Protection of Retinal Pigment Epithelium From Oxidative Injury by Glutathione and Precursors

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Purpose. This study was performed to examine the effect of exogenous glutathione (GSH) or its precursor amino acids on oxidative injury in cultured human retinal pigment epithelium (RPE).

Methods. Cultured human RPE cells were suspended in Krebs-Henseleit medium, and 150 μM t-butylhydroperoxide was added. Cell viability was assessed by 0.2% trypan blue exclusion 30, 60, and 120 minutes after the addition of GSH or its amino acid precursors.

Results. Added GSH provided protection at concentrations of 0.01 mM and higher. The amino acid precursors for GSH, glutamate, cysteine, and glycine also protected against injury, but this required at least 0.1 mM of each amino acid. Inhibition of intracellular GSH synthesis by inclusion of 1 mM buthionine sulfoximine eliminated the protection by added amino acids but did not alter the protection by added GSH.

Conclusions. These results indicate that protection by the amino acid precursors is mediated through synthesis of GSH, and they also show that exogenous GSH can provide protection against oxidative injury. Invest Ophthalmol Vis Sci. 1993;34:3661-3668.

Age-related macular degeneration, the leading cause of blindness in elderly Americans,12 causes visual loss through photoreceptor damage, usually secondary to injured retinal pigment epithelium (RPE) cells. The RPE plays a critical role in the maintenance of photoreceptors by phagocytosis and degradation of shed photoreceptor debris. The biochemical basis for the injury to the RPE is not understood clearly and may involve multiple factors such as degenerative changes in Bruch’s membrane, damage to the choroidal vasculature, and oxidative injury.34 The possibility that oxidative injury is involved is suggested by the high concentrations of polyunsaturated fatty acids in the photoreceptor outer segments, high oxygen partial pressure in the RPE-photoreceptor complex, and intense light focused on the macular area.

Glutathione is an important antioxidant that protects against injury by acting as a reductant of peroxides in a reaction catalyzed by glutathione peroxidase.5 Two types of peroxidase activities have been described. The major activity in most cell types is catalyzed by selenoenzymes, which are active on fatty acid hydroperoxides, phospholipid hydroperoxides, cholesterol hydroperoxides, and hydrogen peroxide.67 Considerable glutathione (GSH) peroxidase activity also is associated with some isoforms of glutathione S-transferase.8 Several studies with a number of different cell types have shown that GSH depletion increases the risk of oxidative injury.910 In addition, GSH can be used to detoxify reactive aldehydes generated from lipid peroxidation11 and can support detoxification of free radicals by ascorbate- and vitamin E-dependent mechanisms.12 In this latter process, reduction of free radicals by α-tocopherol results in generation of the α-chromonoxyl radical, which is reduced back to α-tocopherol by ascorbate. The semidehydroascorbate generated by this reaction is in equilibrium with dehydroascorbate; GSH reduces these oxidized forms back...
to ascorbate\(^{18}\) and thereby can suppress free radical injury.

In the current study, we investigated whether exogenous GSH and its amino acid precursors can protect RPE cells from oxidative injury resulting from the chemical oxidant t-butylhydroperoxide (t-BHP). It is a relatively stable hydroperoxide that has been used extensively in vitro studies of oxidative injury.\(^{14}\) Also, t-BHP permeates cell membranes readily and is metabolized by GSH peroxidase. Thus, it provides a convenient and reproducible model for study of oxidative injury in cultured human RPE cells.

**MATERIALS AND METHODS**

RPE cell cultures were established from human donor eyes obtained through the Georgia Lions Eye Bank. Cultures were established from 13 human eyes with a mean donor age of 49.6 years. Information as to race was not available from the eye bank.

Cells were detached from globes less than 72 hours old with 0.25% trypsin—ethylenediaminetetraacetic acid (Gibco, Grand Island, NY). After incubation at 37°C for 1 hour, Dulbecco's minimal essential medium (Gibco, Grand Island, NY) supplemented with fetal bovine serum (20%) was added to the trypsin—cell mixture, and the cells were reincubated.\(^{15}\) Primary cell cultures were established in 25 cm\(^2\) Primaria tissue culture flasks with low-glucose (1000 mg/ml) Dulbecco's minimal essential medium with sodium pyruvate (110 mg/ml) supplemented with fetal bovine serum (20%), amphotericin B (3 \(\mu\)g/ml), and gentamicin sulfate (50 \(\mu\)g/ml). Cells were maintained at 37°C under 95% \(\mathrm{O}_2\) and 5% \(\mathrm{CO}_2\), and the medium was changed every 2 to 3 days. After reaching confluence, cells were passed into 75 cm\(^2\) flasks every 7 to 14 days. Cultures were tested routinely for mycoplasma contamination. All experiments were performed on cells after the third to sixth passage.

For suspension, the cells initially were washed three times with Hank's balanced salt solution, and a 0.1% trypsin—ethylenediaminetetraacetic acid solution (Sigma Chemical Company, St. Louis, MO) was added. After no more than 5 minutes at 37°C, an equal volume of culture medium was added, and the cells were collected by centrifugation. Cells were resuspended in Krebs-Henseleit medium (Sigma) (NaCl 13.8 g/l, KCl 0.72 g/l, KH\(_2\)PO\(_4\) 0.26 g/l, MgSO\(_4\) 7H\(_2\)O 0.59 g/l, NaHCO\(_3\) 4.0 g/l, CaCl\(_2\) 2H\(_2\)O 0.75 g/l [pH 7.4]) supplemented with 12.5 mM HEPES buffer. There was no cell shrinkage when cells were exposed to this mixture, no cell death was observed during centrifugation.

Cells were preincubated with 0.25 mM acivicin, an inhibitor of the above enzyme,\(^{16}\) for 15 minutes before the experiment was started. To inhibit GSH synthesis from its amino acid precursors, cells were preincubated with 1 mM buthionine sulfoximine (BSO), an inhibitor of \(\gamma\)-glutamyl synthetase.\(^{17}\) GSH or amino acids were added 5 minutes before the addition of 150 \(\mu\)M t-BHP. The viability of cells was assessed by the percentage excluding 0.2% trypan blue. Similar experiments on cells in monolayer showed similar characteristics and were verified further in some experiments by lactic dehydrogenase release. Cells were studied in suspension to allow multiple time course samplings under identical conditions.

To determine whether GSH synthesis from the precursor amino acids was required for protection, cells were pretreated with BSO. Because \(\gamma\)-glutamylcysteine synthetase is normally feedback inhibited, cells were pretreated with 250 \(\mu\)M diethylmaleate (Sigma) for 20 minutes to deplete GSH. After pretreatment, diethylmaleate was removed before subsequent studies. Levels of intracellular GSH were measured by high-performance liquid chromatography after derivatization with 40 mM iodoacetic acid and 1-fluoro-2,4-dinitrobenzene (1% v/v), as described by Reed, Babson, Beatty et al.\(^{18}\) Dinitrophenyl derivatives were separated on a 3-aminopropyl column (Custom LC, Houston, TX) and detected at 365 nm. GSH was quantified by integration relative to standards.

Statistical analyses were performed by repeated measures analysis of variance with the Biomedical Statistical Software (Los Angeles, CA).

**RESULTS**

To determine the concentration range over which t-BHP induced oxidative injury in RPE cells, cell viability was measured 2 hours after treatment with concentrations of t-BHP ranging from 5 to 500 \(\mu\)M. The results (Fig. 1) showed that RPE cell viability decreased as the t-BHP concentration was increased over this concentration range. To optimize the ability to study the effects of potentially protective agents, additional studies were performed with 150 \(\mu\)M t-BHP, a concentration that caused approximately a 50% loss of cell viability over this time course (Fig. 1, inset).

The effect of precursor amino acids for GSH synthesis on t-BHP—induced cell death was evaluated by adding glutamate, cysteine, and glycine as a mixture 5 minutes before the addition of t-BHP. Addition of the mixture of the three amino acids, each at 0.01 mM, resulted in no detectable protection; addition of 0.1 and 1.0 mM each showed a concentration-dependent protective effect (Fig. 2). When cysteine was omitted from this mixture, no protection was observed at either 0.1 or 1.0 mM glutamate and glycine. Similarly, cysteine alone did not provide protection. Thus, the data showed that the three amino acid precursors all...
FIGURE 1. Effect of varying concentrations of t-BHP on RPE viability 2 hours after treatment. The t-BHP concentration is shown in micromolar units. The averages for experiments ± SE are given. The insert shows the effect of 150 μM t-BHP on RPE viability. △: control (N = 10); ▼: cells + 150 μM t-BHP (N = 8). Values for incubation with t-BHP were significantly different (P < 0.05) from control.

FIGURE 2. Protection of RPE cells from 150 μM t-BHP-induced cell death by amino acid constituents of GSH. □: 0.01 mM glycine, glutamate, and cysteine (N = 11); ▲: 0.1 mM amino acid (N = 11); ▼: 1.0 mM amino acid (N = 11). Averages for experiments ± SE are given. Values for incubation with 0.1 and 1.0 mM amino acid were significantly different (P < 0.05) from 0.01 mM and control. In areas in which the error bar is missing, the standard error is less than the size of the symbol.
must be present to protect against t-BHP-induced toxicity.

Pretreatment of suspended RPE cells with diethylmaleate depleted intracellular GSH levels and stimulated GSH synthesis (Fig. 3). Incubation of diethylmaleate-treated cells with amino acids resulted in recovery of cellular GSH within 60 minutes. This recovery of GSH was prevented by inclusion of BSO. Thus, BSO effectively inhibits GSH synthesis by RPE cells. Viability studies performed with BSO-pretreated cells showed a significant reduction in the ability of added amino acids to protect against t-BHP-induced cell death (Fig. 4). Consequently, the results indicate that protection by the amino acid precursors of GSH involves use of the precursors for GSH synthesis.

To determine whether the direct addition of GSH could protect against t-BHP-induced injury, cells that were not pretreated with diethylmaleate were preincubated without or with a near-physiological GSH concentration (0.01 mM). Cells without GSH showed a nearly 50% loss in cell viability at 2 hours. Those pretreated with 0.01 mM GSH showed little loss in cell viability and were protected significantly relative to cells without GSH (Fig. 5). Higher GSH concentrations also provided protection, but when the GSH concentration was decreased to 0.001 mM, this protective effect was lost (Fig. 6). Addition of BSO did not affect the ability of exogenous GSH to protect against t-BHP-induced injury.

**DISCUSSION**

Age-related macular degeneration is the leading cause of blindness in the elderly American population. Vi-
FIGURE 4. Effect of 1 mM BSO and amino acid constituents on t-BHP-induced cell death \( (N = 13) \); 1.0 mM glycine, glutamate, and cysteine were used. Values for incubation with amino acids were statistically different \( (P < 0.05) \) from t-BHP at 120 minutes; the addition of 1 mM BSO to incubations with amino acids showed no statistical difference from those with t-BHP alone.

FIGURE 5. Protection of RPE cells from t-BHP injury by exogenous GSH. ▲: cells + 150 μM t-BHP \( (N = 34) \); □: cells + 0.01 mM GSH + 150 μM t-BHP \( (N = 11) \). Averages for experiments ± SE are given. Values for incubation with GSH were significantly different \( (P < 0.05) \) from those for incubation with t-BHP alone at 120 minutes.
Visual loss results from either geographic atrophy of the RPE or exudation from blood vessels that break through Bruch’s membrane and proliferate beneath the pigment epithelium. The pathognomonic change in age-related macular degeneration is the formation of abnormal excrescences beneath the RPE called drusen. These facts have combined to imply a critical role for abnormalities in RPE function in the pathogenesis of macular degeneration.19-22

The RPE is responsible for the constant uptake and digestion of shed photoreceptor outer segments. Photoreceptor death results from RPE cell death or dysfunction. Therefore, healthy RPE metabolism is essential to the photoreceptors and, thus, to vision. RPE health relies on its ability to metabolize numerous potential toxins, such as the free radicals and peroxides that could exist in the photoreceptor debris.23 The light and oxygen necessary for visual function also can cause and potentiate free radical and peroxide formation.

Thus, RPE dysfunction could result if there was an inadequate ability to detoxify free radicals or peroxides. Although the biochemical mechanisms responsible for macular degeneration have not been established clearly, inadequate protection from this form of cellular injury is an appealing hypothesis. De La Paz and Anderson25 recently reported an age-related increase in retinal tissue peroxidation in the macular area. Their results suggest that the posterior region of the human retina is susceptible to lipid peroxidation. Peroxides generated during lipid peroxidation normally are metabolized by glutathione peroxidase, an enzyme that is dependent on the availability of GSH. In many cell types exposed to oxidative injury, GSH can become limiting either because of insufficient GSSG reduction or GSSG export from the cells.24 Although GSH is normally present in the retina,25 the intracellular supply may be inadequate for the oxidative load under some conditions. Considerable variation in dietary sulfur-containing amino acids occurs in humans, and up to 16-fold variations in tissue GSH concentrations have been reported for other human tissues.26

In the current studies, we have imposed an oxidant load on cultured human RPE cells and shown that the amino acid precursors of GSH protect against oxidant-induced injury. Also, the additional GSH protected against t-BHP-induced toxicity. Protection occurred at near-physiological concentrations (0.1 mM amino acids, 0.01 mM GSH), but the data suggest that two different mechanisms may be involved. Protection by the amino acids is prevented by inclusion of BSO, an inhibitor of GSH synthesis. In contrast, protection by GSH was not inhibited by BSO.

Thus, amino acid–dependent protection appears to involve intracellular GSH synthesis, but GSH-dependent protection does not. This conclusion also is supported by the observation that only 0.01 mM GSH is required for protection, whereas 0.1 mM amino acids are required. These results indicate that GSH
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degradation and resynthesis cannot account for the protection by added GSH. The nonenzymatic reaction in the incubation medium of GSH with t-BHP is very slow (data not shown); thus, extracellular metabolism of t-BHP does not appear to contribute significantly to the protection. Direct measurement of the GSH peroxidase-catalyzed reduction of t-BHP in the cells and extracellular medium after 30 minutes incubation showed that 98.6 ± 0.2% (N = 3) of the activity was associated with cells, further supporting this interpretation.27 Another possibility is that the added GSH provided protection at the external surface of the cells, by being taken up intact or by another uncharacterized mechanism. For instance, cell membranes contain enzymes, transporters, and receptors with critical thiol groups that could be inactivated by oxidation. Thus, extracellular antioxidants may be essential for preserving membrane function and cell viability. In other tissues, exogenous GSH has been found to be protective by uptake-dependent and uptake-independent mechanisms.28-31

Several investigators have shown that antioxidants can protect the retina from oxidative damage resulting from hyperoxia, light, or chemical oxidants. Winkler and Giblin27 isolated rat retina and demonstrated an important role of GSH in protecting the retina from oxidative damage. When the GSH level was lowered with diamide (an oxidant of glutathione), the receptor potential of the retina was decreased significantly.

Li, Tso, Wang, and Organisciak33,34 showed that rats are less susceptible to retinal light damage when their diets are supplemented with ascorbate. Studies of monkeys fed a diet deficient in vitamin E have shown macular degeneration with focal outer-segment products of lipid peroxidation such as 4-hydroxy nonenal. It is speculated that this effect was caused by lipid peroxidation proceeding without protection by the antioxidant, vitamin E.36,37 Stone, Henderson, Howard et al38 reported that dietary supplementation with vitamin E protected rats from hyperbaric oxygen-induced retinal damage.

The current studies establish the importance of intracellular GSH in protection against oxidant-induced injury in the RPE. Such protection may occur through a variety of mechanisms, including well-characterized GSH peroxidase-dependent reduction of hydroperoxides, direct reaction with free radicals, and, possibly, direct reaction with toxic aldehyde products of lipid peroxidation such as 4-hydroxy nonenal. The results also show that exogenous GSH can offer protection by a mechanism independent of intracellular GSH synthesis. This protection could occur through an extracellular reaction with toxic products released by the cells or by indirectly maintaining cellular antioxidants. This latter possibility could occur if ascorbate-dependent reduction of oxygen-centered radicals results in the cellular release of semidehydroascorbate or dehydroascorbate. GSH reduces these oxidized forms back to ascorbate,39 which could be taken up by the cells to maintain antioxidant functions. This may be important for maintenance of vitamin E because ascorbate appears to have a major role in reducing the α-chromanoxyl radical back to the functional antioxidant α-tocopherol.12 Thus, GSH also offers protection through interactions with other antioxidants.

Key Words
retinal pigment epithelium, glutathione, antioxidant system, age-related macular degeneration

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