Phagocytosis and H₂O₂ Induce Catalase and Metallothionein Gene Expression in Human Retinal Pigment Epithelial Cells

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Purpose. Reactive oxygen intermediates have been implicated in the aging process and degenerative diseases of the eye, including retinopathy of prematurity, cataractogenesis, and macular degeneration. The purpose of this study was to investigate the effect of phagocytosis of photoreceptor outer segments and the addition of exogenous H₂O₂ on catalase and metallothionein expression in human retinal pigment epithelial cells.

Methods. Confluent RPE cells were treated with bovine photoreceptor outer segments or H₂O₂ for either 6 or 18 hours. Slot blot hybridization was used to assess catalase and metallothionein gene expression after 6 hours. Catalase enzyme activity and metallothionein content were measured after 18 hours.

Results. Phagocytosis or the addition of H₂O₂ increased catalase enzyme activity and metallothionein twofold above control levels. The addition of n-acetyl cysteine abrogated the inductive effect caused by either stress. Catalase and metallothionein gene expression, measured by slot blot hybridization, also were measurably induced by either stress. Phagocytosis of photoreceptor outer segments increased extracellular H₂O₂ concentration nine times above control.

Conclusions. The response of the retinal pigment epithelial cells to phagocytosis was indistinguishable from the response observed after the addition of exogenous H₂O₂. The generation of H₂O₂ during phagocytosis may act as an intracellular signal in retinal pigment epithelial cells that leads to increased levels of key antioxidant enzymes and other proteins important for protecting the cells from oxidative damage. Invest Ophthalmol Vis Sci. 1995;36:1271–1279.

One of the important functions of the retinal pigment epithelial (RPE) cell is the phagocytosis and degradation of retinal outer segments (ROS). In dystrophic rats, a defect in the ability of pigment epithelial cells to phagocytose rod outer segment results in degeneration of the sensory retina.¹ There also is evidence that a defect in the RPE contributes to age-related maculopathy in humans.² This normal process of RPE uptake and degradation may be an oxidative stress to the RPE. Peroxisomal beta-oxidation of these long chain fatty acids, mitochondrial respiration, and numerous oxidases produce reactive oxygen intermediates, which include hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH).³ Superoxide (O₂⁻) production has been reported by porcine RPE.⁴ Studies in our laboratory have revealed that, in human RPE, catalase activity decreases with age, and the decrease is greater in eyes with signs of macular disease.⁵ Catalase is an iron-dependent enzyme that scavenges H₂O₂ either catalytically by the dismutation of H₂O₂ directly to H₂O and molecular oxygen or peroxidatically with the aid of a hydrogen donor.⁶ An age-related decrease in catalase has been correlated with an increase in H₂O₂ production in the adult housefly,⁷ suggesting that cellular defenses against oxygen-free radicals deteriorate with age.

Recently, we reported⁸ high levels of metallothionein (MT) in human RPE cells that also showed an age-related decline. Metallothionein is a small, metal-binding, acute-phase stress protein whose synthesis is
Units catalase activity/mg Protein

<table>
<thead>
<tr>
<th>Hydrogen Peroxide Concentration</th>
<th>Units Catalase Activity/mg Protein</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.250</td>
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<tr>
<td>0.250 mM</td>
<td>100</td>
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<td>0.500 mM</td>
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FIGURE 1. Catalase and metallothionein induction by H₂O₂. Retinal pigment epithelial cells were incubated in CMF-12 medium with 0.25 mM H₂O₂ or 0.5 mM H₂O₂. After 18 hours, cells were harvested and catalase activity or metallothionein concentration was determined. Values are mean ± SEM. Results statistically different from control levels are indicated. (A) Catalase activity (n = 7, *P = 0.05). (B) Metallothionein content (n = 6, *P = 0.01).

Human donor tissue (donor ages, 3 to 82 years; mean age, 47.73 years; n = 15) was obtained through the National Disease Research Interchange (Philadelphia, PA) by 24 hours after death. Retinal pigment epithelial cells were isolated as described. Cells were grown in Coon’s modified Hams F-12 nutrient medium. The medium was supplemented with 24 mM sodium bicarbonate, 5% fetal bovine serum (HyClone, Logan, UT), 2.0 mM glutamine, 0.20 mM ascorbic acid, 100 μg/ml streptomycin, 100 U/ml penicillin, 5 μg/ml bovine insulin, 10 ng/ml transferrin, 5 ng/ml NaSeO₃, and 10 ng/ml epidermal growth factor. Cells were passage in a standard fashion using 0.25% porcine trypsin.

Retinal Pigment Epithelium Isolation and Culture

MATERIALS AND METHODS

Unless otherwise specified, all reagents were obtained from Sigma Chemicals (St. Louis, MO).

induced by heavy metals, glucocorticoids, and numerous stress-related factors. It participates in the detoxification of heavy metals and the maintenance of zinc and copper homeostasis. Metallothionein contains a high number of thiol groups and may play a direct role in protection against oxygen-free radical damage by scavenging OH⁻ radicals.

Previous studies suggest the importance of antioxidants in protecting the RPE cell from the oxidative damage that occurs during normal physiologic processes. We now report the induction of MT and catalase in human RPE cells by H₂O₂ and phagocytosis of bovine ROS.

FIGURE 2. Catalase and metallothionein induction by phagocytosis. Retinal pigment epithelial cells cultured in CMF-12 medium until confluent were fed a single dose of bovine rod outer segments (20 μg/ml, 5 × 10⁶ rod outer segment particles per milliliter) or latex beads (50 × 10⁶ beads per milliliter). After 18 hours, cells were harvested, and catalase activity or metallothionein content were determined as described. Values are mean ± SEM. Results statistically different from control levels are indicated. (A) Catalase activity (n = 8, *P = 0.0001). (B) Metallothionein content (n = 9, *P = 0.01).

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**Units catalase activity/mg Protein**

**FIGURE 3.** Catalase and metallothionein induction by H₂O₂ and phagocytosis of bovine rod outer segments are inhibited by oxygen-free radical scavenger n-acetyl cysteine. Cells cultured in CMF-12 medium were given a single dose of H₂O₂ (0.5 mM) or fed bovine rod outer segments (20 µg/ml) in the presence of n-acetyl cysteine (20 mM) and were incubated for 18 hours. Cells were harvested, and catalase activity and metallothionein concentration were determined. Values are mean ± SEM. Results statistically different from control levels are indicated. (A) Catalase activity \((n = 7, \ast P < 0.05)\). (B) Metallothionein concentration \((n = 6, \ast P < 0.001)\).

(EDTA) in calcium- and magnesium-free Hank’s balanced salt solution (Gibco-BRL, Gaithersburg, MD). Third- or fourth-passage cells with epithelioid morphology, observed by phase-contrast microscopy, were used approximately 1 week after plating. Medium was changed the day before experimental manipulations. Each experiment was conducted using duplicate or triplicate samples with at least three cell lines from different donors.

**Preparation of Cellular Protein**

Cultured human RPE cells were collected using trypsin/EDTA, sonicated (VirSonic 50; Virtis, Gardiner, NY) for 30 seconds in 100 µl ice-cold 20 mM Tris-HCl buffer (pH 7.8). After centrifugation (Eppendorf Centrifuge 5415; Brinkmann, Westbury, NY) at 13,000g for 10 minutes, the supernatants were collected for protein determination based on the method of Bradford (BioRad, Hercules, CA).²⁸

**Bovine ROS Isolation, Storage, and Feeding**

Bovine ROS were isolated using sterile technique from fresh bovine eyes (AnTech, Tyler, TX) using a discontinuous sucrose gradient.²⁹ Purified ROS were stored at 4°C in 5 mM Tris-acetate buffer, pH 7.40, containing 65 mM NaCl, 17% sucrose, 2 mM MgCl₂, 1 mM N,N-bis-2-bis carboxymethylamino-(ethyl) glycine, 100 µg/ml streptomycin, 100 U/ml penicillin, and 100 U/ml catalase. Before use, the ROS were centrifuged at 9000g for 10 minutes, and the storage medium was removed. The ROS were used within 2 weeks of isolation. Bovine ROS were presented to human RPE cells in growth medium at a protein concentration of 20 µg/ml for either 6 or 18 hour at 37°C. Latex beads (50 × 10⁶ beads/ml) were fed to cultured RPE cells in a similar fashion. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Catalase Enzyme Assay**

Catalase activity in cell homogenates was measured by a technique that depends on the first-order decomposition of H₂O₂ by catalase and the subsequent measurement of residual H₂O₂ by reaction with excess potassium permanganate (KMnO₄).³⁰ Residual KMnO₄ was measured at 480 nm. Catalase activity was determined by comparison to a standard curve using purified catalase and expressed in units of catalase per milligram of soluble protein.

**Cadmium—Hemoglobin Assay for Metallothionein**

Metallothionein was quantitated using the procedure of Eaton and Toal.³¹ The protein concentration in each sample was adjusted to 50 µg/200 µl. After heating (100°C, 2 minutes), the supernatant was added to 200 µl of a solution containing 1 µCi/ml cadmium-109 (New England Nuclear, Boston, MA) and 1 µg/ml total cadmium.

**Statistical Analyses**

Statistical analyses were performed using the Student’s \(t\)-test on the Statistical Analysis Software program (SAS Institute, Cary, NC).

**Slot Blot Hybridization**

Total cellular RNA (20 µg) was isolated using TRIzol reagent (Gibco-BRL, Gaithersburg, MD) and blotted onto a nylon membrane (Gene Images, Cleveland, OH). After blotting, the filters were dried (80°C, 2
FIGURE 4. H$_2$O$_2$ and phagocytosis of bovine rod outer segments (ROS) induce catalase and metallothionein mRNA synthesis. Cells cultured in CMF-12 medium were fed a single dose of H$_2$O$_2$ (0.5 mM) or bovine ROS (20 /g/ml) for 6 hours. Total RNA was isolated by TRIzol reagent and analyzed by slot blot hybridization. (A) Catalase slot blot: lane 1, untreated retinal pigment epithelial cells; lane 2, bovine ROS; lane 3, H$_2$O$_2$ (0.5 mM); lane 4, H$_2$O$_2$ (0.25 mM); lane 5, H$_2$O$_2$ (0.125 mM); lane 6, phorbol myristate acetate (50 ng/ml). (B) Metallothionein slot blot: lane 1, untreated retinal pigment epithelial cells; lane 2, bovine ROS; lane 3, H$_2$O$_2$ (0.125 mM); lane 4, H$_2$O$_2$ (0.25 mM); lane 5, H$_2$O$_2$ (0.5 mM). Blots were then hybridized with a probe for /?-actin as a control for total RNA concentration (C).

Evaluation of Transcription Factors in Retinal Pigment Epithelium

Nuclear extracts were prepared from cultured human RPE cells treated with various agents for 4 hours in T-75 flasks according to the method of Westfall et al. Cells were washed in cold phosphate-buffered saline, lysed in HEPES (10 mM, pH 7.9) at 4°C containing 15 mM MgCl$_2$, 10 mM KCl, 0.5 mM 1,4-dithiothreitol (DTT), 0.1% (wt/vol) NP-40, and nuclei were collected by centrifugation. Nuclear proteins were extracted in high salt buffer containing 20 mM HEPES, pH 7.9, 1.5 mM MgCl$_2$, 0.5 mM DTT, 0.2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride. The nuclear fractions were diluted in 20 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride and stored at —70°C. The protein concentrations of the extracts were determined by the Bradford procedure.

Electrophoretic Mobility Shift Assay

Double-stranded consensus oligonucleotides NF-kB (5'-AGTTGAGGGTTTCCCCAGGC 3') and AP-1 (5'-CGCTTGATGAGTCAGCCGGAA 3') (Promega, Madison, WI) were end-labeled by T4 polynucleotide kinase and (gamma-32P) adenosine triphosphate. The binding reactions were carried out by incubating nuclear extracts (10 /g) with (gamma-32P) labeled NF-kB or AP-1 consensus oligonucleotides (~50,000 counts per minute) in a buffer containing 10 mM Tris, pH 7.4, 100 mM KCl, 5 mM MgCl$_2$, 5% glycerol, 1 mM DTT, and 4 /g of poly(dI-dC)·poly(dI-dC) (Pharmacia Fine Chemicals, Piscataway, NJ) for 20
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Catalase enzyme activity in cultured human RPE cells was increased 150% and 400% after 18 hours by the addition of 0.25 mM and 0.5 mM H₂O₂, respectively (Fig. 1A). H₂O₂ (0.25 mM or 0.5 mM) also significantly increased MT content by 140% and 220% (Fig. 1B). Phagocytosis of a single dose of bovine ROS also increased catalase activity significantly above control. The addition of latex beads increased catalase activity above control; however, the result was not statistically significant (Fig. 2A). Phagocytosis of latex beads or bovine ROS increased MT 150% and 300%, respectively (Fig. 2B).

The induction of catalase and MT by H₂O₂ and phagocytosis was inhibited by 20 mM N-acetyl cysteine (NAC) (Figs. 3A, 3B) and also by treatment with 1 mM actinomycin D (data not shown).

mRNA Expression

Phagocytosis of bovine ROS increased catalase and MT mRNA 2.8-fold and 3.8-fold, respectively, when compared to control (Figs. 4A, 4B). Catalase and MT mRNA also were increased by the addition of exogenous H₂O₂: 0.5 mM H₂O₂ induced catalase and MT mRNA 2.4-fold and 0.25 mM H₂O₂ 1.6-fold, compared to control (Figs. 4A, 4B), whereas 0.125 mM H₂O₂ did not induce either catalase or MT mRNA (Figs. 4A, 4B). Catalase mRNA was induced 1.4-fold by the treatment of RPE cells with 50 ng/ml phorbol myristate acetate (PMA), known to induce H₂O₂ production in neutrophils (Fig. 4A).

H₂O₂ Production

The mechanism by which phagocytosis increased catalase and MT expression became more evident by the demonstration that phagocytosis of bovine ROS increased extracellular H₂O₂ production ninefold. Phagocytosis of latex beads increased extracellular H₂O₂ twofold (Fig. 5).

Transcription Factors

To demonstrate further that phagocytosis of bovine ROS and H₂O₂ treatment are an oxidative stress to RPE cells, we used an electrophoretic mobility shift assay to demonstrate the activation of NF-kB-specific and AP-1 DNA binding activity in RPE cell nuclear extracts. Cultured human RPE cells were treated with paraquat, bovine ROS, latex beads, H₂O₂, PMA, and lipopolysaccharide for 4 hours at 37°C. Human RPE contained low constitutive levels of NF-kB, and activation was increased by phagocytosis of bovine ROS and latex beads, lipopolysaccharide, paraquat, H₂O₂ (Fig. 6), and PMA. Similarly, AP-1 was activated by H₂O₂, PMA, IL-6 + dexamethasone, and phagocytosis of bovine ROS (Fig. 7). Specificity of binding was demonstrated by the addition of a 200-fold excess of unlabelled nuclear regulatory factors NF-kB and AP-1 (Figs. 6, 7). The activation of AP-1 by H₂O₂ required a higher concentration than did NF-kB (0.5 versus 0.15 mM, respectively). The activation of NF-kB resulting from phagocytosis was decreased by NAC. AP-1 activation also was decreased by NAC, but to a lesser extent (Fig. 8). These results show that NF-kB and AP-1 activation by phagocytosis may be mediated through reactive oxygen intermediates.

DISCUSSION

Human RPE cells have high concentrations of catalase and MT. This may be because of the rich oxygen environment and the strong metabolic demands of ingestion and digestion of photoreceptor outer segments, which are associated with the generation of

![FIGURE 5. Extracellular H₂O₂ production is increased by phagocytosis of bovine rod outer segments or latex beads. Retinal pigment epithelial cells incubated in Earle’s balanced salt solution containing 0.56 mM phenol red and 20 U/ml of horseradish peroxidase were fed a single dose of bovine rod outer segments (20 μg/ml) or latex beads (50 × 10⁶ beads per milliliter). H₂O₂ content was measured after 4 hours against a standard curve. Values are mean ± SEM. Results statistically different from control levels are indicated. (n = 7, *p ≤ 0.001).](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933191/ on 11/26/2018)
reactive oxygen intermediates. Previous studies have shown that long-term feeding of ROS increases cellular autofluorescence and catalase activity in RPE, and the uptake of latex beads stimulates $O_2^-$ production in porcine RPE cells. The current study confirms that ROS phagocytosis is an oxidative stress and points to an increase in intracellular $H_2O_2$ as the reactive oxygen species. Ingestion of nonmetabolizable latex beads increases extracellular $H_2O_2$ and induces catalase and MT. However, ingestion of oxidizable ROS increases $H_2O_2$ and induces catalase and MT to a much greater degree. This suggests a dual source of $H_2O_2$. It is possible that phagocytosis activates a nicotinamide-adenine dinucleotide phosphate oxidase producing $O_2^-$. The RPE cell contains high superoxide dismutase activity that would quickly dismutate $O_2^-$ to $H_2O_2$. Phagocytosis of ROS would produce additional $H_2O_2$ by peroxisomal and mitochondrial $\beta$-oxidation of ROS lipids and the activation of other cellular oxidases. We reported finding both nicotinamide-adenine dinucleotide phosphate oxidase and palmitoyl Co-A oxidase activities in cultured RPE cells. The contribution of these systems to total $H_2O_2$ production in the RPE remains to be determined.

The induction of catalase and MT by phagocytosis and $H_2O_2$ is regulated at the level of transcription as shown by the increase in mRNA and the inhibition by actinomycin D. Catalase and MT induction by phagocytosis, $H_2O_2$, and the subsequent inhibition by NAC demonstrate that human RPE cells respond to intracellular oxidative stress, suggesting regulation by a reactive oxygen intermediate-mediated mechanism. N-acetyl cysteine is a nontoxic drug that readily enters cells and serves as a scavenger for reactive oxygen intermediates and as a precursor for glutathione, a major intracellular thiol and reactive oxygen intermediate scavenger in eukaryotic cells. Conversely, NAC does not efficiently quench superoxides, but it has been shown extensively to act as a repressor of ultraviolet light, 12-O-tetradecanoylphorbol-13-acetate, and $H_2O_2$ induction of c-fos, c-jun, and the activation of nuclear factors NF-kB and AP-1. Recent studies suggest that production of the hydroxyl radical, derived from hydrogen peroxide, is required for activation of NF-kB. Induction of MT by oxidative stress and during acute-phase responses possibly is regulated by nuclear regulatory factors that bind to the promoter regions of specific genes. For example,
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During acute-phase responses, AP-1 is thought to induce MT II A gene expression through a functional AP-1 site in its promoter. The catalase gene contains an Sp1 site in its promoter region. This possibility is supported by a recent report that marginal zinc deficiency in cultured RPE cells decreased catalase activity. Our data showed that cultured human RPE cells also contain low constitutive levels of NF-kB, another transcription factor activated by reactive oxygen intermediates and requiring zinc for DNA binding.

We have shown age-related declines in catalase activity and MT concentration in macular RPE. This decrease with age may produce deleterious effects to the human macula, which may be unable to mount a sufficient defense against oxidative stress. The decreased ability of cells to respond to stress during aging could be the result of a decrease in transcriptional regulation by specific nuclear regulatory factors. Further studies are in progress to determine how antioxidants and other factors mediate RPE response to reactive oxygen intermediates generated during phagocytosis of photoreceptor outer segments, which may be critical in protecting the RPE from lifelong oxidative stress.

Key Words
catalase, metallothionein, human retinal pigment epithelium, oxidative stress and gene expression

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