 ± 8.7	+6.70
Control 24 hours	133.18 ± 12.3	
24 hours	149.1 ± 11.4	+11.9
Control 48 hours	139.98 ± 16.2	
48 hours	209.63 ± 17.9	+47.6*
Control 72 hours	152.4 ± 3.8	
72 hours	228.0 ± 5.7	+49.62*

MMP-2 mRNA expression was normalized to 18S transcript content. Data are expressed as a percentage of the control. Results are mean nanograms ± SEM of three independent experiments run in triplicate on cultured cells.

* Statistically significant at $P < 0.05$.

Regulation of TIMP-2 by Repetitive Transient Oxidant Injury in RPE Cells

We evaluated the effect of repetitive transient oxidant injury on the potential posttranslational regulation of TIMP-2 activity as well as protein expression. Results observed from TIMP-2 activity were of a magnitude similar to those shown in cells that were repeatedly exposed to acute oxidant (Fig. 5). In addition, minimal modifications in levels of TIMP-2 protein were observed after repetitive transient oxidant injury. Only, a small (not statistically significant) increase was observed 72 hours after 100 μM HQ exposition (Fig. 5).

Regulation of Collagen Type IV by Repetitive Oxidant-Mediated Injury in RPE Cells

Regulation of collagen synthesis and secretion by oxidants has been shown in a variety of nonocular[29–31] and ocular tissues.[32] Various studies have shown that RPE cells can synthesize collagen type IV, the most important collagen type present in the basal lamina.[40–42] For the repetitive acute oxidant injury, the third and fourth injuries for 6 hours induced an approximately 1.7 and 2.5-fold increase (163.14% ± 9.13%, $P < 0.05$; 254.21% ± 8.57%, $P < 0.01$)

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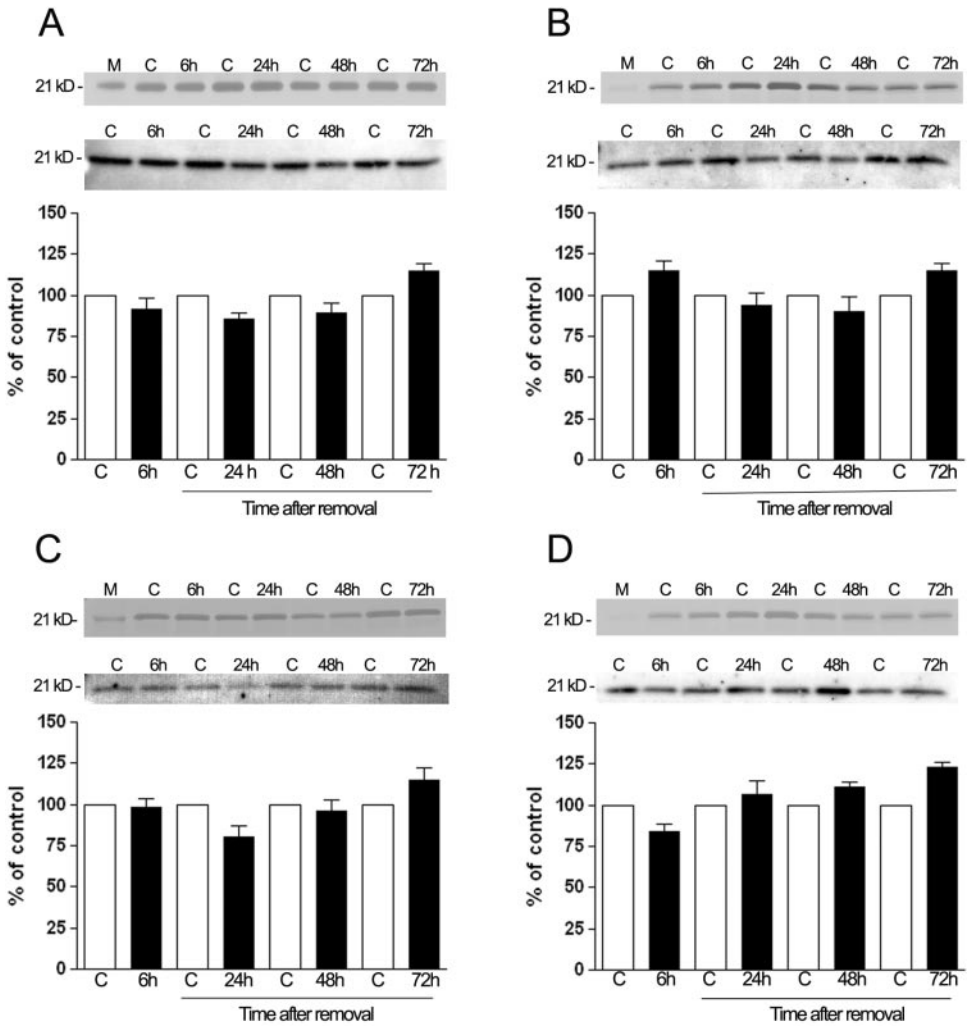

FIGURE 5. TIMP-2 activity was unaffected by repetitive transient exposure to oxidant injury. Incubation of ARPE-19 cells with 100 μM HQ did not induce any significant change in TIMP-2 activity or protein expression in the first (**A**), second (**B**), third (**C**), and fourth (**D**) injuries. *Top*: Reverse zymogram from a representative experiment. The remainder of the notation is as described in Figure 4, except that differences were not significant.

in collagen type IV respectively (Fig. 6A). In contrast, the first two acute injuries did not induce changes in collagen accumulation. We also observed an approximately 2.4-fold increase (243% ± 37.19%, $P < 0.05$) in collagen after the last acute injury by immunostaining (Figs. 7A–C).

During repetitive transient oxidant injury, collagen type IV did not increase during the first or the second exposures to the oxidant. In contrast, collagen type IV increased by approximately 65% 6 hours after removal of the oxidant during the third injury, but quickly recovered to normal levels 24 hours after the injury (Fig. 6D). A further increase in this protein was observed during the last acute injury (Fig. 6E). We found an approximately 2.9 increase (287% ± 21.42%, $P < 0.01$) 6 hours after removal of the oxidant. This increase did not recover to normal levels during the following 72 hours after removal of the HQ (Fig. 6E). Equivalent increase in collagen type IV was observed by immunostaining during the last transient exposure to HQ (Figs. 7D–F).

We also performed real-time RT-PCR on total RNA extracts, to determine the impact of oxidant injury on collagen α1 (IV) mRNA expression. Minimal modifications in the levels of mRNA were observed after repetitive acute and transient oxidant injuries (Tables 4, 5). However, a small increase of 39.7% was observed 72 hours after the last transient injury (Table 5). In summary, both nonlethal repetitive acute and transient oxidant injuries greatly increased collagen type IV accumulation in RPE without significantly affecting collagen α1 (IV) mRNA expression.

Effect of Chronic Oral HQ Exposure on Sub-RPE Deposits and BrM Changes in 16-Month-Old, Female C57BL/6 Mice

First, we confirmed that HQ-treated mice had increased blood levels of HQ, a quinone present in automobile exhaust, industrial pollution, food, and cigarette tar. Mice on a regular fat diet with HQ in the drinking water demonstrated serum levels of 10.4 ± 0.7 ng/mL, whereas control mice showed nondetectable levels.

Based on our observations obtained with HQ in vitro, we hypothesize that the redox molecule HQ might contribute to drusen pathogenesis. We used the 16-month-old C57BL/6 mouse model for dry AMD published by our laboratory,[27–30] but providing an alternative source of oxidant stimulus by replacing exposure to blue light with exposure to HQ. In addition, mice received a regular fat diet instead of a high-fat diet. We evaluated the impact of HQ on the development of sub-RPE deposits, by using TEM. As expected, mice not exposed to HQ showed normal morphology of the RPE, BrM, and choriocapillaris endothelium (Fig. 8A). Some specimens demonstrated mild frequency of any BLD. None of the eyes in this group demonstrated moderate BLD.

Animals exposed to HQ showed pathologic changes in the RPE and BrM characterized by moderate BLD. Our grading system demonstrated that 83% of eyes exhibited moderate BLD with a mean severity score of 7.31 ± 3.48, significantly greater than the severity score 1.45 ± 1.18 in animals not exposed to

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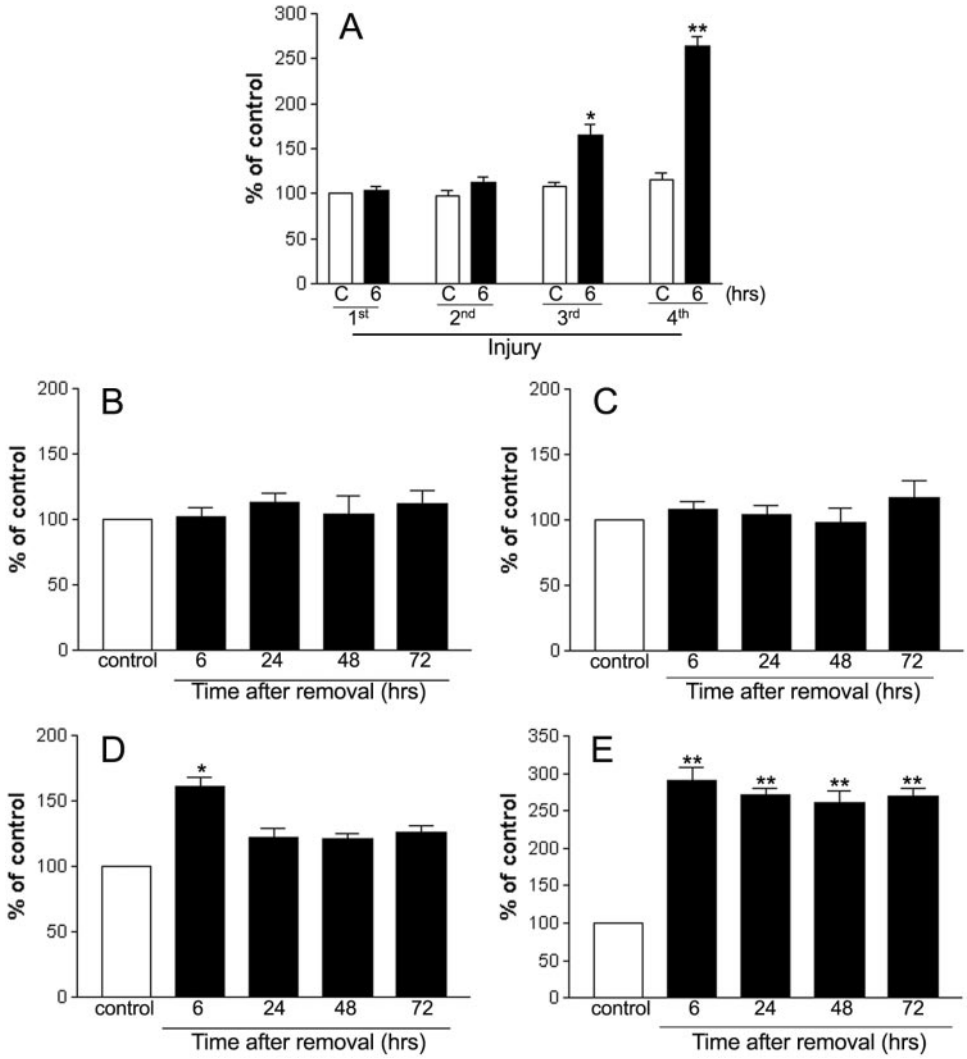

FIGURE 6. Quantification of collagen type IV accumulation in ARPE-19 cells by ELISA. HQ repetitive acute oxidant-mediated injury upregulated collagen type IV accumulation in ARPE-19 cells after the third and fourth exposures to 100 μM HQ for 6 hours but, did not induce changes in collagen type IV accumulation in cells treated with the same concentration of HQ every 3 days during the first 2 weeks (**A**). HQ repetitive transient oxidant-mediated injury upregulated collagen type IV accumulation in ARPE-19 cells after the third and fourth exposures to HQ (**D**, **E**), but did not induce changes in collage type IV accumulation in cells treated with same concentration of HQ every 5 days during the first two exposures (**B**, **C**). Data are expressed as a percentage of control and shown as the mean ± SEM of three independent experiments run in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$: statistically significant differences compared with the control.

HQ (Table 6). The BrM was thickened, with coated vesicles, membranous profiles, and banded structures, (Figs. 8B, 8C), typical of those described in some human AMD specimens. In addition, some animals showed occasional blebs (Table 6, Fig. 8C). These findings indicate that HQ may induce basal laminar deposits in mice and that a high-fat diet is not contributory.

DISCUSSION

Prolonged oxidative injury has been suggested as one of the causes of a number of retinal pathologic conditions, including AMD. In the present study, the cellular responses and ECM turnover regulation of RPE cells to repetitive nonlethal oxidant injury with HQ were examined in vitro, and the impact of this compound in drinking water on the development of sub-RPE deposits was evaluated in vivo. The results in vitro indicated that exposure of ARPE-19 cells to repetitive nonlethal oxidative injury induces cell membrane blebbing. In addition, changes in MMP-2 activity and collagen type IV accumulation, responses that might have direct involvement in RPE disease pathogenesis, were detected. These responses were most pronounced on repetitive transient oxidant exposure, but were also significant after repetitive acute oxidant exposure. In vivo, exposure to HQ induced formation of sub-RPE deposits, thickening of BrM, and accumulation of deposits within BrM.

Although oxidants derived from visible light exposure or those derived from endogenous metabolism are more frequently implicated in RPE injury,[5,6] we postulate that toxic substances associated with Western lifestyle may directly contribute to the formation of drusen and late forms of AMD by an increase in oxidative stressors. Based on this idea, we evaluated the redox molecule HQ, a major preoxidant component of cigarette smoke, automobile exhaust, and certain processed foods, on the contribution of drusen pathogenesis.

HQ competes with the normal substrate of mitochondrial oxidases associated with electron transport and undergoes redox cycling with its corresponding semiquinone radical. As a result, HQ metabolism in the mitochondria generates oxidant products including superoxide, hydroxyl radical anions, and hydrogen peroxide,[43] which in turn damages mitochondrial membranes and leaks into cytoplasm participating in protein oxidation and/or lipid peroxidation.[44–46] A repetitive period of treatment with a nonlethal dose of oxidative stimulus was chosen to evaluate cellular responses of the RPE cells and to determine the impact of a prolonged injury on regulation of molecules important to ECM turnover maintenance.

The present study demonstrates that RPE cells that are repeatedly exposed to an oxidant show intensive bleb formation, which was well tolerated by the RPE, as shown in this and other studies by our group.[31] Cellular blebbing observed in the present study is a well-defined injury response that may occur in both physiological and pathologic situations and can be part of the apoptosis pathway.[47] Nonlethal blebbing occurs in vivo and may be a common cellular injury response in certain

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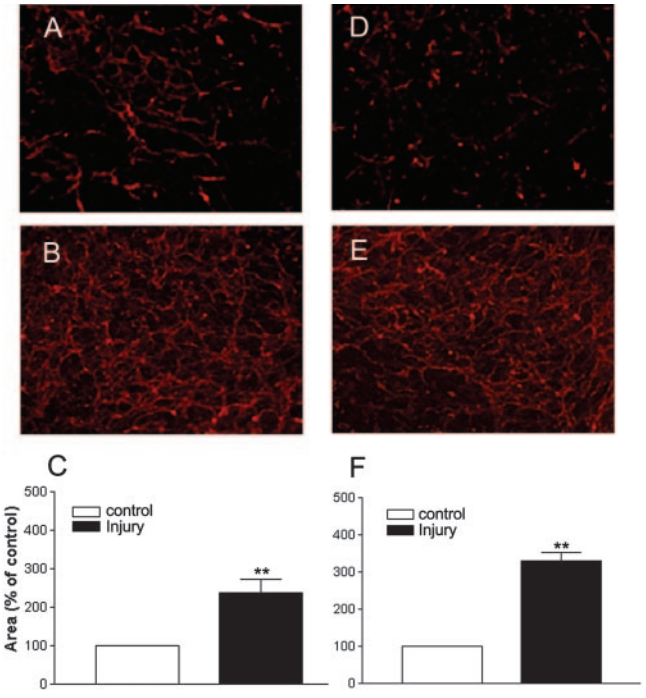

FIGURE 7. Repetitive acute and transient oxidant-mediated injuries with HQ-upregulated collagen type IV accumulation in GFP-ARPE-19 cells. (**A**, **B**) Immunostaining for collagen type IV during the last acute injury from a representative experiment before (**A**) and after (**B**) injury. (**C**) Average of results of three independent experiments run in triplicate on cultured cells. $^{**}P < 0.01$, statistically significant difference compared with the control. (**D**, **E**) Immunostaining for collagen type IV during the last transient oxidant injury from a representative experiment before (**D**) and (**E**) after injury. *Bottom*: as in (**C**). Magnifications, ×10.

diseases characterized by extracellular deposit accumulation, such as glomerulonephritis.[48,49] Several observations of human AMD and in animal models suggest that blebbing may contribute to sub-RPE deposits.[50]

In this work, blebbing occurred at a nonlethal HQ concentration in RPE cells without either cell death or proliferation, similar to our past findings and those of other groups.[31,51,52] These results confirm previous observations that blebbing can be different from programmed cell death[53,54] and may be an early response to nonlethal injury activated independently of apoptosis.[54]

The cellular mechanism(s) by which the balance in ECM turnover is altered in AMD remains unknown. However, strong evidence supports the hypothesis that MMPs, their tissue inhibitors, and collagens may play an important role in the pathogenesis of deposit accumulation in diverse disorders such as renal disease and atherosclerosis.[18–21,55] Not surprisingly, dysregulation of these molecules in AMD pathogenesis has been the topic of recent research. The RPE synthesizes collagens types I through IV, fibronectin, and many other molecules crucial for the formation and repair of its basement and Bruch's membranes.[40–42] Furthermore, we and others have shown that the RPE synthesizes MMPs, especially MMP-2, crucial for the degradation and turnover of extracellular matrix, and that MMP-2 synthesis, release, and activity may be regulated by physiological stimuli. Active MMP-2 is the major RPE enzyme for the degradation of collagen I, collagen IV, and laminin, all important components of BrM.[56,57] This study expands our previous work to demonstrate the capacity of repetitive oxidant injury to dysregulate MMP-2 activity and, by consequence, collagen IV accumulation. The accumulation of collagen IV cannot be explained through transcriptional regulation, given that its mRNA levels remain unaltered by the nonlethal oxidative stimulus. Dysregulation of MMP-2 activity and collagen type IV are likely to play a crucial role in early stages of dry AMD. For example, a recent study by Leu et al.,[58] revealed that,

TABLE 4. Regulation of Collagen α1 (IV) mRNA by Repetitive Acute Oxidant-Mediated Injury in ARPE-19 Cells

		Collagen α1 (IV) mRNA	
Injury	**Times**	**Amount (ng)**	**% Change**
1st	Control	97.14 ± 1.4	
	6 hours	99.85 ± 2.3	+2.78
2nd	Control	98.73 ± 8.2	
	6 hours	109.32 ± 7.1	+10.72
3rd	Control	104.27 ± 11.8	
	6 hours	120.3 ± 13.6	+15.37
4th	Control	117.9 ± 1.17	
	6 hours	132.7 ± 2.14	+12.5

Collagen α1 (IV) mRNA expression was normalized to GAPDH mRNA signal. Data are expressed as the percentage of control. Results are mean nanograms ± SEM of three independent experiments run in triplicate on cultured cells.

TABLE 5. Regulation of Collagen α1 (IV) mRNA by Repetitive Transient Exposure to Oxidant Injury in Cultured ARPE-19 Cells

	Collagen α1 (IV) mRNA	
Times	**Amount (ng)**	**% Change**
1st Injury		
Control 6 hours	93.6 ± 12.2	
6 hours	92.51 ± 11.6	−1.2
Control 24 hours	95.21 ± 10.3	
24 hours	101.10 ± 7.5	+6.18
Control 48 hours	97.81 ± 13.6	
48 hours	104.14 ± 14.1	+6.47
Control 72 hours	102.3 ± 10.3	
72 hours	109.75 ± 6.4	+7.28
2nd Injury		
Control 6 hours	99.42 ± 1.2	
6 hours	105.27 ± 2.13	+5.88
Control 24 hours	98.68 ± 7.8	
24 hours	103.52 ± 3.7	+4.90
Control 48 hours	101.18 ± 11.4	
48 hours	108.5 ± 8.7	+7.23
Control 72 hours	106.53 ± 4.7	
72 hours	114.7 ± 3.8	+7.66
3rd Injury		
Control 6 hours	93.8 ± 12.6	
6 hours	97.5 ± 15.1	+3.94
Control 24 hours	102.6 ± 11.0	
24 hours	107.17 ± 9.8	+4.45
Control 48 hours	111.23 ± 14.4	
48 hours	118.5 ± 10.3	+6.53
Control 72 hours	99.78 ± 7.7	
72 hours	103.43 ± 13.0	+3.65
4th Injury		
Control 6 hours	104.8 ± 14.5	
6 hours	107.61 ± 8.3	+2.68
Control 24 hours	98.9 ± 11.6	
24 hours	99.11 ± 7.1	−0.21
Control 48 hours	109.7 ± 4.9	
48 hours	119.4 ± 6.3	+8.84
Control 72 hours	116.51 ± 8.2	
72 hours	162.7 ± 12.0	+39.7*

Collagen α1 (IV) mRNA expression was normalized to GAPDH mRNA signal. Data are expressed as the percentage of control. Results are mean nanograms ± SEM of three independent experiments run in triplicate on cultured cells.

* Statistically significant at $P < 0.05$.

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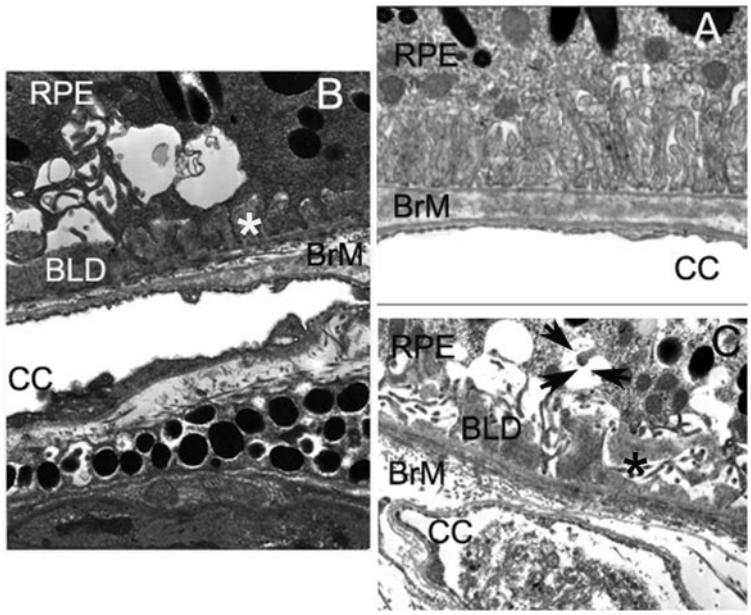

FIGURE 8. Transmission electron microscopy of the outer retina and choroid from a 16-month-old female mouse fed with a regular fat diet for 4 months. (**A**) Outer retina and choroid of a mouse fed a regular diet without oxidant showed a normal RPE, BrM, and choriocapillaris. (**B**) Outer retina and choroid of a mouse fed a regular diet and exposed to HQ (0.8% for 4 months) in drinking water revealed sub-RPE deposits characterized by accumulation of moderately severe BLD with dense granular material between the RPE and its basement membrane (✻), compatible with a high mean severity score. The specimen, also, shows the abnormal choriocapillaris endothelium with increased thickening and loss of fenestration. (**C**) TEM of the outer retina and choroids from another 16-month-old female mouse fed a regular diet and exposed to HQ. The specimen shows moderately thick BLD with banded structures (✻) and occasional blebs (*black arrows*). CC, choriocapillaris, Magnification: (**A**, **C**) ×25,000; (**B**) ×7,200.

in AMD eyes, areas of normal BrM contain demonstrably active MMP-2, but drusen and sub-RPE deposits are "cold" spots for MMP-2 activity, correlating loss of MMP-2 activity with deposit accumulation. Therefore, sub-RPE deposits in human and mouse macular specimens contain basement membrane components, such as collagen type IV.[59,60]

We have recently shown that after both sustained oxidant injury for 24 hours with another nonlethal oxidative injury to the RPE and transient exposure for 6 hours (acute transient oxidant injury phase) followed by a recovery phase (reassessment after removal of injury stimulus during the subsequent 6–72 hours), caused nonlethal blebbing and increased release of pro-MMP-2 with a concomitant decrease in released active MMP-2.[16] In addition, collagen IV accumulation increased only after sustained exposure to the oxidant of 24 hours. However, RPE recovered to normal within 24 to 48 hours after removal of the oxidant.[16] In the cited study, the duration of exposure to the oxidant was very short in relation to the rate of evolution of early disease in AMD. We believe that repetitive prolonged exposure of the RPE to oxidants probably represents more physiologically relevant conditions to bleb-inducing injury in vivo. Thus, in our experimental protocols, we used repetitive nonlethal oxidant exposure for either 4 or 6 weeks. Our results also indicate a complex interrelationship between oxidant-induced injury and MMP-2 activation. Oxidant injury did not affect release of pro-MMP-2, but greatly diminished active

TABLE 6. The Effect of Oral HQ on BLD Severity in Mice

	Frequency of Any BLD		Frequency of Moderate BLD		Mean Severity Score		Mean BrM Thickness		EC Damage or Invasion	
	I	II	I	II	I	II	I	II	I	II
1	Yes	Yes	No	Yes	0.67	8.75	1.2	10.18	No	No
2	No	Yes	No	Yes	0.34	8.12	0.71	7.21	No	No
3	Yes	Yes	No	Yes	2.12	6.38	4.7	10.43	No	Yes
4	Yes	Yes	No	Yes	2.01	9.21	3.52	8.61	No	No
5	Yes	Yes	No	Yes	3.21	8.2	2.40	7.35	No	No
6	Yes	Yes	No	No	0.36	3.18	2.98	8.85	No	No
	83%	100%	0%	83%	1.45 ± 1.18	7.31 ± 3.48	2.58 ± 2.3	8.77 ± 2.13	0%	16.5%

Experimental groups were the following: I received regular fat diet without hydroquinone; II received regular fat diet plus hydroquinone in the drinking water. Any BLD: presence of any homogeneous deposit (focal or continuous) between RPE cell membrane and its basement membrane in at least one (of 10) micrograph from an individual specimen. Moderate BLD: presence of continuous deposits underlying several cells, banded structures (fibrous long-spacing collagen) and/or thickness more than one quarter cells height in at least three micrographs from an individual specimen. Frequency of Moderate BLD: χ^2: group I versus II, $P = 0.01$; Mean Severity Score: *t*-test: group I versus II, $P = 0.001$. BrM mean thickness score: *t*-test: group I versus 2, $P = 0.05$.

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MMP-2. Although direct oxidation of MMP-2 may contribute to some of the observed diminished enzymatic activity after bleb injury, we believe that the accumulation of large amounts of extracellular pro-MMP-2 indicates a more complex form of dysregulation. MMP-2 is secreted as a proenzyme or zymogen (72 kDa) and is sequentially processed to an intermediate, and then to the active 68 kDa form.[36] MMP-2 regulation is complex with possible regulation at the level of transcription, translation or posttranslational processing by endogenous activators and inhibitors including TIMP-2 and membrane type 1-matrix metalloproteinase (MT1-MMP).

Preliminary data suggest that bleb injury interferes with the activation of latent pro-MMP-2 into active MMP-2, perhaps by downregulation of cell surface MT1-MMP and/or its accessory protein TIMP-2 (Elliot S, et al. *IOVS* 2004;45:ARVO E-Abstract 1816). Of note, TIMP-2 has a dual role in MMP-2 regulation, depending on its abundance. Low to modest levels contribute to pro-MMP-2 activation by tethering pro-MMP-2 to MT1-MMP, which is associated with the cell membrane. A neighboring MT-1-MMP then catalyzes the formation of active MMP-2 from the tethered zymogen. High levels of TIMP-2, however, preempt and inhibit all membrane associated MT1-MMP, thereby precluding activation of pro-MMP-2.

This study demonstrates that repeated exposure to an oxidant stimulus does not affect TIMP-2 activity or protein expression, suggesting that HQ may regulate expression of MT1-MMP. Future studies will address the influence of nonlethal oxidant injury on MT1-MMP.

Our results indicate an interrelationship among nonlethal oxidant-induced injury, MMP-2 activity, and collagen type IV accumulation. In addition, they demonstrate good agreement between the responses to repetitive acute and transient exposures to an oxidant, although the diminished MMP-2 activity and increased accumulation of collagen IV were more dramatic after repetitive transient injury for 6 weeks. These data provide evidence that ECM turnover decreases in RPE cells after a more prolonged exposure to nonlethal oxidant injury and may help explain the sub-RPE formation in AMD.

To extend the in vitro observations obtained with HQ in RPE cells to a more physiological environment, we used the experimental mouse model for sub-RPE published by our laboratory and others.[24–26,61] However, we provided an alternative source of oxidant stimulus by replacing exposure to blue light with exposure to HQ. In this study, we observed that mice that were chronically exposed to HQ showed development of sub-RPE deposits and BrM thickening consistent with changes in early stages of human AMD.[62] Findings were of a magnitude similar to those previously observed in mice exposed to high-fat diet plus blue green light[24] and/or HQ in food (Cousins SW, et al. *IOVS* 2003;44:ARVO E-Abstract 1619). We were able to replicate the pathologic changes by feeding mice with a regular fat diet and chronically exposing them to HQ in drinking water. These mice showed similar pathologic changes, irrespective of the fat content in the diet. These observations support the hypothesis that oxidant injury can initiate a process resulting in deposit formation. The morphologic changes observed in this study featured vesicular bleblike structures and moderately thick BLD containing typical homogeneous electron-dense material with banded structures consistent with long-space collagen. BrM was moderately thickened in general, often containing vesicle structures and other inclusions. Although all these changes do not represent authentic progressive AMD, because no typical drusen are formed and no choroidal neovascularization is seen, they represent the morphologic features of the early manifestations of AMD. In addition, it cannot be excluded that features of advanced AMD may develop in these mice after a longer exposure to HQ.

We have previously hypothesized that the RPE is the key target cell in deposit formation. Specially, we proposed that HQ and other oxidants trigger a specific cellular process called nonlethal blebbing.[16,63,64] We have demonstrated that RPE cells that are repeatedly exposed to oxidant showed blebbing of the cell membrane material.[16] In preliminary experiments, we have also demonstrated that mice receiving subconjunctival injections of HQ exhibited a rudimentary form of BLD, often demonstrating small vesicular bleblike structures (Reinoso MA, et al. *IOVS* 2005;46:ARVO E-Abstract 3010).

Our results indicate that two different oxidant stimuli (i.e., blue light and HQ) may induce a common response in the RPE and that a high-fat diet is not an absolute requirement for the development of BLD. Thus, the results suggest the role for blebs in sub-RPE formation and propose HQ as another oxidative injury stimulus to the RPE, which may serve to explain the mechanisms that underlie pathologic BLD deposits in early AMD.

Taken together, the data suggest that repetitive prolonged oxidant exposure can induce cellular responses that may promote sub-RPE deposit accumulation. Active MMP-2 regulates the breakdown and turnover of type IV collagen in the RPE basement membrane and regulates the turnover of other collagens in the inner BrM. In the absence of active MMP-2 and increased accumulation of collagen IV, RPE blebs containing cell membranes, cytosolic proteins, and organelles may be expected to accumulate as deposits between the RPE cell membrane and its basal lamina. Also, excessive amounts of new basement membrane may accumulate over these trapped blebs, causing drusen. Furthermore, it is possible that therapies that preserve the function of RPE-derived MMPs after oxidant injury may promote deposit clearance and diminish the progression of AMD.

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