Characterisation of Glutathione Export from Human Donor Lenses

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Purpose: To investigate whether human donor lenses are capable of exporting reduced glutathione.

Methods: Human lenses of varying ages were cultured in artificial aqueous humor for 1 hour under hypoxic conditions to mimic the physiologic environment and reduced glutathione (GSH) and oxidized glutathione (GSSG) levels measured in the media and in the lens.

Results: Human donor lenses released both GSH and GSSG into the media. Donor lenses cultured in the presence of acivicin, a γ-glutamyltranspeptidase inhibitor, exhibited a significant increase in GSSG levels ($P < 0.05$), indicating that GSSG undergoes degradation into its constituent amino acids. Screening of GSH/GSSG efflux transporters revealed Mrp1, Mrp4, and Mrp5 to be present at the transcript level, but only Mrp5 was expressed at the protein level. Blocking Mrp5 function with the Mrp inhibitor MK571 led to a significant decrease in GSSG efflux ($P < 0.05$), indicating that Mrp5 is likely to be involved in mediating GSSG efflux. Measurements of efflux from the anterior and posterior surface of the lens revealed that GSH and GSSG efflux occurs at both surfaces but predominantly at the anterior surface.

Conclusions: Human lenses export GSH and GSSG into the surrounding ocular humors, which can be recycled by the lens to maintain intracellular GSH homeostasis or used by neighboring tissues to maintain GSH levels.

Translational Relevance: Early removal of a clear lens, as occurs to treat myopia and presbyopia, would eliminate this GSH reservoir and reduce the supply of GSH to other tissues, which, over time, may have clinical implications for the progression of other ocular diseases associated with oxidative stress.

Introduction

Glutathione is the principal antioxidant in the lens and is a tripeptide synthesized from the amino acids cysteine, glutamate, and glycine. Reduced glutathione (GSH) exists at unusually high concentrations in the lens ($\sim 10$ mM), enabling the lens to withstand oxidative insults by scavenging reactive oxygen species, maintaining protein thiols in a reduced state, acting as a cofactor for antioxidant enzymes, and detoxifying exogenous compounds via GSH conjugation. These high levels of GSH are maintained by direct GSH uptake from the ocular humors, synthesis of GSH from its precursor amino acids, regeneration of GSH from oxidized glutathione (GSSG), and the export of GSH, followed by its degradation into constituent amino acids by $\gamma$-glutamyltranspeptidase (GGT), to ensure GSH turnover.

In cells such as hepatocytes, lung epithelial cells, and astrocytes, GSH efflux is a major component of GSH homeostasis. Liver hepatocytes export GSH into the plasma to set circulating GSH levels, lung epithelial cells release GSH to protect the epithelial surface from extracellular reactive oxygen species, and astrocytes release GSH to provide neurons with a source of cysteine for GSH synthesis. In ocular tissues, cultured human retinal pigment epithelial cells and bovine
lenses\textsuperscript{18} have been shown to release GSH (\textasciitilde 10\% efflux for retinal pigment epithelial (RPE) cells and \textasciitilde 20\% efflux for bovine lenses) as part of the mechanism to recycle the GSH tripeptide by breaking it down into its constituent amino acids. Recently, we showed that rat lenses release reduced GSH (\textasciitilde 5\% of GSH efflux)\textsuperscript{19} and that this export was a controlled process mediated by members of the multidrug resistance-associated protein (Mrp) family. Furthermore, we demonstrated that exported GSH can be degraded into its constituent amino acids by $\gamma$-GGT, with the resultant amino acids being taken up by either the lens or possibly neighboring tissues for the resynthesis of GSH. Culturing lenses in the presence of the oxidative stressor, H$_2$O$_2$, revealed that rat lenses respond by releasing GSSG, which is consistent with other studies showing that lenses exposed to hyperbaric oxygen or osmotic stress preferentially release GSSG as a means of removing unwanted oxidized molecules.\textsuperscript{20–23}

These animal studies suggest that GSH metabolism in the lens is more dynamic than previously appreciated and that the lens may play a role in supplying GSH and its constituent amino acids to other tissues of the eye that interface with the ocular humors in order to maintain overall intracellular redox homeostasis of the eye.\textsuperscript{24} Since differences in antioxidant levels between nocturnal and diurnal animals exist,\textsuperscript{25,26} in this study, we have extended our work to investigate whether human lenses also export glutathione (GSH or GSSG), the potential mechanism(s) that may mediate GSH export, and whether exported GSH or GSSG can be broken down into its constituent amino acids. Our results show that the human lens does export both GSH and GSSG from its anterior and posterior surfaces, supporting the hypothesis that the lens can influence the composition of the aqueous and vitreous humors, as well as potentially the redox status of other tissues in the front and/or back of the eye. The clinical implications of premature removal of the lens on the function and ocular health of neighboring tissues are discussed.

**Methods**

**Reagents**

Phosphate-buffered saline (PBS) was prepared from PBS tablets (Sigma-Aldrich, St. Louis, MO, USA). MK571 was purchased from Cayman Chemicals (Ann Arbor, MI, USA), and acivicin was from Enzo Life Sciences (Farmingdale, NY, USA). The multidrug resistance-associated protein Mrp1 antibody (QCRL-1), Mrp2 antibody (F-6), and Mrp5 antibody (E-10) were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Unless otherwise stated, all other chemicals were obtained from Sigma-Aldrich.

**Human Donor Lenses**

Human lenses were obtained from donor eyes courtesy of the New Zealand National Eye Bank. The human donor lenses used in this study ranged from 25 to 80 years of age and were received on average 24 to 48 hours postmortem. All donor lenses were examined under dark- or bright-field microscopy with an underlying grid to assess for any signs of cataract or damage during the retrieval process. Any lenses with opacities or distortions of the grid lines were excluded from further analysis. Human lens work was conducted in compliance with the Declaration of Helsinki and was approved by the Southern Health and Disability Ethics Committee (reference: 18/STH/26).

**Lens Culture System for Measuring Glutathione Efflux**

Whole human eyes were dissected using sharpened microdissection scissors, and extreme care was taken to avoid contact with the lens surface. Lenses were carefully transferred using glass loops\textsuperscript{27} into 12-well culture plates and incubated in 2.1 mL artificial aqueous humor (AAH; 125 mM NaCl, 0.5 mM MgCl$_2$, 4.5 mM KCl, 10 mM NaHCO$_3$, 2 mM CaCl$_2$, 5 mM glucose, 20 mM sucrose, 10 mM HEPES, pH 7.2–7.4, 300 ± 5 mOsm), supplemented with 1% penicillin/streptomycin/neomycin for 1 hour at 37°C under hypoxic conditions (90\% N$_2$, 5\% CO$_2$, 5\% O$_2$). After culture, lenses were carefully removed and each lens weighed and placed in an Eppendorf tube containing 50 mM EDTA (pH 8.0). Lenses were homogenized and then centrifuged at 13,000 rpm for 20 minutes at 4°C. Samples of AAH and lens homogenate supernatant were collected for glutathione and lactate dehydrogenase (LDH) analysis and then snap-frozen in liquid nitrogen and stored at $-80°C$ for no more than 1 month. For inhibitor-based experiments, lenses were cultured for 1 hour at 37°C under hypoxic conditions in either the absence or presence of 100 μM MK571 using dimethyl sulfoxide (DMSO) as a vehicle control or 1 mM acivicin using DMSO as a vehicle control. After this time period, lenses and AAH were collected as described above and stored at $-80°C$. In order to determine whether GSH/GSSG efflux occurred at the anterior or posterior surface of the lens, specialized wells were designed that contained an insert that divided the well into anterior and posterior compartments (Supplementary Fig. S1). The posterior
compartment was filled with 0.8 mL AAH, which was a sufficient volume of AAH to bathe the posterior surface of the lens. The donor lens was then placed gently into the insert with the posterior surface of the lens facing the posterior compartment. A layer of 6% agarose gel was then placed around the circumference of the lens in order to ensure a tight seal and no leakage between compartments. The anterior compartment was then filled with 1.2 mL AAH, and lenses were incubated for 1 hour at 37°C under hypoxic conditions. Following incubation, lenses and AAH from the anterior and posterior compartment were collected, snap-frozen, and stored at −80°C as previously described.

**Glutathione Assay**

Lens and media GSH/GSSG levels were measured using a biochemical assay adapted from the Glutathione (GSH/GSSG/total glutathione) Fluorometric Assay Kit (BioVision, San Francisco, CA, USA). All samples were thawed slowly on ice to minimize autoxidation of reduced thiols in the samples. Samples were diluted as necessary, combined with 19.5% w/v trichloroacetic acid, and centrifuged for 4 minutes at 13,000 rpm at 4°C to pellet unwanted proteins. Then, 10 μL of sample was added in duplicates to a black 96-well clear bottom fluorescence plate containing master mix (0.4 M NaHCO₃, 6 M NaOH, and 67 mM PBS). Samples were assayed for GSH and total glutathione (GSH + GSSG), and the difference between GSH and total glutathione was used to calculate GSSG levels. Samples for measuring total glutathione were incubated with 10 μL 0.02M tris(2-carboxyethyl)phosphine (TCEP), a reducing agent, for 10 minutes at room temperature. All samples were then derivatized with 10 μL of 1% w/v o-phthalaldehyde (OPA) in methanol for 40 minutes in the dark at room temperature. Fluorescence was measured using a 2300 EnSpire Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA) with excitation at 350 nm and emission at 420 nm. Standard curves for GSH were prepared using the same master mix (0.4 M NaHCO₃, 6 M NaOH, and 67 mM PBS). Samples for measuring total glutathione were incubated with 10 μL 0.02M tris(2-carboxyethyl)phosphine (TCEP), a reducing agent, for 10 minutes at room temperature. All samples were then derivatized with 10 μL of 1% w/v o-phthalaldehyde (OPA) in methanol for 40 minutes in the dark at room temperature. Fluorescence was measured using a 2300 EnSpire Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA) with excitation at 350 nm and emission at 420 nm. In parallel, standard curves for GSH were prepared using the same master mix to calculate sample glutathione concentrations and standard curves for GSSG prepared to assess the reducing capacity of TCEP. Results are expressed as %GSH efflux, which is calculated as the GSH amount in the AAH media (efflux) as a percentage of the total glutathione (GSH + GSSG) amount in the lens and media, and as %GSSG efflux (in GSH equivalents), which is calculated as the difference between total glutathione and GSH amounts in the media (i.e., GSSG efflux), as a percentage of total glutathione (GSH + GSSG) amount in the lens and AAH media.

**LDH Assay**

In order to ensure that glutathione efflux was not a result of leakage from damaged membranes, an LDH assay (Roche Diagnostics Corp, Indianapolis, IN, USA), which is a widely accepted assay for the quantitative determination of cell membrane integrity and cell viability, was employed. LDH standard dilutions were prepared by using type III bovine heart LDH (14 mg protein/mL; Sigma-Aldrich) in either 50 mM EDTA (pH 8.0) or AAH to quantify the lens and media LDH activity, respectively. Lens supernatants were diluted 1:100 in EDTA in order to ensure that absorbance measurements lay within the range of the standard curve. Media were used undiluted. Then, 100 μL LDH standards and samples was loaded into a clear 96-well plate and measured in triplicates. Reaction solution, containing catalyst solution (diaphorase/NAD⁺ mixture) and dye solution (iodotetrazolium chloride and sodium lactate) in a 1:45 ratio, was prepared immediately before use, and 100 μL of the mixture was added to each well. The plate was incubated in the dark at room temperature for 15 minutes and absorbance was measured using a 2300 EnSpire Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA) at a wavelength of 490 nm. Data are presented as percentage LDH release and calculated based on the LDH activity detected in the media as a proportion of the total LDH activity in the lens and media. Prior to LDH analysis, a predetermined LDH “cutoff” value was determined to distinguish between controlled transport and membrane leakage as a result of loss of lens integrity. This involved measuring the release of GSH (expressed as %GSH efflux) plotted against the corresponding %LDH release values (Supplementary Fig. S2A). It can be seen that for GSH efflux, the data cluster tightly below 0.2% LDH release. If we compare our threshold to other studies, albeit animal lenses, which range from 1.75% to 5%, our value is incredibly more stringent than others. Therefore, lenses that exhibited LDH release greater than 0.2% were excluded from the study.

**Reverse Transcription–Polymerase Chain Reaction**

Whole-lens RNA (epithelium and fiber cells) was extracted from a human donor lens and isolated using TRIzol reagent according to the manufacturer’s protocol (Thermo Fisher Scientific, Waltham, MA, USA). RNA concentration and purity were assessed using the NanoDrop 2000 (Thermo Fisher Scientific). Total lens complementary DNA (cDNA) was
synthesized from 1 μg total RNA mixed with 50 μM oligo(dT)20 in 8-μL final reaction volumes. The RNA was denatured at 65°C for 5 minutes, immediately placed on ice to cool, and then combined with 2× First-Strand Reaction Mix and SuperScript III/RNaseOUT Enzyme Mix (Thermo Fisher Scientific) for cDNA amplification. A control reaction (no cDNA synthesis) was also conducted in the absence of SuperScript III/RNaseOUT enzyme. Synthesized cDNA or control reaction (0.5–1 μL) was added to separate polymerase chain reaction (PCR) mixtures containing final concentrations of AmpliTaq Gold Buffer, 2 mM MgCl2, 0.2 mM dNTPs, 1.25 U/μL AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific), and 0.25 μM sense and antisense primers (Table 2). All primers were synthesized and purified by Integrated DNA Technologies (Singapore). The DNA polymerase was heat activated at 94°C for 2 minutes prior to PCR cycling (AmpliGold Bio-Rad T100 Thermo Cycle). PCR amplification was performed as follows: denaturation at 94°C for 30 seconds, annealing at 64°C (Mrp1) or 60°C (Mrp2, Mrp4, Mrp5, and OATP1a2) for 30 seconds, extension at 72°C for 45 seconds for 36 cycles, and a final extension at 72°C for 5 minutes. Amplified PCR products were analyzed by electrophoresis on a 1.2% w/v agarose gel containing SYBR Safe (Thermo Fisher Scientific) and visualized using an UV illuminator. PCR bands were subsequently extracted and purified (PureLink Quick Gel Extraction and PCR Purification Combo Kit; Thermo Fisher Scientific) and DNA sequenced. The primer sets and the expected sizes of PCR products are listed in Table 2.

**Membrane Protein Preparation**

Epithelial and fiber membrane fractions were prepared by dissection of a pair of donor lenses. Epithelial membrane fractions were prepared by gently peeling away the lens capsule and the adherent epithelial monolayer using two sharpened forceps, and the capsule was placed in a tube containing 0.5 mL homogenizing solution (5 mM Tris-HCl [pH 8.0], 5 mM EDTA, and 5 mM EGTA). The remainder of the lens, which was composed of the lens fiber cells, was placed into an Eppendorf tube containing 0.5 mL homogenizing solution. All samples were then centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatant was discarded and the pellet washed three times in storage solution (5 mM Tris [pH 8.0], 2 mM EDTA, and 100 mM NaCl). The fiber membrane fractions were further suspended in 7 M urea and centrifuged, and the pellet was washed twice in 20 mM NaOH. The resultant fiber membrane fractions and epithelial membrane fractions were resuspended in storage solution and stored at −80°C until further use. Rat kidney and brain (control tissues) membrane protein fractions were also prepared by careful excision and homogenization of tissues as described above. Concentrations of protein in the lens membrane samples were determined using the Direct Detect spectrometer (Merck Millipore, Burlington, MA, USA).

**Western Blotting**

Rat control tissue (30 μg/lane), human lens epithelial (20 μg/lane), and human lens fiber (30 μg/lane) membrane protein fractions were loaded on a 10% acrylamide separating gel with 4% v/v stacking gel and then transferred onto the Immuno-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) by electrophoresis for 60 minutes at 170 mA. After transfer, membranes were incubated with blocking solution (5% w/v skim milk powder in 1× Tris-buffered saline with Tween 20 [TBS-T]; 0.1% v/v Tween-20, 20 mM Tris, and 137 mM NaCl [pH 7.6]) at room temperature for 1 hour and then incubated with primary antibodies (Mrp1 [1:200], Mrp4 [1:100], and Mrp5 [1:200]) diluted in 1× TBS-T buffer containing 2 mM EDTA (pH 8.0) and 1% w/v bovine serum albumin overnight at 4°C. After rinsing twice with Milli-Q water (Merck Millipore, Burlington, Massachusetts, USA) and washing three times for 10 minutes in 1× TBS-T buffer, the membranes were then incubated with horseradish peroxidase conjugates (goat anti-mouse, 1:10,000; Abcam, Cambridge, UK) at room temperature for 1 hour. Labeled protein was visualized with the Western Lightning Plus ECL (Perkin Elmer) and developed using the Chemidoc MP imaging systems (Bio-Rad Laboratories).

**Data Analysis**

Statistical analyses were performed with GraphPad Prism, version 7.03 (GraphPad Software, Inc., San Diego, CA, USA). Standard curves for GSH/GSSG were prepared by fitting data to a nonquadratic equation and for LDH by fitting data to a linear regression equation using Microsoft Excel (Microsoft, Redmond, WA, USA). To compare differences between GSH and GSSG efflux from vehicle- and inhibitor-treated lenses or from the anterior and posterior surface of the lens, the Wilcoxon matched-pairs signed-rank test was employed. Unless otherwise stated, all data are presented as the mean ± SEM. P values of less than 0.05 were deemed to be statistically significant and statistical significance displayed as *P < 0.05, **P < 0.01, or ***P < 0.001.
### Results

Transparent donor lenses were cultured in AAH for 1 hour under hypoxic conditions and the lenses and media collected and then frozen at −80°C so that LDH release and GSH/GSSG levels could be measured later. The media were collected in multiple tubes containing 50 mM EDTA to ensure that the samples we measured had undergone only one freeze/thaw cycle to minimize auto-oxidation. We first measured LDH activity to exclude membrane damage as a possible source of glutathione release. Supplementary Figures 2A–D show that any donor lenses that exhibited <0.2% LDH were retained for further analysis of GSH/GSSG levels while lenses that exhibited >0.2% LDH were excluded from further study. Table 1 summarizes the number of donor lenses obtained from the NZ National Eye Bank and the number of lenses that were included for each experiment due to <0.2% LDH activity.

<table>
<thead>
<tr>
<th>GSH/GSSG Release following 1-h Culture</th>
<th>No. of Lenses Measured</th>
<th>No. of Lenses &lt;0.2% LDH</th>
<th>No. of Lenses &gt;0.2% LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Release from lenses of varying ages</td>
<td>Control: 20</td>
<td>Treatment: 9</td>
<td>Control: 19</td>
</tr>
<tr>
<td>Release from lenses cultured in the absence or presence of acivicin</td>
<td>Control: 10</td>
<td>Treatment: 9</td>
<td>Control: 8</td>
</tr>
<tr>
<td>Release from lenses cultured in the absence or presence of MK571</td>
<td>Control: 10</td>
<td>Treatment: 10</td>
<td>Control: 8</td>
</tr>
<tr>
<td>Release from the anterior or posterior surface of the lens</td>
<td>Control: 14</td>
<td>Treatment: 11</td>
<td>Control: 3</td>
</tr>
</tbody>
</table>

Human lenses were cultured in AAH under hypoxic conditions for 1 hour, and LDH levels were measured in the lenses and media and expressed as %LDH release. Lenses that exhibited <0.2% LDH were retained for further analysis, while lenses that exhibited >0.2% LDH were excluded from this study. Treatment refers to incubation of lenses in the presence of either acivicin or MK571.

### Cultured Human Donor Lenses Maintain Higher Levels of Reduced GSH Relative to Oxidised GSH

While it is known that in the lens, GSH levels exist at higher concentrations relative to GSSG, the effects on intracellular GSH/GSSG levels on cultured postmortem lenses were unknown. Lenses that previously were cultured in AAH for 1 hour under hypoxic conditions were thawed on ice and lenses measured for GSH and GSSG levels (n = 20 lenses). All lenses were found to contain a higher GSH to GSSG ratio (Fig. 1). On average, approximately 72% of total glutathione was in the reduced form (0.87 ± 0.07 μmol/g GSH and 0.18 ± 0.01 μmol/g GSSG), confirming that reduced GSH levels in the lens are able to be maintained postmortem and following tissue culture. However, the concentration of reduced GSH was approximately threefold lower than that reported by other groups from donor lenses of comparable age groups.

A previous study has shown that human lenses analyzed 20 hours postmortem exhibited ~48% total glutathione compared with human lenses obtained immediately postmortem, indicating that the time delay from death to lens processing can significantly impact intracellular GSH levels. It is likely that the combination of the older ages of donor lenses coupled with postmortem delays may have had an impact on intracellular GSH levels.

### Both Reduced and Oxidized Forms of Glutathione Are Released From Human Lenses under Isosmotic Conditions

To determine whether GSH and/or GSSG is exported from the lenses assayed above, AAH media collected from lenses cultured for 1 hour under hypoxic conditions were thawed on ice and GSH and GSSG concentrations measured in the media. Figure 2A reveals the amount of GSH and GSSG released from cultured donor lenses after 1 hour. The findings show considerable variability between lenses in terms of GSH versus GSSG release with 9 lenses displaying a greater amount of GSH release compared with GSSG,
Figure 1. Measurement of intracellular GSH/GSSG levels. Human lenses were cultured in AAH under hypoxic conditions for 1 hour, and intracellular GSH and GSSG in donor lenses were measured \((n = 20\) lenses). Note: The paired lenses are from the same donor at each age.

While 10 lenses exhibited a greater amount of GSSG release compared with GSH (Fig. 2A).

The amount of GSH released relative to the total amount of GSH in the lens and media was calculated and expressed as a percentage (%GSH efflux). Lenses released a variable range of GSH from as low as 2% to as high as 12% (Fig. 2B). Pooling the data together revealed the mean value of %GSH release to be 5.06% ± 0.59% \((n = 19\) lenses). This was slightly higher than the %GSH efflux found in rat lenses, which was reported to be approximately 2%, and lower than %GSH efflux in bovine lenses, which was approximately 10%.

The amount of GSSG released relative to total glutathione in the lens and media was calculated and expressed as a percentage (%GSSG efflux). Lenses displayed significant variability of %GSSG efflux, from as low as 4% to as high as 39% (Fig. 2C). The mean value of GSSG efflux was calculated to be 20.27% ± 2.52% \((n = 19\) lenses), which was approximately four times that of %GSH released. Taken together, these results indicate that human donor lenses release a greater proportion of intracellular GSSG than GSH into the media, and this most likely reflects the need for these donor lenses to maintain a reduced intracellular state by removal of GSSG.

Glutathione Released by Human Lenses Undergoes Degradation by GGT

In order to determine whether GSH released by the lens undergoes degradation by γ-GGT, lenses were cultured in the absence or presence of 1 mM acivicin, an inhibitor of γ-GGT \((n = 6\) lenses per group). Measurements of GSH/GSSG released by donor lenses treated with acivicin were highly variable, with three lenses displaying slightly increased GSH release in the presence of acivicin and the other three lenses showing decreased GSH release in response to acivicin treatment (Fig. 3A). Calculation of the mean amount of reduced GSH released from vehicle-treated control and acivicin-treated donor lenses showed no significant changes between the two groups \((n = 6\) lenses, Fig. 3B), suggesting that γ-GGT did not appear to break down reduced GSH released by these donor lenses. In contrast, lenses treated with acivicin all displayed an increase in GSSG release \((n = 6\) lenses, Fig. 3A). The mean GSSG amount released from acivicin-treated lenses revealed a significant increase relative to vehicle-treated lenses (1.3-fold increase, \(P < 0.05\); Fig. 3B). This suggests in human lenses, GSSG, but not GSH, released from the lens undergoes degradation by γ-GGT into its constituent amino acids.

GSSG Release from Human Donor Lenses Is Partially MRP Mediated

Having confirmed that human lenses release both GSH and GSSG, the identity of efflux transporters involved was next investigated. In tissues such as the liver, brain, and kidney, GSH conjugate removal is predominantly mediated by the efflux transporters Mrp1/2/4/5 and the uptake/bidirectional transporter Oatp1a1/1a2. To determine which, if any, of these transporters were present in human donor lenses at the transcript or protein level, reverse transcription–PCR (RT-PCR) was performed using Mrp and Oatp isoform-specific primers (Table 2) and Western blotting carried out on lens epithelial and lens fiber membrane protein preparations using Mrp-specific antibodies (Fig. 4). PCR revealed that of the five candidates known to mediate GSH/GSSG efflux, Mrp1, Mrp4, and Mrp5 were present, but Mrp2 and Oatp1a2 were absent at the transcript level (Fig. 4A). However,
Figure 2. Measurement of extracellular GSH and GSSG. Human lenses were cultured in AAH under hypoxic conditions for 1 hour, following which GSH and GSSG levels in the media were quantified. (A) Measurement of GSH and GSSG in the media. (B) Extracellular GSH levels were measured and divided by the total amount of GSH in the lens and media and expressed as %GSH efflux. (C) Extracellular GSSG levels were measured and divided by the total amount of GSSG in the lenses and media and expressed as %GSSG efflux (n = 19 lenses). Note: The paired lenses are from the same donor at each age. However, only one of the lenses from a 37-year-old donor could be analyzed for GSH/GSSG levels as the other lens exhibited LDH release greater than 0.2%.

Western blotting revealed that while all transporters were present in the positive control (kidney or brain), only Mrp5 was expressed at the protein level in the lens, with Mrp5 expression being only detected in the epithelial membrane protein fraction (Fig. 4B).

To determine whether Mrp5 was functional and responsible for mediating GSH/GSSG efflux, lenses were cultured in the absence or presence of 100 μM MK571, a generic MRP inhibitor (n = 8 lenses per group). GSH release was variable, with five lenses releasing more GSH in response to MK571 treatment and three lenses exhibiting a decrease in GSH release following MK571 treatment (Fig. 5A). Calculation of the mean amount of GSH released into the media revealed that although lenses showed an approximately 1.30-fold higher release of GSH in response to MK571 treatment relative to control, this was not statistically significant (n = 8 lenses, Fig. 5B), indicating that GSH
Figure 3. Measurement of GSH and GSSG release in the media in the absence or presence of the γ-GGT inhibitor acivicin. Human lenses were cultured in the absence or presence of 1 mM acivicin or AAH vehicle control (0.5% v/v DMSO) for 1 hour under hypoxic conditions and the media measured for GSH and GSSG release. (A) Measurement of GSH and GSSG in the media in the absence (ctrl) or presence (aci) of acivicin. (B) Mean values of GSH and GSSG release in the absence (ctrl) or presence of acivicin (aci). Data are shown as mean ± SEM (*P < 0.05, Wilcoxon matched-pairs signed-rank test). n = 6 lenses for each condition.

efflux in the human lens is not mediated by MRP transporters. In terms of GSSG release, there appeared to be a more consistent pattern, in which all lenses exhibited a decrease in GSSG release as a result of MK571 treatment (Fig. 5A). Calculation of the mean amount of GSSG released into the media revealed a significant 1.7-fold decrease in GSSG release in the presence of MK571 compared with control lenses (n = 8 lenses, P < 0.01; Fig. 5B). These results show that Mrp5 is likely to be partially responsible for mediating GSSG release but not GSH release from human donor lenses.

GSH/GSSG Efflux Occurs at Both the Anterior and Posterior Surface of the Lens

To investigate whether GSH or GSSG release occurs from the anterior or posterior surface of the lens, lenses were cultured within a specialized compartmentalized well from which media samples could be collected from either the top compartment, representing the release from the anterior surface, or the bottom compartment, representing release from the posterior surface. Following culture in AAH under hypoxic conditions for 1 hour, total glutathione (GSH + GSSG) released into the anterior and posterior compartments was first measured (n = 11 lenses). Although the amount of total glutathione into each compartment is highly variable between lenses, it can be seen that in all cases, more total glutathione was released into the anterior chamber compared with the posterior chamber (Fig. 6A). If we then analyze the relative amounts of reduced GSH (no GSSG) released from the anterior versus posterior surface, it can be seen that reduced GSH was detected in both compartments (Fig. 6B), indicating that lenses release GSH from both the anterior and posterior surfaces. In addition, lenses also appeared to exhibit greater reduced GSH release from the anterior
Table 2. PCR Primer Sets and Predicted Product Sizes for Mrp1, Mrp2, Mrp4, Mrp5, and Oatp1a2

<table>
<thead>
<tr>
<th>Protein</th>
<th>GenBank Reference</th>
<th>Sequence from 5' to 3'</th>
<th>Predicted Size, bp</th>
</tr>
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<tr>
<td>MRP1</td>
<td>NM_004996.4</td>
<td>Forward: ATCACCTTCTCCATCCCCCGA</td>
<td>408</td>
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<tr>
<td></td>
<td></td>
<td>Reverse: ATCCACTGTCAGAGGGGAT</td>
<td></td>
</tr>
<tr>
<td>MRP2</td>
<td>NM_000392.5</td>
<td>Forward: TCGAACACTTAGCCGAGTT</td>
<td>538</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GGAATAATGCGCGGCAGAAC</td>
<td></td>
</tr>
<tr>
<td>MRP4</td>
<td>NM_005845.4</td>
<td>Forward: TCGCAAATCCCCCTTGTTCCC</td>
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<tr>
<td></td>
<td></td>
<td>Reverse: GGTGGTGGGGGGTTTCTGATA</td>
<td></td>
</tr>
<tr>
<td>MRP5</td>
<td>NM_005688.4</td>
<td>Forward: GACGGAGATTGGAGAGCGAG</td>
<td>492</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CCCTGCCCTCTTCTTCCAG</td>
<td></td>
</tr>
<tr>
<td>OATP1a2</td>
<td>NM_134431.3</td>
<td>Forward: CTGTCAAACAAGCTGCCAC</td>
<td>445</td>
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<tr>
<td></td>
<td></td>
<td>Reverse: CCAGGTATGCGGCAGGAAAGA</td>
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</tr>
</tbody>
</table>

All PCR primer sets were designed using the online software OligoPerfect (Thermo Fisher Scientific). Primer sets were subsequently analyzed for suitability using the primer-BLAST tool in NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to ensure the oligonucleotides were from protein-coding regions that were conserved and adjacent to introns.

surface compared with the posterior surface. Similarly, measurements of GSSG release revealed that lenses released GSSG from both the anterior and posterior surfaces but predominantly from the anterior surface (Fig. 6C). Calculation of the mean amount of GSH and GSSG released across all donor lenses (Fig. 6D) revealed that GSH and GSSG was predominantly released from the anterior surface relative to the posterior surface (n = 11 lenses, P < 0.005 [GSH], P < 0.001 [GSSG]). Collectively, this suggests that under physiologic conditions, human lenses are potentially capable of releasing GSH/GSSG into the aqueous humor and also into the vitreous, albeit at lower levels compared with the aqueous humor.

Discussion

We have previously hypothesized that due to the high concentrations of GSH in the lens, it may act as a reservoir for GSH that can be used to provide GSH or GSH metabolites to other tissues of the eye to protect against oxidative stress. In support of this hypothesis, we have previously shown that young rat lenses use Mrp transporters to export reduced GSH and that this exported GSH can be degraded by the enzyme γ-GGT to supply the lens with precursor amino acids for uptake and resynthesis of GSH by the lens and/or neighboring tissues. In this study, we have used human donor lenses to translate our work in rat lenses to determine whether a similar export of GSH occurs in ex vivo human lenses. While using human donor lenses is valuable for translating our studies from animals to humans, it should be noted that there are caveats to working with human donor lenses. Unlike animal lenses, in which everything can be controlled in most parts, there are many factors that are out of the researchers’ control when working with human donor lenses. This includes the cause of death, the delay between time of death and lens processing, and the age of the donor. These factors are likely to contribute to the variability seen in our results between donor lenses and represent the inherent biologic variability between individuals and thus donor lenses. Nevertheless, the ability to use human donor lenses in this study has been invaluable in better understanding the molecular mechanisms used by the lens to protect itself from oxidative stress and contribute to redox balance in other tissues of the eye.

Culturing donor lenses under hypoxic conditions to mimic the natural environment of the lens resulted in the release of both GSH and GSSG into the media. Some lenses, however, showed more GSH release, while some donor lenses exhibited more GSSG release (Fig. 2). The release of GSH is consistent with other studies that demonstrated GSH release in rat and bovine lenses. On the other hand, GSSG release has previously been reported in rat and rabbit lenses that were exposed to oxidative stressors. This suggests that the human donor lenses used in this study were under oxidative stress, which most likely contributed to the lower levels of intracellular GSH measured in this study (Fig. 1B), relative to previous studies. Given that on average, a greater proportion of lens GSSG was exported into the media relative to GSH, the release of GSSG by the human lens is likely to reflect a physiologic adaptation to remove unwanted oxidized molecules to maintain a reduced intracellular environment in the lens.

It is well established that GSH efflux is a major component of GSH homeostasis. This is achieved through export of GSH and then degradation of GSH...
Figure 4. Molecular identification of GSH/GSSG efflux transporters in the human lens. (A) Transcripts for Mrp1, Mrp4, and Mrp5 were identified in the human lens (RT+), whereas transcripts for Mrp2 and Oatp1a2 were absent in the lens. No PCR products were seen in control reactions using lens messenger RNA, in which the reverse transcriptase enzyme was omitted (RT−). (B) Total rat kidney (K) or brain (B) membrane proteins (control tissues) alongside lens epithelium (Epi) and lens total fiber (TF) membrane protein fractions were probed with antibodies against Mrp1, Mrp4, and Mrp5. Western blot analysis revealed the presence of Mrp1 as a ∼190-kDa band in the kidney (left), which was absent in the lens epithelium and fibers; Mrp4 as a ∼150-kDa band in the kidney, which was absent in the lens epithelium and fibers (middle); and Mrp5 as a ∼185-kDa band in the brain, which coincided with a band in the lens epithelium (right). Lower bands at ∼55 kDa were seen in the lens TF for Mrp1 and lens epithelium for Mrp4 and Mrp5, which most likely represents nonspecific bands.

into its constituent amino acids by γ-GGT for reuptake and then resynthesis of GSH. Since GSH and GSSG are both substrates for γ-GGT, we incubated lenses in the absence or presence of acivicin, a γ-GGT inhibitor (Fig. 3B). If γ-GGT was involved in the degradation of GSH, we would expect a higher level of GSH to be released in the presence of acivicin, whereas if γ-GGT was involved in the degradation of GSSG, we would expect a higher level of GSSG to be released in the presence of acivicin. Interestingly, we discovered that in human donor lenses, GSSG but not GSH increased in the presence of acivicin (Fig. 3B), suggesting that only GSSG is metabolized by γ-GGT. This is interesting as this finding was in contrast to rat lenses, which demonstrated GSH but not GSSG degradation by γ-GGT. This may be due to substrate availability but also raises the possibility that different species may express different GGT enzymes that have differential affinities for GSH and GSSG. For example, GGT1 and GGT5 have been identified in human tissues and shown to be capable of degrading both GSH and GSSG. However, human GGT1 is known to have a slightly higher affinity for GSSG, while human GGT5 has been found to have a significantly higher affinity for GSH. In addition to GGT1 and GGT5, other human GGT isoforms with high sequence homology to GGT1 and GGT5 have also been identified, such as GGT6 and GGT7. Although their exact expression...
Figure 5. Measurement of GSH and GSSG release in the media in the absence or presence of the MRP inhibitor MK571. Human donor lenses were cultured in AAH for 1 hour in the absence (ctrl) or presence of 100 μM MK571 under hypoxic conditions. (A) Measurement of GSH and GSSG in the media in the absence or presence of MK571. (B) Mean values of GSH and GSSG release in the absence or presence of MK571. Data are shown as mean ± SEM (***P < 0.01, Wilcoxon matched-pairs signed-rank test). n = 8 lenses per condition.

and affinity for GSH versus GSSG are yet to be determined, it is possible that these additional isoforms may be involved in GSSG metabolism in the human lens.

While GSH and GSSG were both shown to be exported from the lens, it was important to determine whether efflux occurs from the anterior and/or posterior surface of the lens. These results show for the first time that GSH and GSSG release occurs from both surfaces of the lens, although this release was greater from the anterior surface. Previous studies have measured total glutathione (GSH + GSSG) concentration in the human aqueous humor to be approximately 2 μmol/L, with GSSG accounting for only 0.3% of the total concentration. Given that the volume of the human aqueous humor is approximately 150 mL, this concentration is equivalent to approximately 0.30 nmol GSH and 0.001 nmol GSSG. Our results showed that approximately 6 nmol GSH and 5 nmol GSSG were released from the anterior surface within 1 hour. These levels of GSH and GSSG are much higher than the reported values for GSH and GSSG in the aqueous and may be a reflection of the fact that this is an artificial culture system and not representative of the in vivo eye, in which GSH or GSSG is able to be taken up by other ocular tissues or drained by the trabecular meshwork. While total glutathione (GSH + GSSG) concentration in the human vitreous humor is unknown, Whitson et al. have measured reduced GSH levels collected from the entire human cadaver vitreous and found it to be approximately 20 μmol/L. Given that the total volume of the human vitreous humor is approximately 4 mL, this corresponds to 80 nmol reduced GSH. Extrapolating our results to this study suggests that the lens would contribute about 9% of the total of reduced GSH measured in the vitreous. This source of GSH from the lens may represent a localized concentration of GSH close to the lens important for protection of the lens from oxidative damage as opposed to GSH.
levels closer to the retina, which are likely to be sourced from the blood supply.

Our previous study on rat lenses demonstrated that GSH efflux, but not GSSG efflux, was primarily mediated by Mrp1 and Mrp5. However, it is known that some Mrps are capable of mediating both GSH and GSSG efflux, and so it was assumed that Mrps would be likely candidates to mediate GSH and GSSG efflux from the human lens. An RT-PCR screen for MRP isoforms in human lenses as well as Western blotting confirmed the presence of MRP5 in the human lens epithelium. Functional experiments using the MRP generic inhibitor MK571 (Fig. 5) indicated that GSSG release across the anterior surface of the lens appears to be partially mediated by Mrp5, which was shown to be localized to the anterior epithelium (Fig. 4B). Since GSSG efflux was not completely inhibited, and GSSH efflux was also shown to occur at the posterior surface (Fig. 6D), this suggests that other efflux transporters may be involved in mediating GSSG (and also GSH) efflux from the lens. Possible candidates to investigate in the future would include hemichannels such as connexin and pannexin hemichannels, which open in response to stressors such as mechanical strains and oxidative insults.59,60 Panx1 and Panx2 are known to be expressed in the lens epithelium and fibers of the mouse lens, and Panx1 in the epithelium of the porcine lens, but the expression of pannexins in human lenses is undetermined. On the other hand, Cx43, Cx46, and Cx50 are known to be expressed in the human lens and have been shown to be permeable to both GSH and GSSG, although Cx46 and Cx50 displayed much lower permeation of GSSG relative to GSH. It would be interesting to test in the future whether Cx43, Cx46, and Cx50 and possible pannexins can function as GSH or GSSG efflux transporters.

It is interesting to speculate as to the possible roles of GSH and GSSG released from the lens. Figure 7 represents an emerging model of the possible fates of GSH and GSSG once released from the lens based on this study and other studies involving human and rodent ocular tissues, as well as the potential implications of this in terms of intertissue GSH metabolism between the lens, the ocular humors,
Figure 7. Emerging model of intertissue GSH metabolism between the lens and the ocular humors. The human lens exports GSH (A) and GSSG (B) under isotonic conditions. (A) The release of GSH into the aqueous humor may be used for uptake by the anterior tissues of the eye, such as the corneal endothelium or trabecular meshwork (TM). GSH released into the vitreous humor can be used to reduce DHA back to ascorbic acid (AsA) to maintain high AsA concentrations localized close to the posterior lens. GSH in the ocular humors may also be oxidized into GSSG in response to high oxidative stress (dotted arrow). (B) GSSG efflux into the aqueous humor may be mediated by MRP5 transporters expressed in the lens epithelium. GSSG in the aqueous humor may be transported to the anterior tissues, such as the cornea or ciliary body, removed from the eye by TM, or degraded into its precursor amino acids via GGT. In the vitreous humor, lenticular GSSG may be degraded by GGT activity and amino acids reused by the lens to maintain GSH homeostasis.

the different tissues of the eye. In the anterior eye, circulating GSH in the aqueous humor could be accumulated by the corneal endothelium, in which putative GSH transporters have previously been identified in rat lenses, or filtered out via the trabecular meshwork to protect against oxidative stress, in which reduced GSH levels in calf trabecular meshwork are reported to be \( \sim 0.4 \mu \text{mol/g wet weight} \) (Fig. 7). On the other hand, GSH released by the lens into the vitreous humor may be used to help drive the local regeneration of ascorbic acid. Human vitreous contains \( \sim 1.5 \text{ mM ascorbic acid} \), which is used to consume oxygen to ensure oxygen levels are low in the eye but, in doing so, becomes oxidized to dehydroascorbate. Since GSH is an essential cofactor in the regeneration of DHA back to ascorbic acid, the localized export of GSH from the lens could be used to regenerate ascorbic acid to ensure the local consumption of oxygen close to the lens to minimize oxidative damage and prevent cataract formation.

There are several possible roles for GSSG following its export from the lens (Fig. 7). First, GSSG appears to be broken down by GGT into its precursor amino acids (Fig. 3B), which could be used for either potential reuptake of amino acids into the lens to resynthesize GSH or for uptake by other tissues such as the cornea, ciliary body, and trabecular meshwork via amino acid uptake transporters that have previously been identified in rat ocular tissues. Second, intact GSSG in the aqueous humor may be drained from the eye via the trabecular meshwork and subsequently removed through the episcleral venous system. Third, GSSG may be accumulated by the corneal endothelium, which is known to contain a highly active GSH redox cycle, in which GSSG is preferentially taken up and rapidly converted to GSH by GSH reductase. In fact, the barrier function of rabbit corneal endothelium is actually better maintained by having GSSG in the medium compared with GSH. This demonstrates the resourceful nature of the lens in removing oxidized compounds and recycling them into useful molecules that can then be used by the lens or neighboring tissues.

**Translational Relevance**

Taken together, this study shows that human donor lenses are able to export GSH and GSSG into its surrounding ocular environment. While further work
is required to gain a deeper understanding of the interaction between the lens and the ocular humors, our present work indicates that the lens is not just a passive optical element but a highly dynamic and active tissue that interacts with its neighboring tissues through modifying the environments in which these tissues function. If we accept that the lens actively contributes to the ocular environment, then it is interesting to speculate on what effect the removal of the lens would have on the function of other neighboring ocular tissues. A long-term effect of lens removal may be to inadvertently reduce the availability of GSH and GSH metabolites in the aqueous humor. Since lens removal is now being performed on younger patients due to our increasing diabetic population, and clear lens extraction is being advocated to correct for presbyopia or myopia in middle-aged patients, over time, these changes may increase the susceptibility of nearby tissues to oxidative stress and subsequent ocular pathologies. If this is correct, the actual loss of the biologic lens may have longer-term consequences for overall ocular health than currently appreciated. This study thus lays the foundation for further investigations to determine whether an association between lens removal, long-term oxidative stress, and an increased risk of ocular pathologies is truly valid.

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References

Glutathione Export from the Lens


