Synthesis and Transport of Glutathione by Cultured Human Retinal Pigment Epithelial Cells

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Purpose. To characterize synthesis and transport of glutathione (GSH) by cultured human retinal pigment epithelial (RPE) cells.

Methods. Cultured human RPE cells were depleted of glutathione, then incubated with various concentrations of cysteine, glutamate, and glycine or with glutathione. High-performance liquid chromatography was used to measure intracellular glutathione with time.

Results. After depletion with diethylmaleate, intracellular glutathione was resynthesized from the amino acid precursors within 60 minutes. The addition of buthionine sulfoximine, a known inhibitor of GSH synthesis, completely eliminated the observed increase. Similar incubation with exogenous GSH failed to increase the intracellular GSH concentration.

Conclusion. Cultured human RPE cells are able to rapidly synthesize glutathione from exogenously administered amino acids, but they are incapable of direct GSH uptake.


Tissues of the eye are constantly exposed to a variety of forms of oxidative insult. This form of injury has been suggested to play a role in the pathogenesis of several ocular conditions, including cataract, age-related macular degeneration, and retinopathy of prematurity.1–3 The retinal pigment epithelium may be particularly sensitive to oxidative damage due to high oxygen partial pressure, intense light exposure, and high concentration of polyunsaturated fatty acids in the adjacent photoreceptor outer segments.4

Glutathione (GSH) is a naturally occurring tripeptide that functions as an antioxidant by supporting glutathione peroxidase-dependent reduction of hydrogen peroxide and organic peroxides.5 GSH is normally present in high concentration in the retina and retinal pigment epithelium (RPE)6 and may be depleted during periods of oxidative stress.7

Recently, we examined the effect of exogenous GSH or its precursor amino acids on oxidative injury in cultured human RPE.8 Our results showed that either 0.1 mM amino acids or 0.01 mM GSH protected against oxidative damage induced by t-butylhydroperoxide. In the present study, we have examined the ability of the RPE both to synthesize and to transport glutathione. The results demonstrate that retinal pigment epithelial cells effectively synthesize glutathione but do not take it up from the extracellular medium.

MATERIALS AND METHODS

RPE Cells

RPE cells were harvested from human autopsy eyes obtained through the Georgia Lions Eye Bank. Cultures were established from human eyes from nine donors with a mean donor age of 40.5 years. Appropriate approval for their use in this project was obtained from the Eye Bank and the Emory University Human Investigations Committee. A circumferential incision was made through the sclera approximately 1 mm posterior to the ora serrata. The vitreous was aspirated, and the retina was gently separated from the RPE. After washing the eyecup with calcium- and magnesium-free balanced salt solution, the eye was filled with 0.25% trypsin (Gibco, Grand Island, NY) in balanced salts solution.9 After 1 hour, the trypsin solution was replaced with Dulbecco’s minimal essential medium (DMEM, Gibco), and the RPE cells were aspirated with
The cells were washed with HBSS three times, and the medium was changed every 4 to 5 days.

Cells were passed into 75 cm² flasks every 7 to 14 days after reaching confluence, and third- to sixth-passage cells were used for experiments. Primary cultures and subcultures were routinely tested for mycoplasm contamination (Genetic Probe Mycoplasma T.C. II, San Diego, CA). For each experiment, the cells were equally distributed among the wells of a six-well plate and allowed to grow to confluency under the conditions described above so that identical wells could be used for experimental variations, controls, and replicate analyses. Confluent wells contained an average of 1 million cells.

GSH Synthesis
Each well was washed three times with 1 ml of Hank’s balanced salt solution (HBSS), then 2 ml of 0.25 mM diethylmaleate (DEM) in Krebs-Henseleit solution was instilled to deplete cellular GSH.10 This procedure results in rapid depletion of cytoplasmic GSH due to formation of a covalent bond of GSH with DEM. The depletion of GSH relieves feedback inhibition of GSH on γ-glutamylcysteine synthetase, the rate-limiting enzyme for GSH synthesis. The cells were incubated for 20 minutes at 37°C in 95% air/5% CO₂. The DEM was removed, and the cells were again washed with HBSS three times. Then 2 ml of Krebs-Henseleit solution containing 0.5 mM glutamate, 0.5 mM glycine, and 0.1 mM cysteine was instilled. The cells were incubated at 37°C in 95% air/5% CO₂ for up to 60 minutes. Wells were sampled as follows: before DEM, after incubation with DEM and washing with HBSS three times, 2 ml of 0.25 mM DEM was added to each remaining well. In two experiments, buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase,11 was used to inhibit irreversibly GSH synthesis. After incubation with DEM and washing with HBSS three times, 2 ml of 0.2 mM buthionine sulfoximine in Krebs-Henseleit solution was added, and the cells were incubated for 20 minutes at 37°C in 95% air/5% CO₂. The cells were washed with HBSS three times, and the amino acids were added as in the other experiments. There were no other changes in the protocol.

GSH Transport
Identical experiments were conducted using 0.1 mM exogenous GSH instead of amino acids. The cells were preincubated with 0.25 mM DEM and 0.2 mM BSO (to inhibit any synthesis) for 20 minutes, washed three times with HBSS, then incubated with 0.1 mM GSH for up to 60 minutes. Wells were sampled as in the synthesis experiments.

To assess any possible inhibitory effect of BSO upon transport, cells were preincubated with DEM (as described above) and acivicin 0.25 mM (to inhibit GSH breakdown,12 thereby preventing transport of amino acids and resynthesis of GSH), then washed and incubated with 0.1 mM GSH as above. This concentration of acivicin produced more than 99% inhibition of γ-glutamyltransferase, the enzyme that degrades GSH. This pretreatment had no effect on either protection against t-butylhydroperoxide injury by extracellular GSH or on the intracellular GSH in the presence of extracellular GSH as described in Figure 7. Cells were also incubated for 60 minutes with 0.1 mM GSH without prior depletion of GSH by DEM.

To determine whether cells released GSH, they were incubated without extracellular GSH. The medium was removed at various times and was analyzed for GSH and GSSG. To verify that cells released GSH instead of GSSG, they were incubated with cystine, an amino acid that is only poorly transported into cells (see below) but that reacts with GSH to produce the Cys-SG mixed disulfide.13 The mixed disulfide was analyzed by high-performance liquid chromatography (HPLC) as described below.

GSH and Cysteine Analysis
The amounts of GSH and cysteine per well were determined by HPLC as described by Reed et al.14 After aspiration of the incubation medium and washing with HBSS three times, 400 μl of HBSS and 100 μl of 70% perchloric acid were added. Each well was scraped thoroughly to ensure complete lysis of the cells, and, after centrifugation, the supernatant was derivatized using 40 mM iodoacetic acid and 1-fluoro-2,4-dinitrobenzene.14 The N-2,4-dinitrophenyl derivatives of S-carboxymethyl GSH, glutathione disulfide (GSSG), S-carboxyaminocysteine, and the mixed disulfide of cysteine and GSH were separated on a S-aminopropyl column (Custom LC, Houston, TX) and detected at 365 nm. The amounts of GSH and cysteine in each sample were quantitated relative to standards by integration. Recovery of GSH and cysteine by this procedure is 95% to 98%, as judged by a method of addition of known quantities of GSH and cysteine to cell preparations. In all samples, GSSG was below threshold for detection.

RESULTS
Initial experiments were performed to determine the amount of GSH in cultured RPE cells and to determine whether the addition of amino acid precursors would increase cellular GSH. Culture dishes containing 10⁶ cells were washed three times with amino acid-
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free Hank's balanced salts medium and were analyzed for GSH content. It was impractical to perform cell counts on each dish, but by controlling culture conditions, we were able to obtain uniform cell numbers on replicate plates. The average GSH content for dishes from nine cultures was 11.4 nmol/10⁶ cells with a standard error of 1.6 nmol/10⁶ cells. Thus, the cells contain a substantial GSH content as cultured, and the content is reproducible in cells from different donors and over the range of passages used.

Incubation of cells with 1 mM each of the precursor amino acids for GSH synthesis showed that only a small and not statistically significant increase in GSH was obtained with this high concentration of amino acids (Fig. 1). Thus, culture media contain ample amounts of GSH precursors to maintain maximal or near-maximal GSH concentrations. However, incubation of cells in balanced salts media without precursor amino acids resulted in a substantial decrease in cellular GSH over 2 hours (Fig. 1). This indicates that the cellular GSH content is maintained only if precursor amino acids are available to the cells.

Previous studies have shown that cysteine is often a limiting precursor for GSH synthesis. To determine whether the decrease in GSH in the balanced salts solution could be due to limited cysteine within the cells, we measured cellular cysteine content. The results showed that cellular cysteine was initially 0.22 ± 0.07 nmol/10⁶ cells (n = 6) and decreased to non-detectable concentrations during a 2-hour incubation without cysteine in the medium (Fig. 2). Inclusion of 1 mM cysteine in the medium resulted in a substantial increase in cellular cysteine (up to 4.0 nmol/10⁶ cells) within the first hour (Fig. 2). Thus, the results show that the cysteine concentration in cultured RPE cells is highly variable and is dependent upon available cysteine in the medium.

To determine the dependence of intracellular cysteine on extracellular cysteine concentration, we incubated cells with 0 to 1 mM cysteine for periods up to 2 hours. In all cases, uptake was very rapid and intracellular cysteine reached a steady state by about 15 minutes (data not shown). The steady-state intracellular cysteine concentration increased over this extracellular concentration range without reaching a true maximal value (Fig. 3). The intracellular concentration appears to be most dependent upon extracellular concentration below 0.5 mM.

The effect of cysteine supply on GSH synthesis was studied by measuring recovery of cellular GSH after its depletion with diethylmaleate. In a typical experiment, cells were treated with 0.25 mM diethylmaleate for 20 minutes to decrease GSH and then were incubated with precursor amino acids for periods up
Figure 3. Effect of varying extracellular cysteine concentration on intracellular cysteine concentrations in cultured human RPE cells. Experiments were performed as described in Figure 1, except that cysteine concentration was varied. Glutamate and glycine concentrations were 0.5 mM. Data are reported for 60-minute incubations, which were comparable to 15-minute, 30-minute, and 120-minute incubations run at the same time. Data are mean ± SEM for two to three separate experiments for each concentration.

Figure 4. Representative HPLC tracings of human RPE cell extracts showing GSH and cysteine concentrations after pretreatment with diethylmaleate to deplete GSH and incubation with amino acids to allow GSH synthesis. (A) Top panel: Cells were washed three times with amino acid-free Hanks balanced salts medium, extracted, and analyzed by HPLC as described in Methods. The GSH derivative is visible as the large peak at 44.5 minutes. No cysteine derivative was detectable in these cells; normally, it would be detected at about 35 minutes. Lower panel: Cells were extracted and analyzed 20 minutes after addition of 0.25 mM diethylmaleate. (B) Series of chromatograms showing recovery of GSH during incubation with 1 mM cysteine. From bottom, zero time after depletion of GSH and before the addition of cysteine, 15 minutes, 30 minutes, and 60 minutes after the addition of 1 mM cysteine. Note the increase in cellular cysteine (35-minute peak), as well as recovery of GSH (44.5-minute peak).

to 2 hours (Fig. 4). The DEM treatment decreased cellular GSH by 84% (Fig. 5) and had no apparent effect on cysteine concentration or uptake (date not shown). By 60 minutes with 1 mM amino acids, cellular GSH had recovered (Fig. 5). This recovery of GSH concentrations was completely prevented by inclusion of 0.2 mM buthionine sulfoximine, an inhibitor of the first enzyme in the GSH synthetic pathway, γ-glutamyl cysteine synthetase.

Rates of GSH accumulation in the cells with concentrations of cysteine (0.1 to 0.5 mM) that result in submaximal cysteine concentrations were comparable to the 1 mM incubation (Fig. 6), indicating that the supply of cysteine for GSH synthesis is adequate for maximal synthesis even at below-maximal cellular cysteine concentrations. A half-maximal synthetic rate was obtained with 0.01 mM extracellular cysteine, a concentration that maintained intracellular cysteine in the range of the untreated cell cultures (0.1 to 0.3 nmol/10⁶ cells).

To determine whether RPE cells could take up and use cystine for the synthesis of GSH, we performed the same DEM pretreatment described above and incubated with 1 mM cysteine. The results showed that cellular cysteine was not increased and that a negli-
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FIGURE 5. GSH accumulation in cells after depletion with diethylmaleate and incubation with amino acid precursors, in absence (●) and presence (○) of 0.2 mM buthionine sulfoximine. Experiments were performed and analyzed as shown in Figure 4. Results are mean ± SEM for eight separate experiments.

FIGURE 6. Effect of extracellular cysteine concentration on GSH accumulation in cultured human RPE cells. Experiments were performed and analyzed as described in Figure 4, except that extracellular cysteine concentration was varied. Rates were calculated as initial rates from plots as shown in Figure 5. Glutamate and glycine concentrations were 0.5 mM.

A considerable increase in cellular GSH occurred during a 60-minute incubation (data not shown). Thus, cysteine is not effectively taken up and used for GSH synthesis. Our previous studies showed that supply of the amino acid precursors for GSH synthesis improved RPE cell resistance to oxidative injury induced by t-butylhydroperoxide. In these studies, exogenous GSH also protected against oxidative injury, suggesting that GSH may be taken up by RPE cells. To determine whether net uptake of GSH occurs, cells were treated with DEM to lower the cellular content of GSH and incubated with 0.1 mM GSH. The results show that no increase in cellular GSH occurred even after 1 hour of incubation (Fig. 7). Because cultured RPE cells may have transport systems on the basal surface that are not accessible to GSH in the medium, we also examined transport in resuspended cells. The results also showed no detectable uptake. Thus, human RPE cells apparently do not have the capacity to take up exogenous GSH.

To determine whether RPE cells release GSH, cells were incubated in medium containing 1 mM amino acid precursors for GSH, and the medium was removed after 15 to 60 minutes. HPLC analysis did not show GSH but revealed a peak that co-eluted with the mixed disulfide of cysteine and GSH. Under these conditions, approximately 60% of the cysteine added was recovered as cystine. Because GSH reacts nonenzymatically with cysteine to produce the mixed disulfide, the results indicated that GSH efflux does occur. This was confirmed by performing experiments without amino acid precursors in the medium, which allowed recovery of GSH in the incubation medium, and by the addition of cystine to the incubation medium, which allowed recovery of the mixed disulfide. Estimates of rates of efflux from the amount of mixed
disulfide recovered indicate that efflux occurred at about 0.18 nmol/10⁶ cells per minute, a rate comparable to that of GSH accumulation in the cells (0.17 nmol/10⁶ cells per minute; see Fig. 5). This indicates that the rate of GSH accumulation in the cells is only about 50% of the actual rate of GSH synthesis.

**DISCUSSION**

The human retinal pigment epithelium is constantly exposed to high levels of light and oxygen, creating an environment likely to support free radical and peroxide formation. De la Paz and Anderson¹⁶ reported an age-related increase in retinal tissue peroxidation in the macular area, suggesting that the posterior region of the retina is susceptible to lipid peroxidation. Peroxides generated during lipid peroxidation are normally metabolized by glutathione peroxidase, an enzyme that is dependent upon glutathione availability. Thus, if retinal pigment epithelial health depends upon its ability to detoxify free radicals and peroxides, this health, in turn, may be related to its ability to maintain cellular GSH.

Cellular GSH is supplied by three mechanisms, synthesis from amino acid precursors, uptake from the extracellular medium, and reduction of GSSG. During oxidative stress, reduction of GSSG back to GSH may become limiting and result in cellular loss of GSSG. Thus, maintenance of cellular GSH is ultimately dependent upon GSH synthesis or uptake. Synthesis is thought to occur in all mammalian cell types. In contrast, uptake of GSH occurs in only a small number of cell types.¹⁷

In previous studies, we imposed an oxidant load upon cultured human RPE cells and demonstrated that near-physiological concentrations of the amino acid precursors of glutathione and exogenous glutathione itself protect against oxidant-induced injury.⁸ Protection by the constituent amino acids was prevented by inclusion of BSO, an inhibitor of GSH synthesis. Thus, the protection by amino acids was not due to a nonspecific thiol effect of cysteine. Furthermore, under these conditions, cysteine alone did not protect, but required the presence of, the other precursor amino acids. Protection by GSH was not inhibited by BSO, suggesting a different mechanism of protection.

In the present study, we have attempted to define further the mechanism by which GSH may protect the RPE from oxidative injury. Cultured human retinal pigment epithelial cells were able to synthesize glutathione from micromolar concentrations of exogenously administered constituent amino acids at a rate of synthesis sufficient to restore depleted intracellular GSH levels in 1 hour. This ability to replenish depleted intracellular GSH levels was inhibited when GSH synthesis was blocked by buthionine sulfoximine. These experimental results support the importance of GSH synthesis in the ability of RPE cells to protect against oxidative injury.

In addition, GSH synthesis is dependent upon cysteine. Maximal synthesis could be supported by submaximal cysteine concentrations; half-maximal synthetic rate was obtained with cysteine concentration as low as 0.01 mM. There was no relationship between the ability to synthesize GSH and the extracellular concentration of cystine, suggesting that cystine is not taken up by the RPE cells. These results suggest that alterations in blood cysteine may be important in determining whether RPE cells are supplied with adequate amounts of cysteine. In recent studies of GSH and its oxidized forms in human blood plasma, we found that the oxidized:reduced ratio is substantially increased in individuals 60 to 93 years than in younger controls.¹⁸ Thus, a similar increase in cysteine:cysteine ratio could result in an inadequate supply of cysteine to the RPE cells. Direct studies of blood plasma cysteine concentrations in aging humans are, therefore, needed to determine whether this is critical for maintaining cellular GSH.

However, our previous results⁸ also suggested that GSH-dependent protection does not require GSH synthesis because only 0.01 mM GSH was required for protection whereas 0.1 mM amino acids were required. Various possible mechanisms include GSH being taken up intact by the cells, GSH protecting at the external surface of the cells, or by another uncharacterized mechanism. In other tissues, glutathione has been shown to protect against oxidative injury by both uptake-dependent¹⁹ and uptake-independent mechanisms.²⁰

In the current studies, the results show that when RPE cells were depleted of GSH, they were not capable of taking up GSH from the culture medium. We also examined whether imposing an oxidative stress with /-butylhydroperoxide could stimulate GSH uptake. These results were also negative (data not shown). Thus, they eliminate GSH uptake as a mechanism of the protection seen in the previous studies. An alternative mechanism may involve release of GSH into the extracellular media, which protects against oxidative challenge. The ability of RPE cells to release GSH was demonstrated in the current study.

The ability to extrapolate these results to understand better the relationship between GSH and the retinal pigment epithelium is limited by our use of tissue culture methodology and by the fact that such studies do not necessarily reflect what goes on in the human eye. However, by using early passage human RPE cells with the cells studied in monolayer, we closely approximated the in vivo situation. Furthermore, the HPLC analytical procedure allowed us to quantify accurately GSH and GSSG levels. The results clearly show that the RPE cells can synthesize GSH. We can
also say confidently that there is no net glutathione transport under GSH-depleted conditions.

In other cell types exposed to oxidative injury, GSH can become limiting. Although GSH is normally present in the retina, the extracellular or intracellular supply may be inadequate for oxidative loads. If GSH plays an important role in protecting against oxidative injury in the clinical setting, it is critical to determine whether one should supplement precursor amino acids to allow replenishing of intracellular GSH or to determine how best to augment extracellular GSH levels. Considerable fluctuations in dietary sulfur-containing amino acids occur in humans. In addition, Batist and coworkers have reported large variations in human tissue GSH concentrations, but data are not available concerning GSH concentrations in human RPE in vivo.

In summary, this study provides four salient observations with regard to glutathione and retinal pigment epithelium:

1. Human RPE cells are capable of synthesizing GSH from its amino acid precursors at a rate sufficient to replenish intracellular GSH under depleted conditions within 1 hour.
2. Human RPE cells are incapable of taking up and using cystine for GSH synthesis.
3. Human RPE cells do not have the capacity to take up exogenous GSH.
4. GSH efflux occurs from human RPE cells at a rate approximately one-half that of GSH synthesis.

Key Words
retinal pigment epithelium, glutathione, antioxidant systems, age-related macular degeneration, amino acids, sulfur containing

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