EXPERIMENTAL CELL RESEARCH 214, 242–249 (1994)

Evaluation of Oxidative Processes in Human Pigment Epithelial Cells Associated with Retinal Outer Segment Phagocytosis

MICHAEL V. MICELI,*,†,[1] MARK R. LILES,* AND DAVID A. NEWSOME*,†

*Sensory and Electrophysiology Research Unit, Touro Infirmary, New Orleans, Louisiana 70115; and †Department of Ophthalmology, Tulane University School of Medicine, New Orleans, Louisiana 70112

To investigate the nature of the oxidative event that occurs during phagocytosis of retinal outer segments (ROS) by cultured human retinal pigment epithelial (RPE) cells, cells were incubated with isolated bovine ROS labeled with either the fluorescent probe carboxy-SNAFL-2 or the nonfluorescent, oxidizable probe 2',7'-dichlorodihydrofluorescein (H_2DCF). The increase in fluorescence following phagocytosis was measured by a flow cytometer. Other measurements included: oxygen consumption using a Clark-type oxygen electrode, extracellular superoxide release by superoxide dismutase inhibitable lucigenin chemiluminescence, intracellular hydrogen peroxide (H_2O_2) production, and the effect of catalase inhibition on cellular thiobarbituric acid-reactive substances (TBARS) caused by phagocytosis. The activities of the enzymes NADPH oxidase and palmitoyl-CoA oxidase were also measured. H_2DCF attached to bovine ROS was oxidized during phagocytosis with a time course suggesting oxidation subsequent to ROS uptake. Measurements of oxygen consumption showed a time-dependent increase of 10%, 4 h after ROS feeding, attributable to a doubling of the cyanide-resistant oxygen consumption. Intracellular H_2O_2 production also doubled 4 h after ROS phagocytosis. ROS uptake by RPE cells produced no significant extracellular superoxide, while extracellular superoxide production was readily demonstrated in a control macrophage cell line. Enzyme activity measurements showed that incubation of RPE cells with ROS doubled catalase activity without affecting superoxide dismutase or glutathione peroxidase activities. Inhibition of catalase during ROS uptake increased TBARS by 66%. Other enzyme activity measurements showed that human RPE cells possess both NADPH oxidase and palmitoyl-CoA oxidase activities. We conclude that ROS phagocytosis subjects RPE cells to an oxidative event on the same order of magnitude as measured in a macrophage. The event is not an extracellular macrophage-type respiratory burst and may be due to intracellular H_2O_2 resulting from an NADPH oxidase in the phagosome or from β-oxidation of ROS lipids in peroxisomes. Irrespective of case, the enzyme catalase appears to be essential in protecting the RPE cell against reactive oxygen species produced during phagocytosis. © 1994 Academic Press, Inc.

INTRODUCTION

Neutrophils and macrophages are known to produce reactive oxygen species during a respiratory burst in order to kill invading bacteria during phagocytosis [1]. The retinal pigment epithelium (RPE) is a single cell layer located between the sensory retina and choroid which provides many important metabolic functions essential to the visual process, one of which is the daily phagocytosis and degradation of spent rod and cone outer segments [2]. Disruption of this process has been shown to result in degeneration of the sensory retina in experimental animal models [3], and there is evidence that a defect in the RPE contributes to age-related maculopathy (ARM) in humans [4].

This normal process of retinal outer segment (ROS) uptake and degradation may be an oxidative stress to the RPE. Superoxide (O_2) production has been reported in porcine RPE; however, this process has not been well characterized and its relationship to the macrophage respiratory burst is not known [5]. Our laboratory has shown that RPE catalase activity decreases with increasing age in the human [6]. The RPE has the highest catalase activity of any ocular tissue measured and contains high concentrations of nonenzymatic antioxidants [6–8]. These antioxidants seem to be essential for protecting the RPE cell against oxidative damage occurring as a result of normal physiologic processes [9, 10]. Due to the possible link between aging, free radical damage, and ARM in the human, the ability of the RPE to protect itself from oxidative damage may have implications for the understanding of the etiology of an important age-related human disease.

The objective of the present study is to further characterize the oxidative processes associated with phagocytosis and degradation of ROS by the RPE.

[1] To whom reprint requests should be addressed at the Sensory and Electrophysiology Research Unit, Touro Infirmary, 1401 Foucher St., New Orleans, LA 70115. Fax: (504) 897-7637.

0014-4827/94 $6.00

MATERIALS AND METHODS

Chemicals. Unless otherwise noted, all chemicals and reagents were obtained from Sigma Chemical Company (St. Louis, MO). Carboxy-SNAFL-2, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF), and latex beads were obtained from Molecular Probes (Eugene, OR). H_2DCF (the non-fluorescent reduced form of 2',7'-dichlorofluorescein) was purchased as the unconjugated succinimidyl ester. Carboxy-SNAFL-2 was also purchased as the succinimidyl ester. ^{14}C-labeled palmitoyl-CoA was obtained from Amersham Corp. (Arlington Heights, IL). The peroxisome proliferators clofibrate and clofibric acid were from ICN Biochemicals (Costa Mesa, CA) and Research Biochemicals Inc. (Natick, MA), respectively.

Cell culture of RPE and RAW 264.7 cells. Human donor tissue was obtained through the National Disease Research Interchange (Philadelphia, PA) by 24-h postmortem. RPE cells were isolated as previously described [11]. Cells were grown in Coon's modified Ham's F-12 nutrient medium with 3.0 μM zinc. The medium was supplemented with 24 mM sodium bicarbonate, 5% fetal bovine serum (FBS) (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 $NaSeO_3$, and 10 ng/ml epidermal growth factor. Cells were passaged in a standard fashion using 0.25% porcine trypsin with 1 mM EDTA in Ca^{2+}, Mg^{2+}-free (CMF) Hanks' balanced salts solution (HBSS) (GIBCO). RAW 264.7 cells, a transformed murine monocyte–macrophage cell line, were obtained from American Type Culture Collection (Rockville, MD) and maintained in the growth medium described above. This phagocytic cell, which exhibits a superoxide respiratory burst, has been used extensively in phagocytosis studies and studies of the macrophage respiratory burst and was used as a positive control [12, 13].

ROS isolation. Retinal ROS were isolated using sterile technique from fresh bovine eyes (AnTech Inc., Tyler, TX), using a discontinuous sucrose gradient [14]. Purified ROS were stored at 4°C in 5 mM Tris–acetate buffer, pH 7.40, containing 65 mM NaCl, 17% sucrose, 2 mM $MgCl_2$, 1 mM EDTA, 100 μg/ml streptomycin, 100 U/ml penicillin, and 100 U/ml catalase. Prior to use, the ROS were centrifuged at 9000g for 10 min and the storage medium was removed. The ROS were used within 2 weeks of isolation.

ROS oxidation and uptake assays. To measure oxidative products production, we utilized the probe H_2DCF, which has been used to quantitate H_2O_2 production in neutrophils and macrophages [15–17] and to measure oxidative stress in brain [18]. Isolated bovine ROS were covalently labeled with H_2DCF by adding 100 μg of the succinimidyl ester of 2',7'-dichlorodihydrofluorescein diacetate in 10 μl of anhydrous dimethylformimide (Aldrich Chemical, Milwaukee, WI) to 2 mg of bovine ROS in 500 μl of 25 mM sodium phosphate buffer, pH 8.0, containing 65 mM NaCl and 17% sucrose. The buffer had been previously bubbled with 100% N_2 for 15 min. The ROS were labeled for 30 min at room temperature in the dark with gentle stirring. The labeled ROS were spun down at 9000g for 10 min and washed three times. H_2DCF-labeled ROS were used within 24 h because the fluorochrome is unstable upon storage. Uptake assays were performed in growth medium which had been treated overnight with 10 ml/liter of Chelex 100 chelating resin (Bio-Rad). This was done to remove transition metals and greatly reduced auto-oxidation of the probe during incubation. The Chelex-treated medium was repleted with 1 mM Ca and 1.2 mM Mg before use. The assay was performed by incubating 10 μg/ml of labeled ROS protein with RPE cells plated in 12-well plates. Incubation was for 1–4 h at 37°C. Since ROS themselves are slightly fluorescent and to control for increases in cellular fluorescence due to auto-oxidation of the probe during handling, 10 μg/ml of H_2DCF-labeled ROS was incubated in duplicate wells without cells. At the end of the incubation, cells were washed five times with CMF-HBSS, trypsinized, pelleted, and resuspended in 500 μl of phosphate-buffered saline (PBS), pH 8.0, containing 10 mM glucose. Analysis was carried out on the living cells using an Epic's Profile flow cytometer with excitation at 488 nm and emission at 515–530 nm. ROS incubated without cells were collected by centrifugation and also analyzed in the flow cytometer. The contribution of auto-oxidized H_2DCF labeled-ROS to total cellular fluorescence was subtracted.

To measure relative phagocytic rates of RPE cells and macrophages, bovine ROS were labeled in a fashion similar to that described above using the succinimidyl ester of the fluorescent dye carboxy-SNAFL-2. The cells were harvested and analyzed by flow cytometry with excitation at 488 nm and emission at 550–610 nm. This assay has been characterized previously [19].

O_2 consumption measurements. O_2 consumption of RPE cells and macrophages was measured using a Clark-type polarographic electrode. Cells were grown as described above and plated in T-75 flasks. Typically, 2–3 mg of cellular protein was used per measurement from one or two confluent T-75 flasks. Cells were fed 100 μg of bovine ROS protein in 5 ml of growth medium, 30 min or 4 h prior to trypsinization and measurement. Measurements were obtained in Coons' medium with no additives in a 2.0 ml volume. O_2 content of the medium was taken as 406 nmol/O/ml [20]. After the baseline measurements were obtained for RPE cells, the oxygen consumption in the presence of 1 mM NaCN was determined as a measurement of the nonmitochondrial O_2 consumption. Cells were collected by centrifugation and measured for protein (Bio-Rad) after the oxygen measurements.

Chemiluminescence assay. Superoxide production was measured using the chemiluminescence probe *N,N'*-dimethyl-9,9'-biacridinium binitrate (lucigenin). Lucigenin chemiluminescence involves the superoxide-mediated generation of excited *N*-methylacridone, via a stepwise electron reduction and oxygenation pathway. At neutral pH, lucingenin is present as a membrane impermeant divalent cation and has been employed clinically to examine the release of extracellular superoxide from activated leukocytes [21, 22]. RPE cells or RAW 264.7 cells were plated in glass scintillation vials 7–14 days prior to analysis and maintained in growth medium as described above. Both RPE cells and RAW 264.7 cells attach readily to glass and maintain normal morphology in the vials. One day prior to analysis, cells were fed 3 ml of phenol red-free (PRF) DMEM (Sigma) containing 24 mM $NaHCO_3$, 25 mM Hepes, pH 7.4, and 5% FBS. ROS stimulation of superoxide release was measured two ways. First, to measure the response of the cells to the ROS stimulus during the first minutes, cells were removed from the incubator and allowed to cool to room temperature in dim light (5 min) and 100 μM lucigenin in DMSO was added at time zero. The vials were swirled, and the background photon emission was monitored every 0.25 min for 5 min using the single photon monitor of a Beckman LS 5000 TE scintillation counter. The final concentration of DMSO was kept at 0.1% or below to avoid scavenging reactive oxygen species. After 5 min, 30 μg of bovine ROS was added in 15 μl of PRF-DMEM (challenge) and the photon emission monitored for another 5 min. At this time 100 U of horseradish superoxide dismutase (SOD) was added in 30 μl of PBS and the emission was monitored for an additional 5 min. The difference between the counts with and without SOD is reported as SOD-inhibitable chemiluminescence and is expressed as counts/0.25 min/mg protein. Second, to measure the generation of superoxide by the cells at longer time points after the ROS challenge, RPE or RAW 267.4 cells were fed with PRF-DMEM as above. The day of the experiment the cells were treated with 30 μg bovine ROS in 15 μl of PRF-DMEM at time zero and returned to the incubator. Vials were removed from the incubator at 30 min, 1 h, and 4 h, 100 μM lucigenin was added, and the photon emission was monitored for 5 min. SOD (100 μM) was then added and the photon emission was monitored for another 5 min. The SOD-inhibitable chemiluminescence was determined from counts before and after SOD addition.

Lipid peroxidation end product determination. Lipid peroxidative products were measured using the thiobarbituric acid (TBA) test for malondialdehyde [23]. Because TBA is not specific for malondialdehyde, results of this analysis were expressed in terms of TBA-reactive substances (TBARS). Samples of crude RPE homogenate were added

to a reaction mixture of 15% glacial acetic acid, 0.4% SDS, and 0.3% TBA. This mixture was heated in a 95°C water bath for 30 min. Each sample was then centrifuged for 10 min at 1000*g*. The supernatant was removed and the absorbance at 540 nm recorded with an Elisa plate reader (Titertek Multiskan MCC 340). Results were based on an extinction coefficient for MDA of $1.5 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed in terms of pmol TBARS/mg protein.

Enzyme activity assays. Catalase activity in cell homogenates was measured by a technique which depends upon the first-order decomposition of H_2O_2 by catalase and the subsequent measurement of residual H_2O_2 by reaction with excess potassium permanganate ($KMnO_4$) [24]. Residual $KMnO_4$ was measured at 480 nm. Catalase activity was determined by comparison to a standard curve using purified catalase and expressed in units catalase/mg soluble protein.

SOD activity was determined using a nitro blue tetrazolium (NBT) reduction assay in which hypoxanthine and xanthine oxidase were used to produce superoxide anions [25]. One unit of SOD activity is defined as the quantity of cellular protein or purified enzyme producing 50% inhibition of NBT reduction, which was recorded at 560 nm. The following curvilinear regression model was generated using purified CuZn SOD and was used to express results from cellular proteins in terms of units SOD/mg soluble protein:

$$Y_{\%\ \text{inhibition}} = 5 + 2\ \log e^{X_{\text{\# protein}}}.$$

Glutathione peroxidase (GPX) activity was measured using an NADPH reduction assay [26]. Soluble cell proteins were added to a reaction mixture containing reduced glutathione, glutathione reductase, and NADPH in a phosphate buffer. The reaction was initiated by adding 5 m*M* H_2O_2, the absorbance decrease at 340 nm was recorded, and the activity in the absence of cellular protein was subtracted. Results were based upon a molar extinction coefficient for NADPH of $6.22 \times 10^3 M_{-1}\ cm_{-1}$. One unit of GPX is defined as μmol NADPH oxided/mn, and results are expressed in mUnits GPX/mg soluble protein.

NADPH-oxidase activity was measured by the superoxide-dependent, SOD-inhibitable reduction of cytochrome C [27]. Macrophages and RPE cells were activated by preincubation at 37°C with phorbol 12-myristate 13-acetate (PMA, 3.0 μg/ml in CMF-PBS) for 5 min. Chilled PBS (4°C) was added to stop the action of PMA, and the cells were scraped off their flasks with a Teflon cell scraper. Cells were pelleted, resuspended in a Tris-buffered sucrose solution (0.34 *M*), and hand-homogenized with a 2-ml Corning Pyrex tissue grinder on ice for 1 min. The homogenate was thn centrifuged at 27,000*g* for 20 min at 4°C to isolate the membrane fraction. The pellet was solubilized in 0.25% sodium deoxycholate for 20 min on ice, centrifuged at 100,000*g* for 1 h at 4°C. The resulting supernatant contained the solubilized NADPH-oxidase system and was stored at −80°C for no longer than 48 h before analysis. The cytochrome C-reduction assay was conducted at 28°C, with the reaction mixture consisting of 130 μ*M* sodium phosphate buffer (pH 7.0), 80 μ*M* ferricytochrome C (Type VI), 25 μg solubilized membrane protein, and 100 m*M* NADPH to initiatethe reaction. The reduction of cytochrome C was monitored at 550 nm in the absence and presence of 30 μg SOD, and the superoxide-independent reduction was subtracted from the superoxide-dependent rate. A millimolar extinction coefficient for ferricytochrome C of 21.1 $mM^{-1}\ cm^{-1}$ was used to calculate the results.

Palmitoyl-CoA oxidase was measured using ^{14}C-labeled palmitoyl-CoA [28]. This method relies on the conversion of acid-insoluble [^{14}C]-palmitoyl-CoA to acid-soluble [^{14}C]acetyl-CoA by the activity of palmitoyl-CoA oxidase. Cells grown in 12-well plates were harvested by scraping and homogenized in a 50 m*M* Tris buffer (pH 8.0) with 250 m*M* sucrose, 0.5 m*M* EGTA, and 0.5% Triton X-100. Homogenates were incubated in a reaction mixture containing cofactors necessary for peroxisomal β-oxidation and ^{14}C-labeled palmitoyl-CoA. After incubation for 30 min, ice-cold perchloric acid was added to each sample, and the precipitate was removed by centrifugation. A total of 500 μl of supernatant was counted by liquid scintillation counting, and results were expressed in dpm/mg protein.

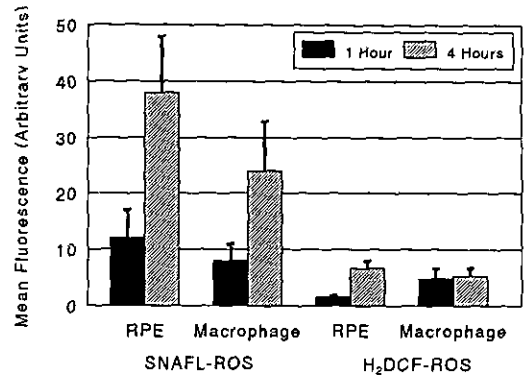

FIG. 1. Measurement of ROS uptake and ROS oxidation by RPE and RAW 264.7 cells. Fluorescence was measured by flow cytometry and is the average of six experiments except four for SNAFL-ROS uptake by RAW 264.7 cells. Error bars are ±SEM. The increase of fluorescence with time is statistically significant at $P < 0.05$ for each pair except for H_2DCF-ROS oxidation by macrophages as determined by paired *t* tests.

RESULTS

ROS Uptake by RPE Cells Produce Reactive Oxygen

We first determined whether we could demonstrate that phagocytosis of ROS produced reactive oxygen. Figure 1 shows the results of incubating RPE and RAW 264.7 cells with ROS-labeled with the oxidizable probe H_2DCF. Incubation of both cell types with H_2DCF-labeled ROS increases the cellular fluorescence, indicating that the probe is oxidized during uptake. Furthermore, H_2DCF-ROS fluorescence increased from 1 to 4 h in RPE cells, which did not occur in the macrophages. To determine whether this was due to relative differences in phagocytosis, RPE and RAW 264.7 cells were incubated with SNAFL-labeled ROS. Both cell types showed an increase in cellular fluorescence from 1 to 4 h consistent with a steady uptake of the labeled ROS. The greater fluorescence measured in the RPE was probably due to their greater size. Since the differences in fluorescence between RPE and RAW 264.7 cells seen from 1 to 4 h with H_2DCF-labeled ROS is not due to differences in the amount of ROS internalized, the oxidative processes that occur in RPE cells seem to be different than those that occur in macrophages.

Direct Measurement of Oxygen Consumption Stimulation Resulting from Phagocytosis

To determine the magnitude of the "respiratory burst" in the RPE cell in comparison with macrophages, we directly measured oxygen consumption before and

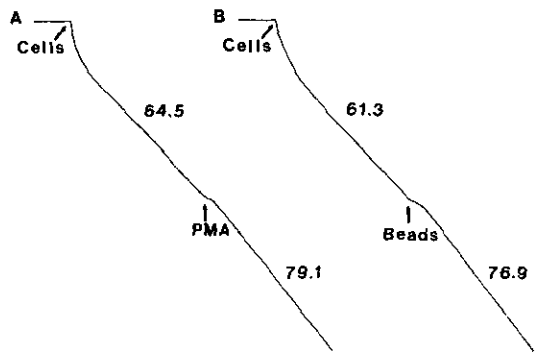

FIG. 2. Oxygraph traces showing the stimulation of O_2 consumption in RAW 264.7 cells upon treatment with (A) PMA or (B) latex beads. Numbers indicate nmol O/min/mg cell protein. The data were similar for stimulation by ROS.

after various stimuli. Measurements on macrophages showed an immediate stimulation of O_2 consumption of 22% upon stimulation with PMA (Fig. 2), latex beads, or ROS. Acute changes in RPE cell oxygen consumption could not be detected when stimulated by these treatments. However, we did measure an increase in O_2 consumption in RPE cells when measured 4 h after ROS feeding (Fig. 3 and Table 1). More importantly, the cyanide-resistant O_2 consumption increased over 100%, to 10% of the total. This shows that the stimulation of O_2 consumption arises from nonmitochondrial oxygen consumption and demonstrates that following phagocytosis of outer segments by RPE a respiratory burst occurs, and on a different time scale than that in the macrophage.

Measurement of Extracellular Superoxide Release

We then measured extracellular superoxide production using a chemiluminescent assay based on lucigenin,

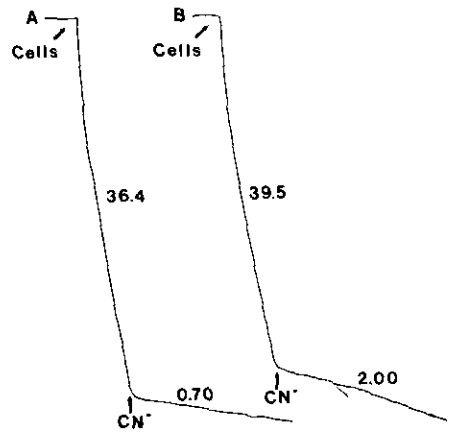

FIG. 3. Oxygraph traces showing the increase in cyanide-resistant O_2 consumption upon treatment of RPE cells with ROS. (A) Untreated, (B) treated for 4 h with 10 μg/ml bovine ROS. Numbers indicate the nmol O/min/mg. The data are summarized in Table 1.

TABLE 1

O_2 Consumption of RPE Cells

+ROS	Endogenous	+CN^-
0 min	33.4	1.43
30 min	35.4*	1.99
4 h	36.5†	3.09‡

Note. Data (nmol O/min/mg) are means ± SEM for $n = 4$ experiments. Statistical significance was determined by ANOVA of repeated measures.

* $P < 0.05$, compared to 0 min.

† $P = 0.08$, compared to 0 min.

‡ $P < 0.01$, compared to 0 min + CN^-.

which is reported to be specific for extracellular superoxide [21, 22]. The results in Fig. 4A indicate that we could readily detect stimulation of superoxide production almost immediately after adding ROS to RAW 264.7 cells, in agreement with the oxygraph experiments. Furthermore, the time course of the superoxide release shows that O_2 peaks at 30 min after ROS addition. This is consistent with the results obtained with H_2DCF-labeled ROS; i.e., in macrophages the respiratory burst occurs during the first hour of stimulation (Fig. 4B). Superoxide production was also readily seen when the cells were stimulated with PMA or latex beads (data not shown). In contrast, the RPE cells showed no significant measurable superoxide release upon stimulation with ROS, or upon prolonged incubation with ROS (Fig. 5A). Neither latex beads nor PMA produced measurable superoxide release in RPE cells (data not shown). These results clearly indicate that the RPE respiratory burst is not a superoxide burst, is entirely intracellular, or both.

Measurement of H_2O_2 Production by 3AT Inhibition of Catalase Activity

Since the RPE oxidative burst seemed to be intracellular, we measured whether H_2O_2 production was stimulated by ROS feeding. This was determined indirectly by measuring the time course of the inhibition of catalase by 3AT. 3AT forms an irreversible inactive complex with the catalase–H_2O_2 compound I [29]. Figure 6 shows that incubating RPE cells with ROS for 4 h doubles the rate of catalase inactivation by 3AT, indicating a doubling of the rate of H_2O_2 production. This effect could not be demonstrated at 30 min post-ROS challenge (data not shown). This is consistent with the doubling of the nonmitochondrial oxygen consumption 4 h after feeding ROS (Table 1).

Effects of ROS Uptake on Antioxidant Enzyme Activities

Since H_2O_2 production is increased in RPE cells after phagocytosis of ROS, we determined the effect of ROS

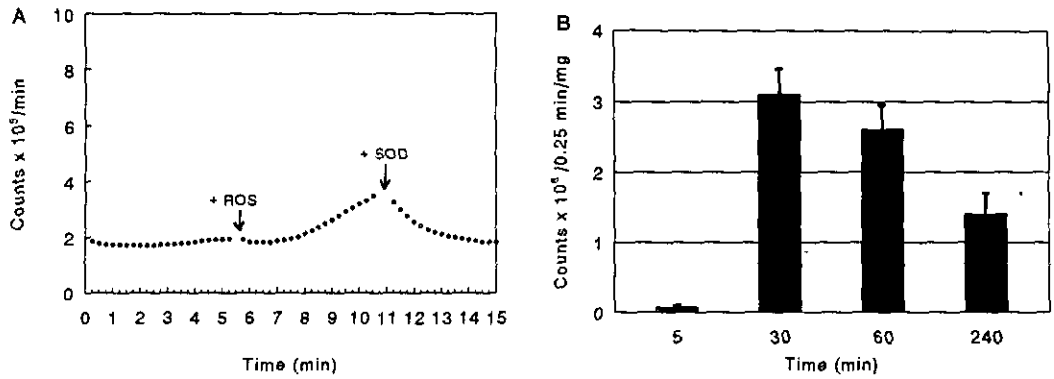

FIG. 4. Superoxide production by macrophages measured with the lucigenin assay. (A) Typical experiment showing stimulation of chemiluminescence upon addition of 10 μg/ml bovine ROS and reduction in chemiluminescence upon addition of 100 U SOD. (B) SOD-inhibitable lucigenin chemiluminescence at different times after ROS challenge. The SOD-inhibitable chemiluminescence was averaged over 5 min. Background counts in the absence of ROS were subtracted from B. Error bars are ±SEM, $n = 3$.

uptake on cellular antioxidant enzymes. Phagocytosis of bovine ROS every other day for a 2-week period induced a twofold increase (relative to control) in the catalase activity of RPE cells (Table 2). No statistically significant differences were observed in the activities of GPX or SOD during ROS phagocytosis. Also, no statistically significant differences in total soluble protein content were observed as a result of ROS uptake.

The effect of selective inhibition of catalase during ROS uptake was also determined. Treatment of cultured human RPE cells with 3AT inhibited cellular catalase activity in a dose-dependent manner, with a concentration of 1 m*M* 3AT producing 97% inhibition after 2 weeks of treatment. The antioxidant enzymes GPX and SOD were unaffected by 3AT treatment, with activities 95 and 94% of control, respectively (Table 2). Likewise, the uptake of labeled ROS by RPE cells was unaltered by 3AT treatment (data not shown).

Measurement of lipid peroxidation indicated a 66% increase in the TBARS of cells treated with 3AT and fed ROS relative to control cells fed ROS alone (Table 2). No increase in TBARS was observed in the cells treated with 3AT only. Our purified ROS contained measurable lipid peroxides (121 pmol TBARS/mg ROS protein); however, the increase seen in the lipid peroxidation end products cannot be accounted for by added ROS, even assuming all ROS are taken up by the cells.

Cultured RPE Cells Contain both NADPH Oxidase and Palmitoyl Co-A Oxidase Activity

It seemed clear that production of H_2O_2 was stimulated by ROS phagocytosis and that catalase was necessary for protection of cellular or ROS lipids resulting from this process. Two potential sources of the increased H_2O_2 are formation of O_2 by an NADPH oxidase and subsequent dismutation by intracellular SOD or the

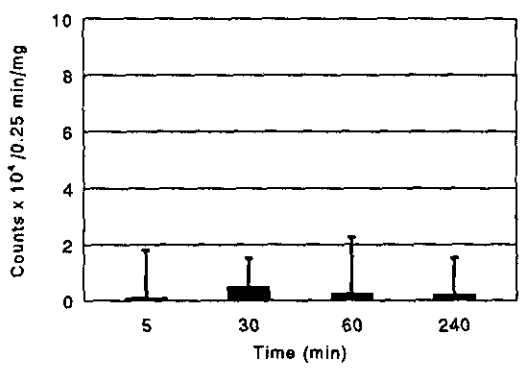

FIG. 5. Lucigenin chemiluminescence of RPE cells upon treatment with 10 μg/ml bovine ROS. RPE cells exhibited no significant measurable SOD inhibitable superoxide production over background upon treatment with ROS. Error bars are ±SEM, $n = 3$. Background counts in the absence of ROS have been subtracted.

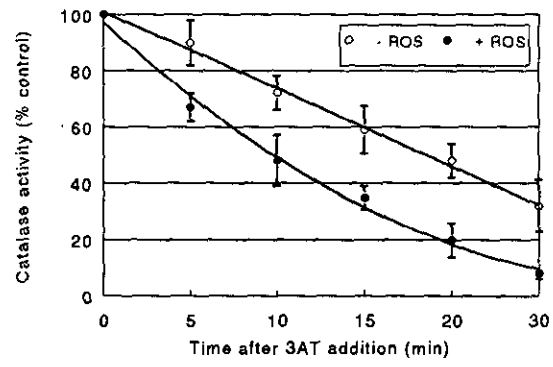

FIG. 6. Time course of the inhibition of RPE cell catalase activity in the presence of 10 m*M* 3AT. Cells incubated with 10 μg/ml bovine ROS for 4 h had twice the rate of catalase inhibition as the untreated cells. The 4-h ROS incubation did not measurably increase catalase activity. Data represent $n = 4$ experiments performed in duplicate, ±SEM, $P < 0.01$ for effect of ROS as determined by ANOVA of repeated measures.

TABLE 2

Analysis of Antioxidant Enzyme Activities and Lipid Peroxidation Products

Group (n)	SOD ($U\ mg^{-1}$)	Catalase ($U\ mg^{-1}$)	GPX ($mU\ mg^{-1}$)	TBARS ($pmol\ mg^{-1}$)	Protein (μg)
Control (4)	165 ± 27	22.8 ± 3.7	285 ± 52	203 ± 16	380 ± 41
Control + ROS (4)	170 ± 37	44.3 ± 6.2*	317 ± 54	239 ± 31	415 ± 49
3-AT (4)	156 ± 33	0.7 ± 0.4†	269 ± 40	195 ± 23	406 ± 27
3-AT + ROS (4)	186 ± 29	1.2 ± 0.6§	293 ± 88	397 ± 60*‡	446 ± 31

Note. Data are reported as means ± SEM. Statistical significance was determined by paired t tests. n, number of cell populations tested.

* $P < 0.05$ (compared to control).

† $P < 0.01$ (compared to control).

‡ $P < 0.05$ (compared to control + ROS).

§ $P < 0.01$ (compared to control + ROS).

production of H_2O_2 by peroxisomal β-oxidation of ROS lipids. In an attempt to determine the relative importance of the NADPH oxidase system vs the peroxisomal β-oxidation system in RPE cells as potential sources of H_2O_2, we measured the activities of the two respective enzyme systems, NADPH oxidase and palmitoyl-CoA oxidase in RPE cells. Not surprisingly, RAW cells had considerable NADPH oxidase activity (6.18 ± 0.72 nmol O_2^-/min/mg, $n = 4$). However, RPE cells also had approximately 50% of the NADPH oxidase activity of the macrophages (2.99 ± 0.37 nmol O_2^-/min/mg, $n = 4$). The RPE cells also had significant palmitoyl-CoA oxidase activity (633 ± 28 pmol oxidized/min/mg, $n = 4$), showing that both enzyme systems are a potential source of reactive oxygen species. Treatment of the RPE cells with clofibrate or clofibric acid, potent inducers of palmitoyl-CoA oxidase in rat hepatocytes [30], slightly increased this activity by an average of 18%. This small increase indicates that RPE cells do not respond to clofibrate or clofibric acid or that palmitoyl CoA oxidase may be constitutive in human RPE cells.

DISCUSSION

Previous evidence has suggested that phagocytosis of ROS by RPE cells is an oxidative stress. This statement is supported by the following: (1) Long-term feeding of ROS increases cellular autofluorescence and increases catalase activity in RPE [31, 32]. (2) Phagocytosis of ROS induces metallothionein and activates NF-κB in RPE cells [33]. (3) Uptake of beads has been reported to stimulate superoxide production by porcine RPE [5]. The present study confirms that ROS phagocytosis is an oxidative stress and points toward an increase in intracellular H_2O_2 as the reactive oxygen species. However, the precise mechanism of reactive oxygen production in the RPE cell is by no means clear. The difficulty of establishing this with certainty is due to the short half-life and interconvertability of many of the key species due to enzymatic and nonenzymatic reactions in the cell. For example, the RPE cell contains high SOD activity which would quickly dismutate O_2^- to H_2O_2. Increases in H_2O_2 could also lead to formation of the highly reactive hydroxyl radical through the Fenton reaction [8].

Macrophages and neutrophils produce a dramatic superoxide respiratory burst during phagocytosis via activation of the NADPH oxidase system. The superoxide produced has been proported to aid in the killing of ingested pathogens and to target ingested proteins for degradation by proteases [1, 34]. This reactive species may also activate an immune response in macrophages or in other components of the immune system [35]. While RPE cells share some common elements with macrophages, the extracellular release of superoxide upon ingestion of rod and cone outer segments would be unwanted due to the potential damage to the surrounding retinal tissue. Retinal outer segments contain unusually high concentrations of unsaturated long-chain fatty acids, in particular 22:6, which would be especially susceptible to peroxidation [36]. Furthermore, the purpose of the production of O_2^- on a scale indicated by the O_2 data is unclear. Intriguingly, we did measure NADPH oxidase activity in RPE membrane preparations at activities approximately 50% of that found in stimulated macrophages. The finding that RPE cells have NADPH oxidase and a previous report of measurable O_2^- production underlines the fact that the role of this system in RPE is still unclear. NADPH oxidase activity has been detected in endothelial cells and fibroblasts [37, 38] and may be involved in the cell's acute phase response by activating cytoplasmic transcription factors [39, 40]. This, coupled with the fact that all cells produce superoxide in mitochondria as a by-product of normal oxidative metabolism, could explain the presence of relatively high activity of NADPH oxidase and the detection of extracellular superoxide under certain conditions in RPE cells [5].

Our data show that inhibition of catalase during ROS feeding increases the content of TBARS in RPE, which

suggests an important role for catalase in protecting the RPE cell during this process, and implicates H_2O_2 as a probable reactive oxygen species. Our data using H_2DCF clearly demonstrates that there is an oxidative event associated with ROS uptake by RPE cells on a magnitude similar to that which occurs in macrophages. The experiments reported here do not prove the participation of H_2O_2 in this event because 2′,7′-dihydrodichlorofluorecein is preferentially, but not exclusively, oxidized by H_2O_2 [41].

It has been demonstrated in other cell types that long-chain fatty acids are preferentially degraded by β-oxidation in peroxisomes, especially when the ATP content in the cell is high and ATP production by mitochondrial β-oxidation is not needed [42, 43]. The demonstration that nonmitochondrial O_2 consumption and H_2O_2 production both double 4 h after challenging cultured RPE cells with isolated ROS is consistent with peroxisomal β-oxidation of ROS lipids. This, coupled with the demonstration of palmitoyl-CoA oxidase activity, a peroxisomal β-oxidation pathway enzyme, and the histochemical demonstration by others that RPE cells contain many peroxisomes [44, 45], suggests that peroxisome participation in the process of lipid oxidation could be a possible source of reactive oxygen species. The likelihood of peroxisomal β-oxidation of very-long-chain fatty acids in RPE cells, coupled with reports that 22:6 fatty acids are recycled back to the photoreceptors after outer segment shedding [46], indicates that control of these alternate pathways and the mechanisms of lipid targeting need to be elucidated. It is also possible that the state of oxidation of ROS lipids may influence lipid targeting and oxidation pathways [47].

We demonstrated in this study that ROS uptake is an oxidative event and that inhibition of catalase during uptake contributes to lipid peroxidation during this process. Since it has been shown previously that catalase declines with age in human RPE [6] and that inhibition of catalase in the lens can lead to cataract formation [48], it is conceivable that oxidative stress may contribute to age-related maculopathy in a similar fashion. The elucidation of how the RPE cell protects against free radical and oxidative damage during phagocytosis could help in understanding this disease process. Since age-related maculopathy is the major cause of vision loss in people over age 60, further study is warranted.

The authors thank Dr. Ion Deaciuc, Department of Physiology, LSU Medical Center, for the use of his oxygraph equipment, Dr. Nancy Bologna, Department of Psychiatry, LSU Medical Center, for her help with the flow cytometry, and Reneé Gumma for her technical assistance. This work was supported by Grant EY-006782 (M.V.M.) from the National Institutes of Health, Bethesda, Maryland.

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Received February 8, 1994
Revised version received May 11, 1994