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Protection from oxidative stress by methionine sulfoxide reductases in RPE cells ☆

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Abstract

We investigated the role of methionine sulfoxide reductases (Msrs) in oxidant-stress-induced cell death in retinal pigmented epithelial (RPE) cells. In RPE cells exposed to varying doses of H_2O_2, gene expression of MsrA and hCBS-1 (the human analog of MsrB2) increased in a dose-dependent and time-dependent manner with maximal increase with 150 μM H_2O_2 in 24 h. H_2O_2 treatment resulted in the generation of reactive oxygen species and activation of caspase 3. Confocal microscopic and protein analysis showed an increase in MsrA expression in cytosol and mitochondria. Silencing of MsrA resulted in caspase 3 induction and accentuated cell death from H_2O_2. Focal, strong immunoreactivity for MsrA was observed in sub-RPE macular drusen from patients with age-related macular degeneration. In summary, our data show that MsrA and hCBS-1 are up-regulated in oxidative stress to counteract injury to RPE.

Keywords: Msrs; RPE; Oxidative stress; Mitochondria; Apoptosis; AMD; Drusen; siRNA

Reactive oxygen species (ROS) oxidize an array of cellular constituents including lipids, amino acids, and nucleic acids and are implicated in aging, initiation and progression of age-related macular degeneration (AMD), and cataract [1,2]. Several antioxidative mechanisms have evolved in mammalian cells to protect oxidative insults that include superoxide dismutase, catalase, and glutathione peroxidase which act by scavenging the superoxide anion and H_2O_2 to prevent ROS-induced damage [3].

Amino acid residues in proteins represent one of the major targets of ROS and cellular oxidants. Among the amino acids, methionine and cysteine are highly susceptible to oxidation, as they possess reactive sulfur-containing side chains that represent the prime targets of ROS [4]. Methionine, both in free and in protein-bound forms, is readily oxidized by ROS, resulting in the formation of two enantiomers [5]. Methionine sulfoxide is reduced back to methionine by methionine sulfoxide reductase A (MsrA) or methionine sulfoxide reductase B (MsrB) depending on whether methionine sulfoxide is present as S- or R-form [6–9]. Oxidation of methionine is considered to be an important cell regulatory event, as it modulates the biological activity of several proteins [4]. The methionine sulfoxide reductase

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(Msr) system has also been shown to participate in protection against oxidative stress by reducing the exposed methionine sulfoxide on proteins that act as reactive oxygen scavengers [10].

The essential role played by MsrA in cell survival has been exemplified by a number of recent studies. Overexpression of MsrA in human T cells and neuronal cells increased the resistance and the lifespan of the cells under conditions that promote oxidative stress [11,12]. Kantorow et al. [13] found that overexpression of MsrA in human lens epithelial cells protected against oxidative stress and increased cell viability. Impairment of MsrA synthesis caused reduction in lifespan in mouse and *Drosophila* [14,15], attenuated virulence in bacterial pathogen [16] or increased sensitivity to a high concentration of exogenously added oxidants [11,17].

Although environmental and genetic factors are known to be involved in the pathogenesis of AMD, the mechanism of disease initiation and progression has not been fully delineated. Extracellular sub-RPE deposits on Bruch's membrane in the macula are one of the risk factors for progression to advanced disease [18]. While the association of oxidative stress with the development of AMD is known, the role of the enzymes that protect free and protein-bound methionine oxidation in the retina has not been studied. The purpose of the present work was to determine the modulation of expression of MsrA and hCBS-1 genes upon exposure of RPE to H_2O_2 in order to determine their role in the ability of cells to adapt to oxidative challenges. We hypothesized that silencing of MsrA would lead to induction of apoptosis. We have examined the mechanism of protection from apoptosis by MsrA in RPE with particular emphasis on the role of mitochondria. The pattern of expression of MsrA in the retina of AMD patients was also investigated.

Experimental

Cell culture and treatment. RPE cells were isolated from human eyes obtained from Advanced Bioscience Resources (Alameda, CA, USA) and cultured in Dulbecco's minimal Eagle's medium (DMEM; Fisher Scientific, Pittsburgh, PA, USA) with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA), and 10% heat-inactivated fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA, USA), as previously described [19]. The Institutional Review Board of the University of Southern California approved our use of cultured human RPE cells. Experiments were performed in confluent RPE cell cultures at the third or fourth passage. Cells were switched from DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% FBS to medium containing 1% FBS overnight and then treated with 50, 100, 150, and 200 μM H_2O_2 for 24 h. Time-dependent expression was studied after treatment of cells with 150 μM H_2O_2 for 2, 4, 8, and 24 h. Immunofluorescence and ROS quantitation studies were conducted using 150 μM H_2O_2.

Cell viability assay. Attached cells were released by treatment with trypsin, pooled with floating cells, washed once with PBS, stained with the LIVE/DEAD viability/cytotoxicity kit (Molecular Probes, Eugene, OR, USA) for 10 min at room temperature, and analyzed using flow cytometry, as described previously [20]. Live and dead cells were distinguished using 2 μM calcein acetyloxymethyl ester and 8 μM ethidium homodimer-1, respectively.

Detection of ROS production. Detection of ROS accumulation in RPE cells was done using a carboxy-H_2-DCFDA staining method (Molecular Probes). DCFDA is a non-fluorescent ester of the dye fluorescein that is cleaved by intracellular esterases and trapped within the cell as the oxidant-sensitive DCF compound [21]. In the presence of ROS, H_2-DCF is rapidly oxidized to highly fluorescent DCF. RPE cells were incubated for 1 h at 37 °C with 5 μM carboxy-H_2-DCFDA dissolved in the culture medium. Cells were examined using a laser scanning confocal microscope (LSM510, Zeiss, Thornwood, NY, USA), and ROS were verified by the presence of green fluorescence. To determine the compartmentalized accumulation of ROS, mitochondria were labeled by a cell-permeable mitochondria-specific red fluorescent dye, CMXRos (Molecular Probes; 500 nM for 30 min), and rapidly evaluated by confocal microscopy. A yellow color is observed when accumulated ROS (green) are colocalized in the mitochondria (red).

Quantitative real-time PCR. Total RNA was isolated from cells using extraction reagent (TRIzol, Invitrogen Life Technologies, Carlsbad, CA) and was quantified by a spectrophotometer. The contaminating genomic DNA was removed with a kit (DNA-free, Ambion, Austin, TX). Reverse transcription was performed with 1 μg RNA and a kit (Reverse Transcription System, Promega, Madison, WI) as per the manufacturer's protocol. The PCR experiments were performed using SYBR Green as the interaction agent (Roche Diagnostics). Each 20 μl PCR contained cDNA template, SYBR Green PCR master mix, and 0.5 μM each gene-specific primer. Reaction conditions were as follows: 6 min at 95 °C followed by 40 cycles of 3 s at 95 °C, 15 s at 55 °C, and 15 s at 72 °C. Quantification analysis of MsrA and $MsrB_2$ (hCBS-1) mRNA was normalized using β-actin or GAPDH as reference. The specificity of PCR amplification products was checked by performing dissociation melting curve analysis. The sequences of primers used for MsrA were forward: 5′-TGGTTTTGCAGGAGGCTATAC-3′, reverse: 5′-GTAGATGG CCGAGCGGTACT-3′ and hCBS-1 ($MsrB_2$) forward: 5′-CCGGAG CAGTTCACGTCAC-3′; reverse: 5′-TGAGCTTCACACTGCTT GCA-3′ [22]. Relative multiples of change in mRNA expression was determined by calculation of $2^{-\Delta\Delta C_T}$. Results are reported as mean difference in relative multiples of change in mRNA expression ± SEM.

Immunofluorescence. Cells were fixed in ice-cold methanol for 15 min and permeabilized in 0.1% Triton X-100 in 1% BSA for 15 min, followed by blocking. Primary (1:75) and secondary antibodies (1:100) were diluted in blocking solution. For visualizing mitochondria, Mito Tracker Red (Molecular Probes) was used. PBS was used for washing between steps. Cells were mounted with fluorescent mounting medium. Images were acquired by confocal microscopy.

Preparation of mitochondrial and cytosolic fractions. Mitochondrial and cytosolic fractions were prepared from non-treated or 150 μM H_2O_2-treated cells. Preparation of mitochondrial and cytosolic fractions was achieved using a commercially available mitochondria/cytosol fractionation kit according to the manufacturer's protocol (BioVision, California). RPE cells were harvested by centrifugation at 600*g* for 5 min and washed with ice-cold PBS. Afterward, the cells were resuspended in a 1.0-ml 1× cytosol extraction buffer mix containing protease inhibitors and dithiothreitol. After incubation on ice for 10 min, the cells were homogenized in an ice-cold tissue grinder. Homogenates were centrifuged at 700*g* for 10 min at 4 °C, and the supernatant was collected. The supernatant was centrifuged at 10,000*g* for 30 min at 4 °C. The resulting supernatant was collected (cytosolic fraction), and the pellet was resuspended in mitochondrial extraction buffer (mitochondrial fraction). The purity of the mitochondria was verified by enzyme enrichment assays provided by the manufacturer. Protein content was quantified with a protein assay (Bio-Rad, Richmond, CA) with BSA as the standard. The cytosolic and mitochondrial fractions were stored at −80 °C.

Western blot analyses. Equal amounts (25 μg) of protein were resolved on Tris–HCl polyacrylamide gels (120 V, Ready Gel; Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose blotting membranes (Bio-Rad, Hercules, CA, USA). For detection of protein expression, membranes were probed with rabbit polyclonal anti-MsrA antibody (Upstate Biotechnologies, NY, USA) at 1:1000 dilutions overnight at 4 °C. After incubation for 2 h with the corresponding secondary antibody tagged with horseradish peroxidase, signals were detected by the chemiluminescence system (Amersham Pharmacia Biotech, Cleveland, OH, USA). Protein band intensity was measured by Scion Image Densitometer Software Package (Scion, Frederick, MD). For caspase 3 expression, membranes were probed with a rabbit polyclonal anti-caspase 3 antibody (Pharmingen).

siRNA selective gene knockdown. A pool of four siRNAs specific for MsrA gene (Accession No. NM_012331) was obtained from Dharmacon Research (Lafayette, CO). We used siPORT Amine as the transfection agent because it delivers siRNA with minimal cytotoxicity. Different concentrations of siRNA were tested in the initial stage of transfection and finally used at a concentration of 100 nM using Ambion's silencer siRNA transfection kit (Ambion, Austin, TX) as per the manufacturer's protocol. As a control for MsrA-targeted siRNA, we used the siCONTROL Non-Targeting Pool, composed of a pool of four siCONTROL Non-Targeting siRNAs (Dharmacon). Maximum silencing with minimal cytotoxicity was obtained with 100 nM siRNA. Cells were harvested 48 h after transfection for assessing protein and transfection efficiency by Western blot of mock-transfected, non-targeted siRNA and MsrA siRNA.

Immunostaining of MsrA in AMD patients. MsrA protein was visualized by immunohistochemistry. Cryostat sections (8 μm) from the macular region of the retina of five donors with non-exudative AMD and five age-matched normal donor eyes (age range 74–95 years) were immunostained with anti-MsrA at 1:75 dilution and biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) secondary antibody. Sections were washed with PBS in between the steps. Complexes were visualized using the Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA) as per the recommended procedure of the manufacturer. Sections were counterstained with hematoxylin and similar procedures were carried out in the absence of primary antibody as control.

Statistical analysis. Results are expressed as means ± SEM. Student's t test was used to determine statistical difference between two group means. Differences were considered statistically significant when $p < 0.05$.

Results

Cell viability with H_2O_2 treatment

Initial studies to assess the cell toxicity of varying amounts of H_2O_2 were performed using the LIVE/DEAD viability/cytotoxicity kit and were validated using the trypan blue exclusion method. Percentage viability averaged 95.4 ± 0.6 in untreated control cells. The viability of cells treated with different doses of H_2O_2 for 24 h showed a gradual decrease with increasing concentrations of up to 200 μM. The viability at 200 μM H_2O_2 was 92.5 ± 1.1 and the maximal decrease in viability observed with 200 μM H_2O_2 was not significantly different from that of the control. As a positive control, we treated cells with 800 μM of H_2O_2 for 24 h and found that the viability declined to 10%.

H_2O_2 treatment and accumulation of ROS

Fig. 1 shows confocal microscopy images from the fluorescence of ROS accumulation in RPE cells of H_2O_2-treated (D–F) and untreated (A–C) controls. The duration and dose of H_2O_2 exposure of RPE in 1% FBS containing DMEM was 24 h and 150 μM, respectively. Treatment with 150 μM of H_2O_2 resulted in pronounced intracellular accumulation of ROS that was mostly localized to the mitochondria.

Dose and time dependency of MsrA and hCBS-1 gene expression

To examine whether oxidative stress alters gene expression of MsrA and hCBS-1, cells were treated with varying doses of H_2O_2 for 24 h and the relative abundance of mRNA was determined using real-time RT PCR. MsrA mRNA expression increased as a function of H_2O_2 concentration up to 150 μM H_2O_2 (Fig. 2). The increase with 150 μM H_2O_2 was 2.5-fold as compared to untreated controls ($p < 0.05$). At doses higher than 150 μM H_2O_2, the increase in mRNA expression began to decline but was still higher than the control, although not differing significantly (Fig. 2A). The trend was similar for hCBS-1 gene expression with H_2O_2 treatment. Similar to MsrA, the maximal increase in gene expression was observed for hCBS-1 at 150 μM H_2O_2 ($p < 0.05$). The level of expression with 100 μM was also significantly different ($p < 0.05$) from that of the control (Fig. 2B). Gene expression of MsrA and hCBS-1 was found to be time dependent. When RPE cells were treated with 150 μM H_2O_2 for varying periods of time, a steady increase in MsrA and hCBS-1 gene expression was observed with maximal increase occurring at 24 h for both genes (Figs. 3A and B).

Cellular localization of MsrA

Subcellular localization of MsrA in RPE cells treated with 150 μM of H_2O_2 for 24 h is shown in Fig. 4A. In the untreated cells, MsrA was predominantly localized to the cytoplasm (Fig. 4A, panels A–D). H_2O_2 treatment caused an increase in cytosolic and mitochondrial MsrA (Fig. 4A, panels E–H). To confirm the above MsrA localization studies, we isolated mitochondrial and cytosolic fractions from cells treated with 150 μM of H_2O_2 and performed Western blot analysis. Consistent with confocal microscopy, protein expression with H_2O_2 treatment increased in both the cytosolic (1.29-fold increase) and mitochondrial (1.75-fold increase) fractions (Fig. 4B).

Increased susceptibility to cell death with H_2O_2 in MsrA-silenced RPE

Silencing of MsrA expression with 100 nM MsrA siRNA resulted in approximately 80% decrease in MsrA

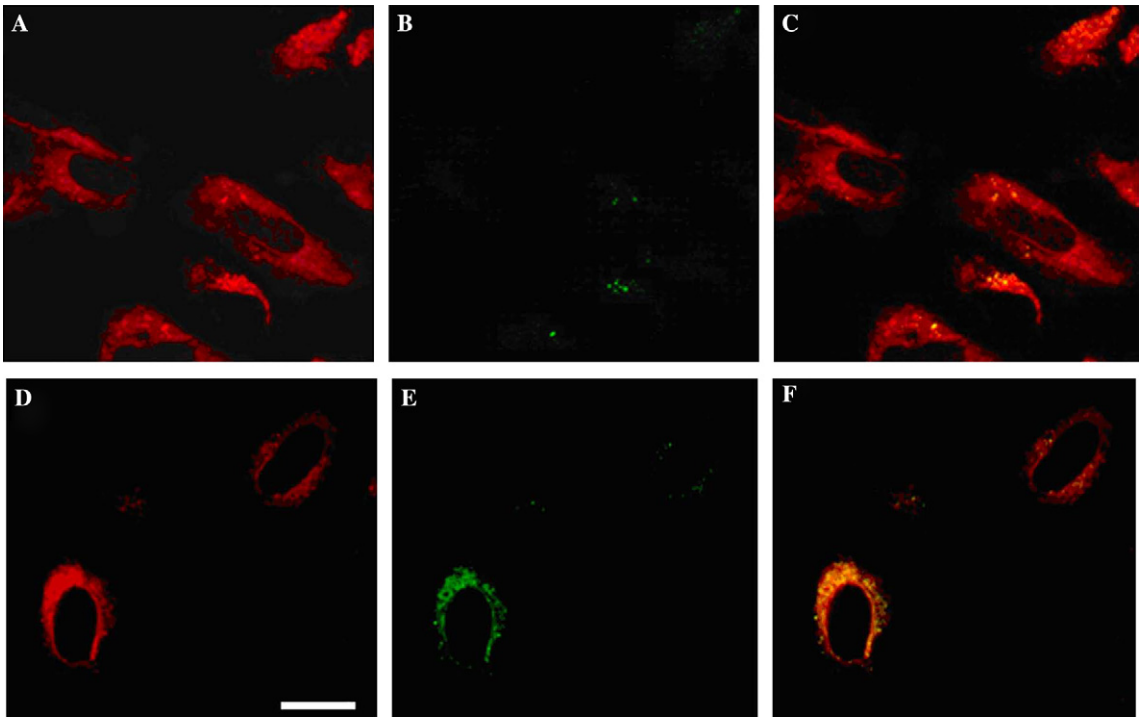

Fig. 1. Accumulation of ROS in mitochondria of RPE cells treated with 150 μM H_2O_2 for 24 h determined by confocal laser scanning microscopy. In each image mitochondria are shown in red, while ROS are shown in green. A,B represent untreated RPE, while D,E represent H_2O_2 treated RPE. Colocalization is illustrated by yellow color in the merged figures (C,F; bar = 50 μm; insets, two fold increased magnification).

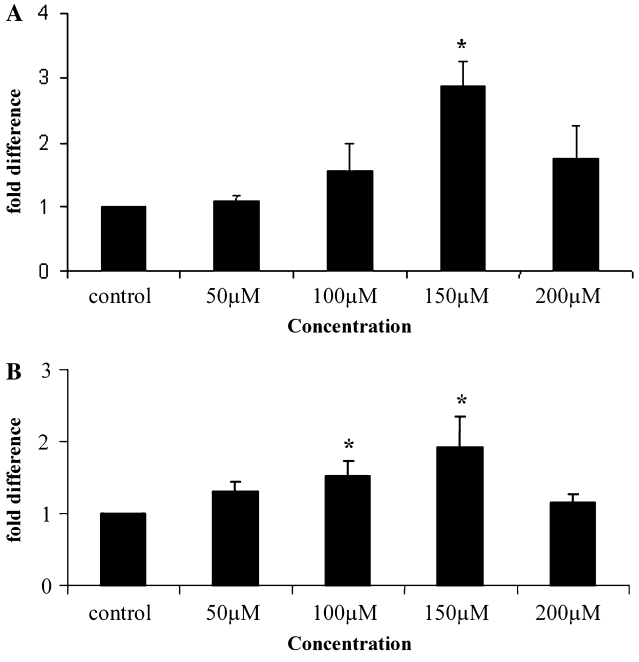

Fig. 2. Real-time quantitative RT-PCR showing the effect of H_2O_2 treatment on MsrA (A) and hCBS-1 (B) gene expression in RPE cells. Results are presented as difference in fold levels compared to untreated cells. Values represent means ± SEM of three independent experiments. *Significantly different ($p < 0.05$) as compared to control.

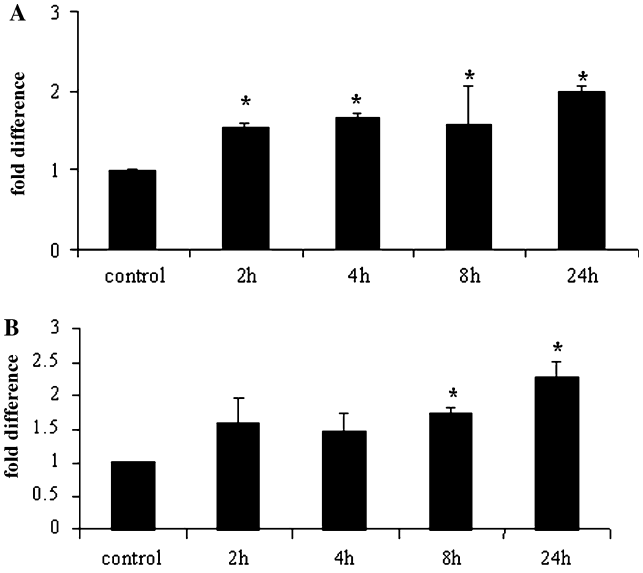

Fig. 3. Time-dependent change in MsrA (A) and hCBS-1 (B) gene expression. RPE cells were treated with 150 μM H_2O_2 for specified times. Results are presented as difference in fold levels compared to untreated cells. Values represent means ± SEM of three independent experiments. *Significantly different ($p < 0.05$) as compared to control.

protein expression (Fig. 5A). After silencing MsrA siRNA, we found a decrease in cell viability with increasing concentrations of H_2O_2. There was no difference in cell viability among untreated controls of MsrA-silenced, mock-transfected and non-targeting siRNA-transfected RPE. As seen in Fig. 5B, silencing of MsrA rendered

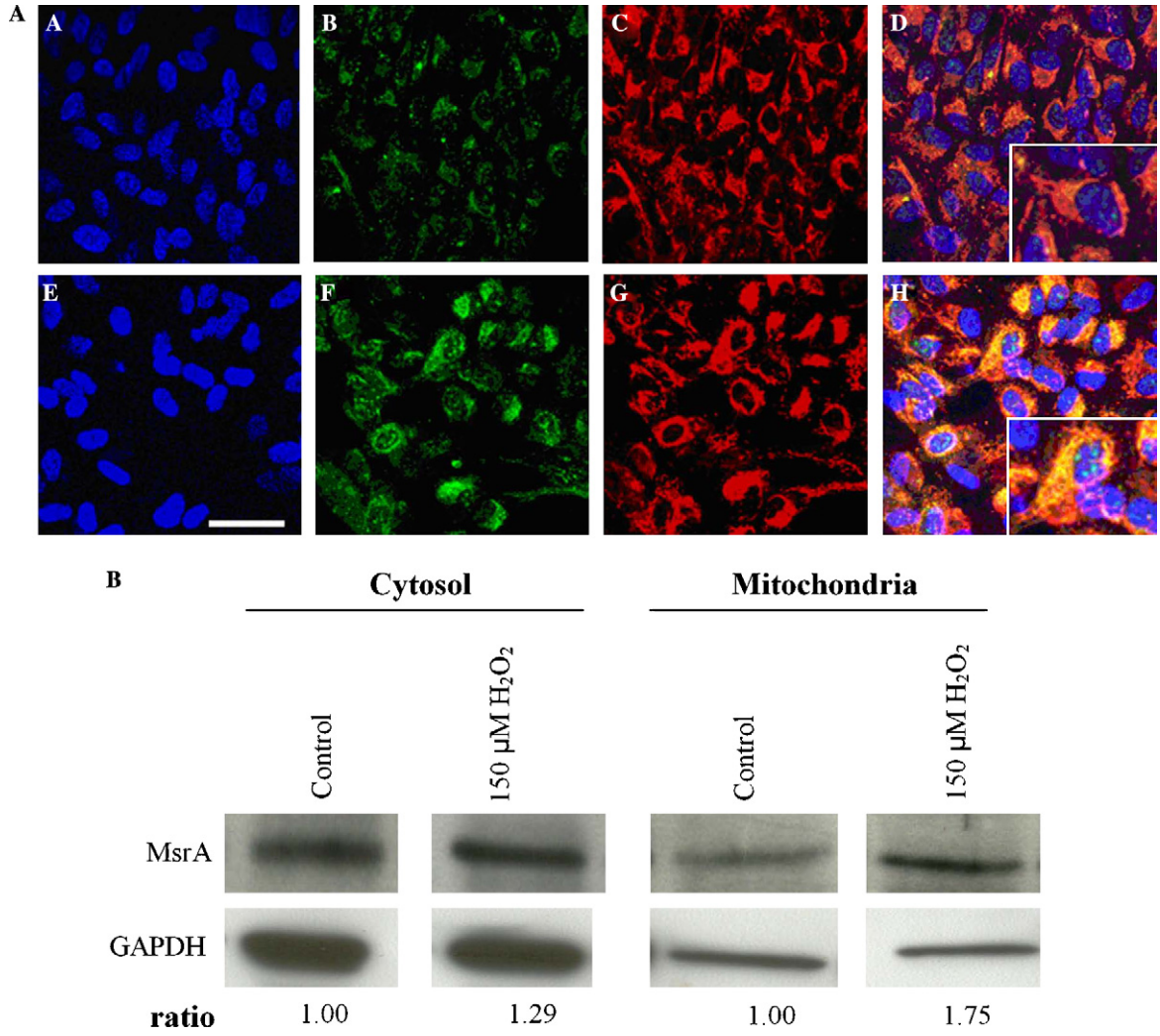

Fig. 4. (A) Localization of MsrA in mitochondria of human RPE cells (bar = 50 μm). Immunoreactivity for MsrA is identified by green fluorescence (panels B,F). Nucleus (panels A,E) and mitochondria (panels C,G) were stained with markers DAPI and Mito Tracker Red, respectively. Cells were treated with 150 μM H_2O_2 for 24 h. Increased expression of MsrA was noticed in H_2O_2 treated cells in the mitochondria and cytosolic regions (panel H; see also inset), when compared to untreated control cells (panel D). (B) Western blot analysis of RPE mitochondrial and cytosolic fractions extracted from RPE cells exposed to 150 μM H_2O_2 and immunoblotted with anti-MsrA antibody as described in experimental. Ratio normalized to GAPDH is shown.

RPE cells more vulnerable to H_2O_2-induced oxidative stress when compared to non-silencing siRNA-transfected cells, and this decrease in viability was highly significant ($p < 0.01$). We ensured that the difference in cell viability observed using the live/dead cell viability assay was not due to the interference of siRNA since no significant difference was observed when transfections were carried out with mock-transfected siRNA.

Evidence for caspase 3 activation

Fig. 6 shows the effect of silencing MsrA and imposing oxidative stress on siRNA RPE with H_2O_2 (150 μM). When MsrA is suppressed in RPE, there was increased expression of caspase 3 as compared to untreated control. Exposure of siRNA RPE further increased expression of the active form of caspase 3 (Fig. 6). This was confirmed by flow cytometry analysis in which siRNA and siRNA-H_2O_2-treated RPE exhibited higher caspase 3 activation as compared to untreated controls (data not shown).

Spatial localization of MsrA in AMD retina

Frozen sections of the macular region of the retina from normal and AMD patients were immunostained for MsrA (Fig. 7). In AMD, an intense staining was noticed in hard as well as in soft drusen (Figs. 7B and C). No apparent expression could be detected in the RPE or choriocapillaris of aged retina (Fig. 7A). No reaction products were observed when the primary antibody was omitted (Fig. 7D).

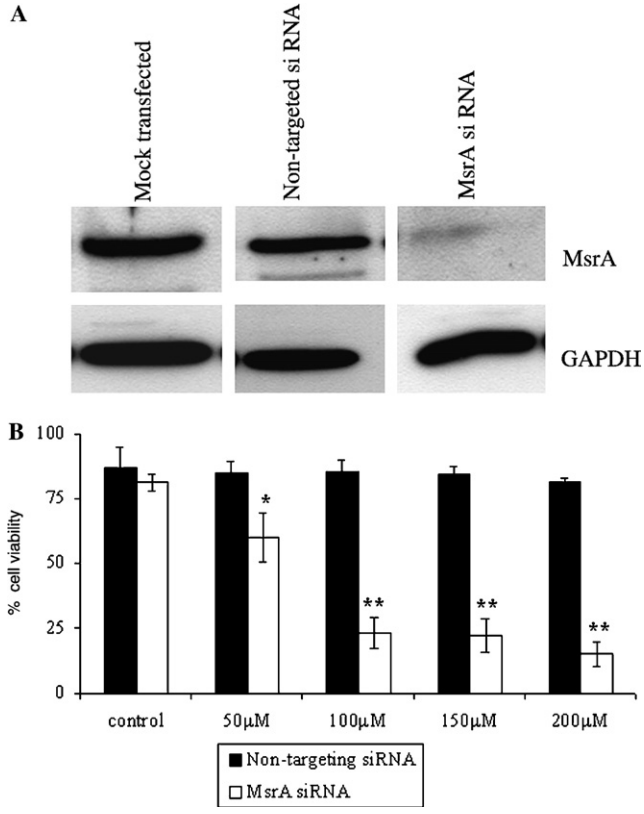

Fig. 5. (A) Western blot analysis of MsrA of RPE cells transfected with 100 nM non-targeting siRNA, mock-transfected and 100 nM MsrA siRNA as described in Experimental. (B) Silencing MsrA gene increased susceptibility of RPE to cell death from H_2O_2 treatment in a dose-dependent manner. Significant difference $^{**}p < 0.01$, $^{*}p < 0.05$ versus controls. Cells were transfected in antibiotic-free DMEM containing 10% FBS and then changed to 1% FBS prior to treatment with H_2O_2. Viability was measured using flow cytometry.

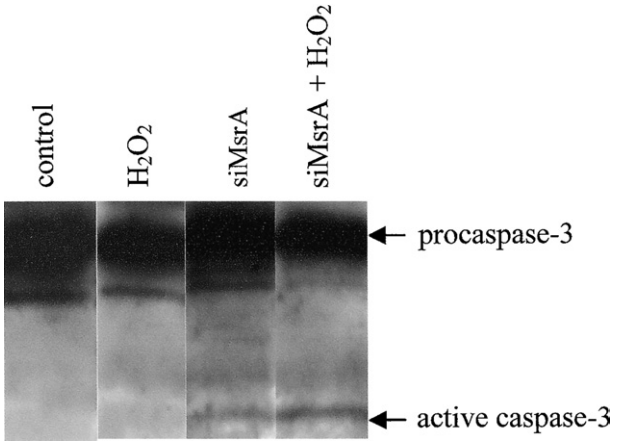

Fig. 6. Western blot analysis showing active pro- and active caspase 3 expression in RPE cells after silencing MsrA and/or exposure to 150 μM H_2O_2. Active caspase 3 expression increased in siRNA containing RPE as compared to control. Oxidative stress due to exogenous H_2O_2 further increased active caspase 3.

Discussion

The data from the present study indicate that induction of oxidative stress in RPE cells by H_2O_2 elicits an array of cellular responses. Evidence for dose- and time-dependent upregulation of MsrA and hCBS-1 was found. This was accompanied by the accumulation of ROS, activation of caspase 3, and increased expression of MsrA. Accordingly, silencing of the MsrA gene rendered RPE cells more sensitive to oxidative stress and increased cell death from exogenously added H_2O_2. MsrA accumulated in drusen in AMD patients.

The methionine sulfoxide reductase family is composed of two structurally unrelated classes of monomeric enzymes, MsrA and MsrB. All other antioxidative enzymes can detoxify oxygen, but only Msrs can participate in the repair of oxidized methionine, an important constituent amino acid of most proteins. Thus, the Msr system is known to be involved in the regulation of protein functioning through the cyclic oxidation/reduction of critical methionine residues, and also in the maintenance of the cellular redox state by reducing the exposed methionine sulfoxide in proteins acting as ROS scavengers [23]. Recent studies have shown the presence of MsrA isoforms in the cytosol and mitochondria to assist in detoxification in both cellular compartments [24]. Further, a couple of new members have recently been added to the Msr family, viz., MsrB3A that is endoplasmic reticulum-specific, and MsrB3B, which was found to be localized to the mitochondria [25,26].

Previous studies in the rat have demonstrated the expression of MsrA in multiple tissues such as liver, kidney, heart, retina, macrophages, and brain [27]. In the present study, we localized MsrA in human RPE cells. The RPE has the highest catalase activity among ocular tissues and contains high amounts of antioxidants to protect them against oxidative stress [28]. The presence of MsrA may serve to protect RPE from protein oxidation and damage and may also help maintain a reduced redox environment in the cell. However, with age, the antioxidative ability of RPE appears to be reduced as evidenced by the decreased catalase activity and decreased hemeoxygenase-1 level [28,29].

Various studies have revealed that ROS from subtoxic concentrations of the physiologic substrate H_2O_2 act as signal transduction messengers and modulate gene expression [30–33]. We examined the regulation of expression of the MsrA and hCBS-1 genes upon exposure to H_2O_2 to determine the role of Msr in the ability of cells to adapt to oxidative stress. The role of MsrA as an antioxidant is well known [34–36] but functional studies on MsrB are scarce. In our present work, we found that both MsrA and hCBS-1 genes were up-regulated upon oxidative exposure, suggesting their probable role as antioxidants by enabling cells to repair their

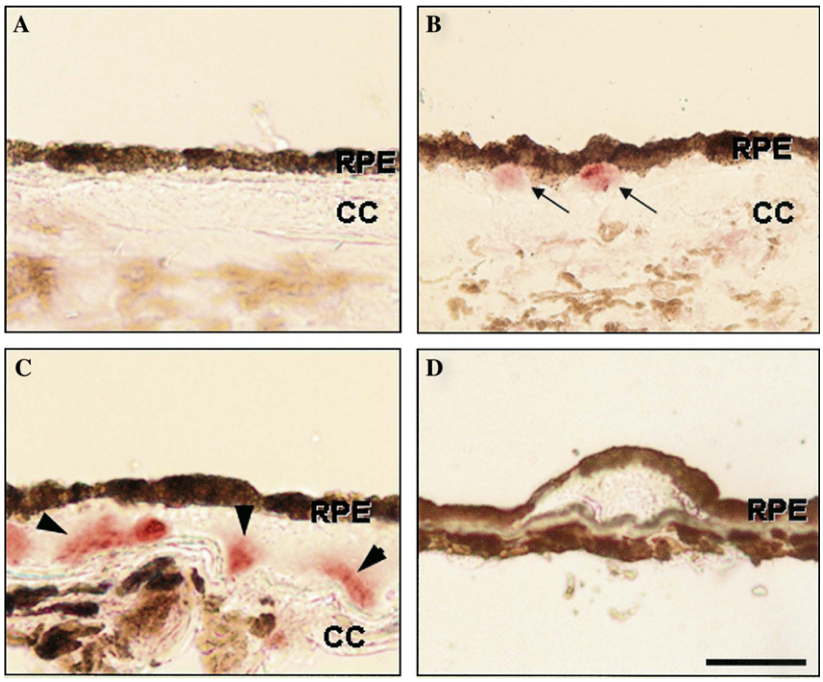

Fig. 7. Immunohistochemical localization of MsrA in the retina of normal human and AMD patients. Retinas of normal and AMD patients were immunostained with anti-MsrA (A–C) or secondary antibody alone (D). MsrA immunoreactivity is observed in the hard (B, arrows) and soft drusen (C, arrowheads). No apparent staining is noticed in the RPE or choriocapillaris (CC). The image is representative of immunostaining performed in five controls and five AMD patients in the age range 74–95 years (bar = 50 μm).

oxidatively damaged proteins. Picot et al. [22] reported that in human WI-38 fibroblast cells exposed to mild oxidative treatment, both MsrA and hCBS-1 genes were up-regulated and concluded that Msrs are not only involved in protein maintenance but also in cellular defense and redox homeostasis.

Confocal microscopic analysis and the MsrA protein expression showed that H_2O_2 increases expression in both cytosolic and mitochondrial compartments. The expression of mitochondria and cytosolic MsrA is in accord with a recent report on the existence of the two subcellular pools in hepatocytes [24].

In addition to MsrA and hCBS-1 genes, our preliminary real-time PCR studies have also revealed that human RPE express MsrB3A and MsrB3B, the two recently identified protein forms that can be targeted to the ER and mitochondria, respectively (P.G. Sreekumar, R. Kannan, and D.R. Hinton, unpublished observations). This may suggest that the reduction of methionine-R-sulfoxides in different cellular compartments is provided by individual MsrB enzymes. The MsrB3s in humans are likely regulated by alternative splicing. Further work will be needed to confirm and address the regulation and significance of these enzymes in retinal pathology as and when specific antibodies become available.

Oxidative stress has been implicated in different types of cell death in the central nervous system and in the eye [37–39]. High levels of ROS disturb the redox balance and directly cause oxidative damage of nucleic acids, lipids, and proteins, which can result in apoptosis [31,40]. The RPE cells are sensitive to oxidative stress as evidenced by the viability results of our study. Silencing of MsrA gene resulted in a significant decrease in cell viability and increased apoptosis. Kantorow et al. [13] found that overexpression of MsrA protected human lens epithelial cells against oxidative stress and silencing rendered cells more sensitive to stress. Furthermore, other studies have shown that overexpression of MsrA renders cells more resistant to oxidative stress [11,12]. Mitochondria are the major target of ROS and H_2O_2 is known to damage mitochondrial DNA (mtDNA) leading to compromised mitochondrial redox functions (for a review, see [41]). It also induces cellular damage through the depletion of ATP, glutathione, and NADPH levels and through DNA strand breakage [3,42]. H_2O_2 also can change the membrane potential permeability resulting in the release of cytochrome *c* from the mitochondria. In our present study using carboxy-H_2-DCFDA staining, we found that ROS accumulated in mitochondria. The ROS in turn is known to trigger an array of apoptotic enzymes leading to cell death. Accordingly, our studies also showed that activation of caspase 3 was involved in cell death from H_2O_2 and the activity of caspase 3 increased further in RPE cells with suppressed MsrA expression.

We found that MsrA was localized in the drusen of AMD patients. Drusen are extracellular deposits that

accumulate below the RPE on Bruch's membrane and are risk factors for developing AMD. The reason and significance of the finding of the accumulation of MsrA in hard and soft drusen remain to be assessed. A general function of MsrA in drusen could be to ensure that methionine sulfoxide residues in proteins do not accumulate as a result of oxidative injury. It is of interest that Nakata et al. [43] recently found large deposits of small heat shock proteins (α-crystallins) in the drusen and suggested that this accumulation may be to prevent further damage to proteins secreted from RPE. The aged control retina did not show expression of MsrA in RPE layer in our immunostaining studies. This may be explained by the finding that MsrA decreases with senescence [22,44,45].

In summary, our data show that H_2O_2 at sublethal concentrations stimulated the expression of Msrs to help the cells to withstand oxidative stress-associated phenomenon. Consistent with an important role for MsrA in oxidative stress, silencing of the MsrA gene resulted in caspase 3 activation and significant apoptosis in RPE. Our data showing extensive deposits of MsrA in drusen suggest it could also play a role in AMD, but the mechanism remains to be elucidated.

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