

Evidence of Dual Mechanisms of Glutathione Uptake in the Rodent Lens: A Novel Role for Vitreous Humor in Lens Glutathione Homeostasis

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PURPOSE. Lens glutathione synthesis knockout (LEGSKO) mouse lenses lack de novo glutathione (GSH) synthesis but still maintain >1 mM GSH. We sought to determine the source of this residual GSH and the mechanism by which it accumulates in the lens.

METHODS. Levels of GSH, glutathione disulfide (GSSG), and GSH-related compounds were measured in vitro and in vivo using isotope standards and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

RESULTS. Wild-type (WT) lenses could accumulate GSH from γ -glutamylcysteine and glycine or from intact GSH, but LEGSKO lenses could only accumulate GSH from intact GSH, indicating that LEGSKO lens GSH content is not due to synthesis by a salvage pathway. Uptake of GSH in cultured lenses occurred at the same rate for LEGSKO and WT lenses, could not be inhibited, and occurred primarily through cortical fiber cells. In contrast, uptake of GSH from aqueous humor could be competitively inhibited and showed an enhanced K_m in LEGSKO lenses. Mouse vitreous had >1 mM GSH, whereas aqueous had <20 μ M GSH. Testing physiologically relevant GSH concentrations for uptake in vivo, we found that both LEGSKO and WT lenses could obtain GSH from the vitreous but not from the aqueous. Vitreous rapidly accumulated GSH from the circulation, and depletion of circulating GSH reduced vitreous but not aqueous GSH.

CONCLUSIONS. The above data provide, for the first time, evidence for the existence of dual mechanisms of GSH uptake into the lens, one mechanism being a passive, high-flux transport through the vitreous exposed side of the lens versus an active, carrier-mediated uptake mechanism at the anterior of the lens.

Keywords: aqueous, cataract, glutathione, homeostasis, lens, oxidative stress, transport, vitreous

In order to protect from oxidation, the lens, like most other cellular systems, maintains very high concentrations (~3–5 mM) of the antioxidant glutathione (GSH).¹ Glutathione has a number of unique functions, the best studied of which is its role as a cofactor for glutathione peroxidase (GPX) enzymes.² Glutathione peroxidase detoxifies H₂O₂ in a reaction that generates a disulfide linkage between two GSH molecules, resulting in production of glutathione disulfide (GSSG). Glutathione disulfide can be restored to its reduced form by an NADPH-dependent reaction catalyzed by glutathione reductase (GR). Glutathione can also protect proteins by acting directly as a free-radical scavenger and metal chelator, conjugating to toxic xenobiotic compounds by glutathione S-transferase (GST) enzymes, and regenerating glutaredoxin (GLX) enzymes, which reduce compounds such as dehydroascorbic acid (DHA).³

The lens GSH pool is thought to be produced by the lens epithelium and immature differentiating fiber cells in a two-step process.⁴ The first and rate-limiting step is the generation of γ -glutamylcysteine (γ -GC) from cytosolic amino acids through an

ATP-dependent reaction catalyzed by γ -glutamylcysteine ligase (GCL). Synthesis is then rapidly completed by the addition of glycine to the peptide through another ATP-dependent reaction catalyzed by glutathione synthase (GS). Glutathione synthesized in metabolically active regions of the lens is then distributed to the remainder of the lens by diffusion through the gap junctions that extensively couple lens fiber cells.⁵ Deficiency in GSH has been linked to age-related nuclear cataract as lens GSH concentration, particularly in the lens nucleus, decreases with age.⁶ This is hypothesized to result from a reduced activity in GSH synthesis enzymes,⁷ as well as a barrier to GSH diffusion into the nucleus that develops with age.⁸ It has been demonstrated that ~1 mM GSH is a critical concentration for the lens nucleus, below which crystallins rapidly form disulfide bonds and aggregate.¹

The lens glutathione synthesis knockout (LEGSKO) mouse was generated to provide a model for the study of glutathione deficiency in the lens and its effect on cataract progression.⁹ Using the lens-specific Cre recombinase MLR10, these mice lack expression of *Gclc*, the catalytic subunit of GCL, within

their lenses. As expected, these mice develop a phenotype that mimics age-related nuclear cataract. Because homozygous knockout lenses were not found to have any residual *Gclc* mRNA, protein, or enzymatic activity, it was expected that these lenses would completely lack GSH. Surprisingly, although LEGSKO lens GSH content is significantly decreased compared to that in wild-type (WT) lenses, it is still maintained at approximately 1 mM. This observation implies that lenses are able to obtain high concentrations of GSH from an exogenous source, that is, the aqueous or vitreous humor.

The concept that the lens may obtain GSH through transport in addition to synthesis is not a novel one and has been reported in a number of studies.^{10–12} Such experiments have led to speculation about the nature of a possible GSH transporter. Although two importers of GSH were previously reported,¹³ they were later determined to be artifacts from the *Escherichia coli* genome and have since been discredited.¹⁴ At the same time, others have suggested that specific GSH transporters do not exist at all and instead that γ -GC can be generated at the external surface of cells by the breakdown of GSH through the enzyme γ -glutamyltransferase (GGT).^{15,16} In this model, it is this precursor rather than intact GSH that is taken up by cells. If this pathway is active in the lens, it could account for the GSH content of LEGSKO lenses, because they still have active GS and could generate GSH from cytosolic γ -GC if they can obtain it from their environment.

We set out to clarify the source and mechanism of GSH uptake in the lens by using an unbiased approach. To this end, we developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays that could accurately and reliably measure GSH, GSSG, and isotopically-labeled compounds simultaneously. Unlike radiologic methods, this mass spectrometric analysis ensures that only intact compounds are measured, providing robust measurements of endogenous GSH levels in addition to labelled GSH derived from uptake and/or synthesis. Because it lacks de novo GSH synthesis in its lens, the LEGSKO mouse provides a unique model for investigating these mechanisms in detail.

MATERIALS AND METHODS

Chemicals

Nonisotopic standards and other reagents of the highest available purity were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Isotopic compounds were obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). γ -Glutamyl-serine-glycine (γ -ESG) and γ -glutamyl-alanine-glycine (γ -EAG) peptides were synthesized at the Dr. Richard Armstrong Laboratory (Vanderbilt University, Nashville, TN, USA) following a published protocol.¹⁷

Animal Work

All animals were used in accordance with the guidelines of the Association for Research in Vision and Ophthalmology for the Use of Animals in Ophthalmology and Vision Research, and experimental protocols for this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University.

LC-MS/MS Analysis

A mass spectrometer (MicroMass Quattro Ultima; Waters Corp., Milford, MA, USA) equipped with an electrospray ionization source coupled to a separation module (Alliance 2695; Waters Corp.) with a reversed-phase C18 column (Discovery HS model; Supelco Analytics, Bellefonte, PA, USA)

TABLE. LC-MS/MS MRM Settings

Compound	Parent Ion, m/z	Product Ion, m/z	Collision Energy, eV
¹³ C ₃ -alanine	93.35	46.54	13
¹⁵ N ₂ -arginine	177.30	62.54	21
Monobromobimane	271.98	193.29	22
GSH	308.65	162.20	20
GSH	308.65	179.34	11
GSH-(<i>glycine</i> - ¹³ C ₂ , ¹⁵ N)	311.03	147.19	22
GSH-(<i>glycine</i> - ¹³ C ₂ , ¹⁵ N)	311.03	165.20	20
GS-B	498.06	435.22	22
GSSG	613.10	355.20	21

GS-B, glutathione-bimane conjugate; GSH, glutathione; GSSG, glutathione disulfide; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring.

was used for LC-MS/MS analysis. Multiple reaction monitoring (MRM) was performed using electrospray ionization in positive ion mode with a cone voltage of 60 V. Precursor and product ions were determined by infusing standards on MS at a concentration of 100 μ M. Precursor and product ions used for MRM are shown in the Table. Also as shown in the Table, two different mass transitions were used for quantitation of both GSH and GSH-(*glycine*-¹³C₂,¹⁵N) in order to improve accuracy and reduce matrix effects.

Formic acid, 0.1%, was used as the ion-pairing agent, and 90% acetonitrile was used as the mobile phase. Compounds were separated using a 12-minute gradient with mobile phase increasing linearly from 2% to 40%. Concentration of compounds in samples was quantitated using external calibration of standards. In measurements of endogenous glutathione content, GSH-(*glycine*-¹³C₂,¹⁵N) was used as an internal standard.

All samples were diluted and homogenized in 0.1% formic acid solution for LC-MS/MS analysis. Samples were spun at 8000 \times g for 10 minutes to precipitate protein and other large debris, and the supernatant was filtered over 0.45- μ m cellulose acetate Spin-X columns (Corning, Inc., Corning, NY, USA).

Cultured Lens Uptake Experiments

Eyes were removed from mice immediately after they were euthanized by CO₂ asphyxiation. Lenses were dissected from eyes by carefully cutting away sclera and removing the lenses with forceps. Lenses were washed three times in sterile phosphate-buffered saline (PBS) to remove vitreous humor and other tissues and then placed in sterile uptake buffer (140 mM NaCl, 25 mM D-(+)-glucose, 10 mM HEPES, 4.2 mM NaHCO₃, 5 mM KCl, 1.3 mM CaCl₂, 0.5 mM MgCl₂, 0.36 mM Na₂HPO₄, 0.44 mM NaH₂PO₄, pH 7.4). Lenses were treated with 1 mM of the GCLC inhibitor buthionine sulfoximine (BSO) and 500 μ M of the γ -GT inhibitor acivicin for 1 hour prior to uptake experiments in order to prevent breakdown and synthesis of GSH during uptake experiments. Uptake buffer pH was adjusted using NaOH to maintain a pH of 7.4 after addition of substrates. Unless otherwise noted, assays were performed at 37°C in 5% CO₂. Uptake assays were stopped by washing lenses three times in ice-cold sterile PBS followed by immediate homogenization of the lenses in ice-cold 0.1% formic acid solution.

Lens Wash Test

Following 1 hour of incubation with 5 mM GSH-(*glycine*-¹³C₂,¹⁵N), lenses were placed in 100 μ L of ice-cold sterile PBS for 60 seconds with gentle agitation. Lenses were then moved into fresh buffer, and this procedure was repeated a total of 6 times. Each wash and the post washed lenses were

analyzed using LC-MS/MS after homogenization and dilution in 0.1% formic acid solution.

Cultured Lens GSH Efflux Assay

Lenses, obtained as stated for cultured lens uptake experiments, were placed in individual wells of a 96-well plate containing 200 μ L of uptake buffer with 500 μ M of acivicin and other inhibitors. Lenses were incubated at 37°C in 5% CO₂ for 1 hour, after which medium was replaced and lenses were incubated for an additional hour. After incubation, a sample of medium was taken for analysis and diluted 1:3 in ice-cold 0.1% formic acid solution.

GSH Uptake Imaging

Glutathione was reacted with monobromobimane to generate a glutathione-bimane conjugate (GS-B) by adding GSH to a final concentration of 10 mM to a solution of 40 mM monobromobimane, 200 mM *N*-ethylmorpholine, 20 mM KOH, pH 8.0, and reacting at room temperature in the dark for 30 minutes. GS-B was isolated from the reaction by phase separation after the addition of methylene chloride. The extraction procedure was repeated four times, after which the aqueous phase maintained a bright yellow coloration, indicating the presence of the bimane conjugate, and the organic phase was completely clear. Purity of the GS-B product was assessed by LC-MS/MS (see Fig. 3A).

Lenses were cultured as described previously and incubated with 1 mM GS-B for 0, 5, 15, or 30 minutes, washed three times in cold PBS, and fixed for 30 minutes in 4% paraformaldehyde. Whole lenses were imaged using confocal microscopy (LSM 510 META model; Carl Zeiss AG, Oberkochen, Germany) with an excitation of 384 nm and emission of 470 nm.

In Vivo Uptake Experiments

Mice were anesthetized using intraperitoneal injection of 3 mg/20 g of body weight ketamine and 0.3 mg/20 g of body weight xylazine. Before injection of substrates into the eye, 1% atropine sulfate drops were applied topically to provide better visualization of the needle and internal eye structures.

Aqueous humor injections were performed by puncturing the cornea with a 27-gauge needle, drawing out endogenous aqueous with an ophthalmic sponge, and injecting approximately 5 μ L of uptake buffer containing GSH-(*glycine*-¹³C₂, ¹⁵N) using a 10- μ L syringe equipped with a 33-gauge needle (Nanofil; World Precision Instruments, Sarasota, FL, USA). A small air bubble was injected with solution in order to prevent solution from leaking out of the puncture site.

Vitreous humor injections were performed by puncturing mouse sclera with a 10- μ L syringe (Nanofil) equipped with a 33-gauge needle at a 45° angle in order to avoid puncturing the lens, and injecting 1 μ L of uptake buffer containing GSH-(*glycine*-¹³C₂, ¹⁵N) directly into the vitreous body.

Vascular Eye Perfusion

Mice were anesthetized by intraperitoneal injection of 3 mg/20 g of body weight ketamine and 0.3 mg/20 g of body weight xylazine, and eye perfusion was carried out based upon established surgical techniques.¹⁸ Briefly, an incision was made in the chest to expose the throat and surrounding tissues. The right common carotid artery was ligated distal to the catheter insertion site. The artery was cut half way across its diameter, and a 32-gauge carotid artery catheter (World Precision Instruments, Sarasota, FL, USA) was inserted. Sutures were tied around the catheterized carotid artery to firmly maintain

the catheter placement. The right internal and external jugular vein were cut fully across to allow for drainage of perfusion fluid. The catheter was connected to a syringe pump (model 11 plus; Harvard Apparatus, Holliston, MA, USA), and perfusion was carried out at a rate of 1 mL/min. Perfusion fluid consisted of a bicarbonate-buffered physiological saline solution (128 mM NaCl, 24 mM NaHCO₃, 4.2 mM KCl, 2.4 mM NaH₂PO₄, 1.5 mM CaCl₂, 0.9 mM MgCl₂, and 9 mM *D*-glucose, pH 7.4) containing 200 μ M GSH-(*glycine*-¹³C₂, ¹⁵N). Mouse body temperature was maintained by using an electric heating pad. Perfusion fluid was heated to and maintained at 37°C for the duration of the perfusion.

Lens Surface Permeability Assay

Lucifer yellow uptake buffer, 100 μ M, was administered to cultured lenses or injected into the anterior chamber of WT mouse eyes. After 1 hour of incubation, lenses were washed three times in PBS and homogenized in PBS. Lens samples were analyzed for fluorescence intensity alongside a lucifer yellow standard curve, with excitation at 485 nm and emission at 528 nm. Permeability coefficients (P_c) were determined by the equation $[P_c = (V/A \times C_i) \times (C_f/T)]$, where *V* is the lens volume (in mL), *A* is the surface area of the cell layer assayed (in cm²), *C_i* is the concentration of lucifer yellow administered (100 μ M), *C_f* is the concentration of lucifer yellow in the lens after incubation, and *T* is the incubation time (in seconds).

Tissue Procurement and Dissection

Frog, fish, and rat eyes were obtained as fresh discarded tissue from various laboratories at Case Western Reserve University. Aqueous humor was collected using a 10- μ L syringe (Nanofil) equipped with a 33-gauge needle by fully puncturing the cornea just above the pupil at a 45° angle and drawing out the fluid. The lens-vitreous-retina mass was removed from the eye by carefully cutting away the cornea and sclera. Vitreous, retina, and lens were separated using prewetted spin columns, following an established method.¹⁹

Fresh bovine and porcine eyes were obtained from local slaughterhouses. Human donor eyes were obtained from the National Disease Research Interchange (Philadelphia, PA, USA). Only healthy eyes without opacities were accepted. Human eyes were from individuals 20 to 60 years of age who had not undergone radiation or chemical therapy.

Aqueous humor was collected from large mammal eyes by using a 1-mL syringe equipped with a 25-gauge needle by injection into the anterior chamber and drawing out the fluid. Vitreous humor and lenses were isolated by cutting along the sclera to access the posterior eye and removing the tissues with forceps.

Statistical Analysis

Bar graphs are expressed as means \pm standard deviations (SD). Line graphs are expressed as means \pm standard error of the means (SEM). Statistical significance of differences in mean values was assessed by Student's *t*-test. Only *P* values <0.05 were considered statistically significant.

RESULTS

Lenticular LEGSKO GSH Is Supplied by Circulating GSH

Using LC-MS/MS methodology, we first confirmed that lens GSH levels were suppressed by >60% (*P* < 0.01) in LEGSKO mouse lenses and that these levels could be further depleted by

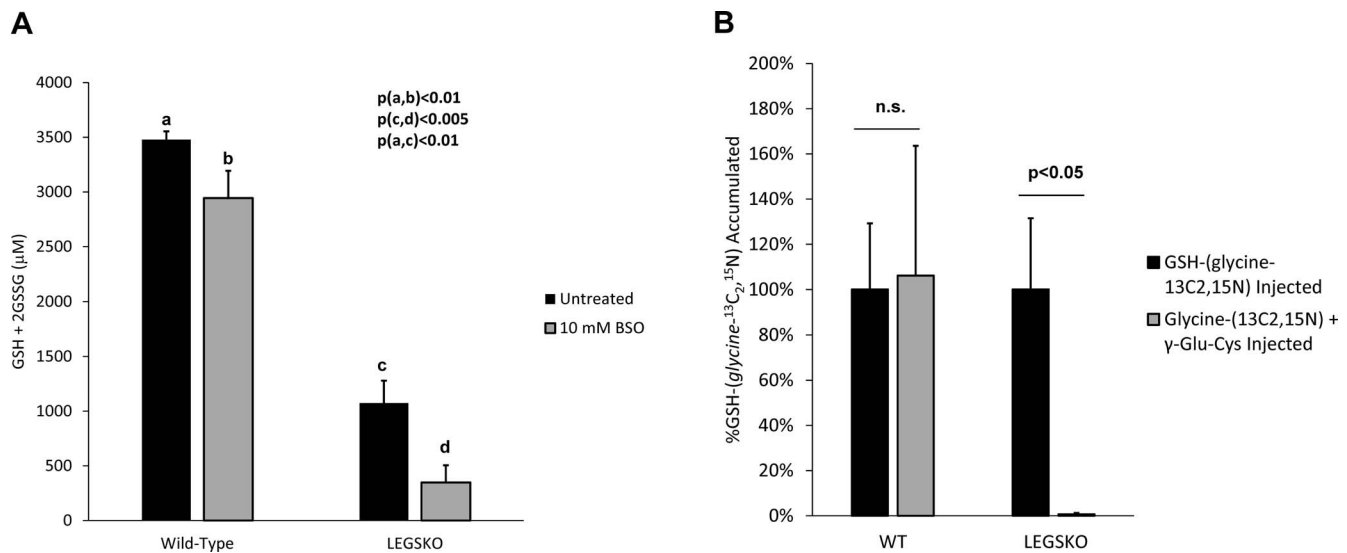


FIGURE 1. Analysis of the source and mechanism of LEGSKO lens GSH. **(A)** Treated mice were supplied exclusively with water containing 10 mM BSO for 2 months before analysis by LC-MS/MS. Wild-type mouse lenses were ~15% depleted of GSH by BSO treatment ($P < 0.01$). LEGSKO mouse lens GSH content was depleted ~70% compared to WT before BSO treatment ($P < 0.01$) and ~90% depleted from WT after BSO treatment ($P < 5 \times 10^{-10}$). **(B)** To test for GSH salvage pathway activity, we injected 250 μM of GSH-(glycine-¹³C₂, ¹⁵N) or glycine-(¹³C₂, ¹⁵N) and γ -GC into the anterior chambers of WT and LEGSKO mouse eyes. After 30 minutes, lenses were removed and analyzed by LC-MS/MS. Wild-type lenses accumulated equal amounts of GSH-(glycine-¹³C₂, ¹⁵N) from both treatments. Conversely, LEGSKO mice only accumulated GSH-(glycine-¹³C₂, ¹⁵N) when it was administered in its intact form ($P < 0.05$). Values are means \pm SD; $n = 4$.

treating mice with BSO, an irreversible inhibitor of GCLC (Fig. 1A). LEGSKO lenses were depleted of GSH by >65% ($P < 0.005$) by using BSO treatment, although WT lenses lost only ~15% of their GSH ($P < 0.01$). It has been reported that BSO has very poor penetration through the blood-brain and blood-eye barriers in adult mice²⁰ and will only largely affect GSH levels in circulation and organs not protected by such barriers. These experiments suggest that the residual GSH levels in the LEGSKO lens are linked to a GSH pool that is rapidly replenished from systemic circulation.

Salvage Pathway Is Not the Source of LEGSKO Mouse Lens GSH

Next, we determined whether a GSH synthesis salvage pathway relying on exogenous γ -GC was functioning in LEGSKO lenses. GSH-(glycine-¹³C₂, ¹⁵N) or equal amounts of γ -GC and glycine-(¹³C₂, ¹⁵N) was injected into the anterior chambers of WT and LEGSKO mouse eyes, and the accumulation of GSH-(glycine-¹³C₂, ¹⁵N) in lenses after injection was determined by LC-MS/MS. Although WT lenses were found to accumulate GSH-(glycine-¹³C₂, ¹⁵N) equally for both injections, LEGSKO lenses only accumulated GSH-(glycine-¹³C₂, ¹⁵N) when it was given in its intact form ($P < 0.05$) (Fig. 1B). This indicates that WT lenses generated GSH-(glycine-¹³C₂, ¹⁵N) by de novo synthesis and confirms the fact that the high residual GSH content of LEGSKO lenses is due to uptake of intact GSH. Because LEGSKO lenses were unable to synthesize GSH from exogenous γ -GC, we conclude that a salvage pathway relying on GGT activity cannot be the source of LEGSKO lens GSH.

Cultured Lens GSH Uptake Occurs by Passive Diffusion

To obtain detailed kinetic measurements of lens GSH uptake, whole lenses were taken from mice and cultured ex vivo. Lenses were pretreated with BSO, an inhibitor of GCLC, and acivicin, an inhibitor of GGT, to prevent any potential

breakdown or synthesis of GSH. These lenses were incubated with isotopically labeled compounds for analysis of uptake by LC-MS/MS. Cultured LEGSKO and WT lenses showed no differences in GSH-(glycine-¹³C₂, ¹⁵N) uptake rates (Fig. 2A). Because this indicates that LEGSKO lenses take up GSH by the same mechanism as WT lenses, WT lenses were used for subsequent experiments.

For comparative purpose, the uptake of ¹³C₃-alanine and ¹⁵N₂-arginine was tested in addition to GSH-(glycine-¹³C₂, ¹⁵N), because both alanine²¹ and arginine²² have been shown to be actively taken up by mammalian lenses. A time course of uptake demonstrated that all three compounds were taken up by the lens with an initial rate period occurring in the first few minutes (Fig. 2B). Subsequent experiments were carried out using initial rate conditions to ensure first-order kinetics. Transport was found to be dose dependent in all cases (Fig. 2C). ¹³C₃-alanine and ¹⁵N₂-arginine uptake rates were saturable and displayed Michaelis-Menten kinetics with estimated K_m values of ~1.9 mM and ~3.3 mM, respectively, and V_{max} of 22 $\mu\text{M}/\text{min}$ and 18 $\mu\text{M}/\text{min}$, respectively. Conversely, GSH-(glycine-¹³C₂, ¹⁵N) uptake was very sluggish and did not appear to be saturable or fit well to the Michaelis-Menten equation, appearing instead to increase essentially linearly with concentration.

To determine whether GSH uptake was mediated by active transport, uptake was tested at both 37°C and 4°C. Whereas ¹³C₃-alanine and ¹⁵N₂-arginine uptake rates were decreased by 75% ($P < 0.05$) and 81% ($P < 0.01$), respectively, lowering temperature had no discernable effect on GSH-(glycine-¹³C₂, ¹⁵N) uptake (Fig. 2D). Furthermore, ¹³C₃-alanine and ¹⁵N₂-arginine uptake rates were significantly higher than the rate of GSH-(glycine-¹³C₂, ¹⁵N) uptake at 37°C ($P < 0.05$) but approximately equal at 4°C (Fig. 2E), suggesting that GSH uptake by the lens is likely a nonspecific and passive process. This was further supported by performing uptake experiments with a 5-fold excess of unlabeled GSH or the GSH analogs γ -EAG or γ -ESG, while maintaining a constant concentration of GSH-(glycine-¹³C₂, ¹⁵N) (Fig. 2F). None of

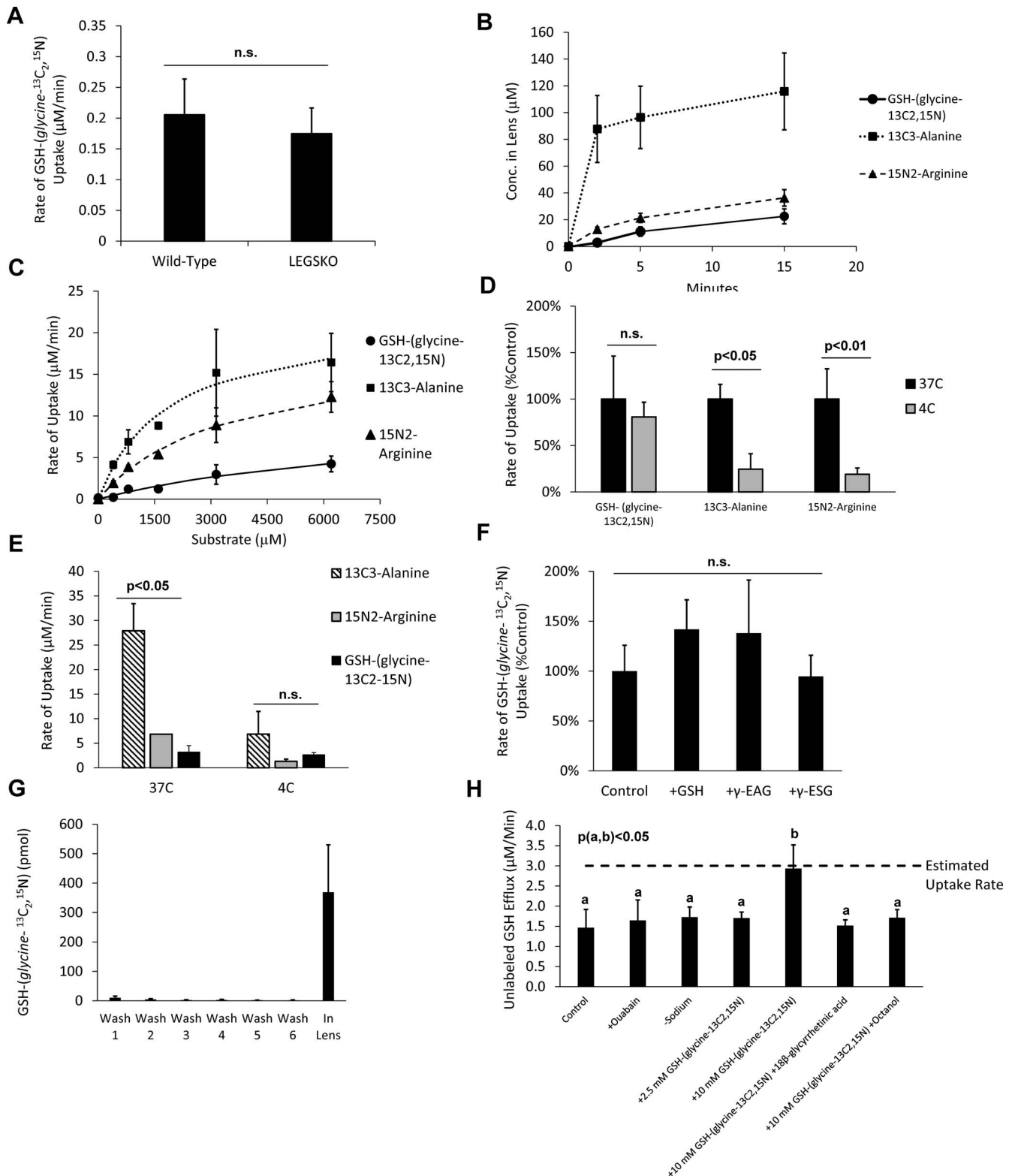


FIGURE 2. Characterization of GSH transport in cultured lenses. All lenses were preincubated with 1 mM BSO and 500 μM acivicin for 1 hour to prevent any breakdown or turnover of GSH-(glycine-¹³C₂,¹⁵N). (A) Wild-type and LEGSKO lenses were incubated with 500 μM GSH-(glycine-¹³C₂,¹⁵N) at 37°C in 5% CO₂ for 1 hour. No significant differences were observed between uptake rates of LEGSKO and those of WT lenses. (B) Wild-type mouse lenses were incubated with 2 mM of each substrate and taken at various time points. GSH-(glycine-¹³C₂,¹⁵N) had the lowest initial uptake rate, followed by ¹⁵N₂-arginine and ¹³C₃-alanine (P < 0.005). (C) Lenses were incubated in various concentrations of substrates and taken for analysis within the initial rate period of uptake. Curves are best fits of the data to the Michaelis-Menten equation. GSH-(glycine-¹³C₂,¹⁵N) showed a significantly lower rate of uptake than ¹³C₃-alanine and ¹⁵N₂-arginine at all concentrations (P < 0.01). (D, E) Lenses were incubated with 2 mM of each substrate at 37°C or 4°C. Lowering temperature had no effect on GSH-(glycine-¹³C₂,¹⁵N) uptake but did significantly inhibit uptake of ¹³C₃-alanine, and ¹⁵N₂-arginine (P < 0.05 and P < 0.001, respectively). ¹³C₃-alanine, and ¹⁵N₂-arginine were taken up at significantly higher rates

than GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) at 37°C ($P < 0.05$), but all compounds were taken up at approximately the same rate at 4°C . (F) Lenses were incubated with 1 mM of GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) at 37°C in 5% CO_2 and 5 mM of GSH, γ -EAG, or γ -ESG. There were no significant differences between the uptake rates of GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) between these groups. (G) To assess how tightly bound to lenses GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) was, lenses were incubated with 5 mM GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) for 30 minutes and then washed 6 times in 100 μL of PBS. Each wash fraction was saved and analyzed for GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) content by LC-MS/MS. (H) The efflux of GSH from lenses was tested by measuring the GSH content of buffer after 1 hour of incubation. This efflux rate was significantly lower than the uptake rate at the GSH concentration found within lenses ($P < 0.05$), was unaffected by ouabain or Na^+ -free medium, and increased to match the rate of uptake when GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) was present in the medium at a higher concentration than within the lens (10 mM) but not when it was present at a lower concentration (2.5 mM). The increased efflux induced by excess GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) was inhibited by the connexin inhibitors 18 β -glycyrrhetic acid and octanol at concentrations of 10 μM and 100 μM , respectively. Values are means \pm SD for bar graphs and \pm SEM for line graphs; $n = 4$.

these compounds had any significant effect on GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) uptake, indicating that lens GSH uptake is not carrier-mediated under conditions of the intact lens immersed in culture medium.

To clarify whether the measured GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) is localized intracellularly or more loosely associated with the

lens, lenses were washed excessively in PBS after uptake assays, and the amount of GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) in each wash was determined by LC-MS/MS (Fig. 2G). The amounts of GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) present in the washes were negligible relative to the amount present in the lens, indicating that the GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) measured in uptake assays was likely

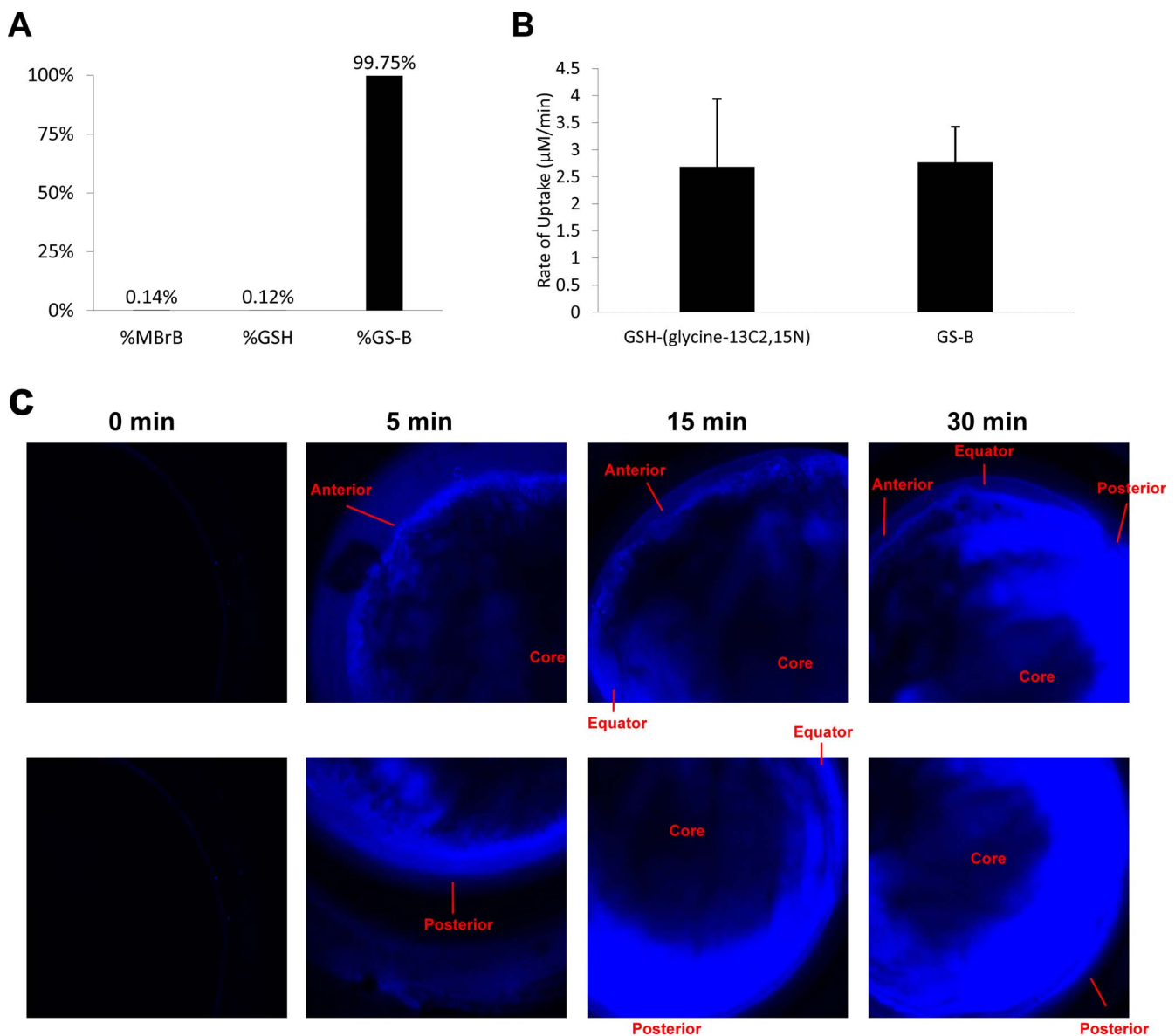


FIGURE 3. Visualization of GS-B uptake in cultured lenses. (A) Purity of synthesized GS-B was tested by LC-MS/MS analysis. The GS-B product was found to be 99.75% pure, with trace amounts of GSH and monobromobimane present. (B) The uptake rates of GS-B and GSH-(*glycine*- $^{13}\text{C}_2$, ^{15}N) were compared by incubating WT lenses with 2 mM of each compound in uptake buffer for 4 minutes and analyzing the intralenticular accumulation of the compound by LC-MS/MS. There were no significant differences in uptake rates. Values are means \pm SD; $n = 4$. (C) Representative images are shown of GS-B uptake by cultured lenses after a 0, 5, 15, or 30 minutes' incubation with 1 mM GS-B.

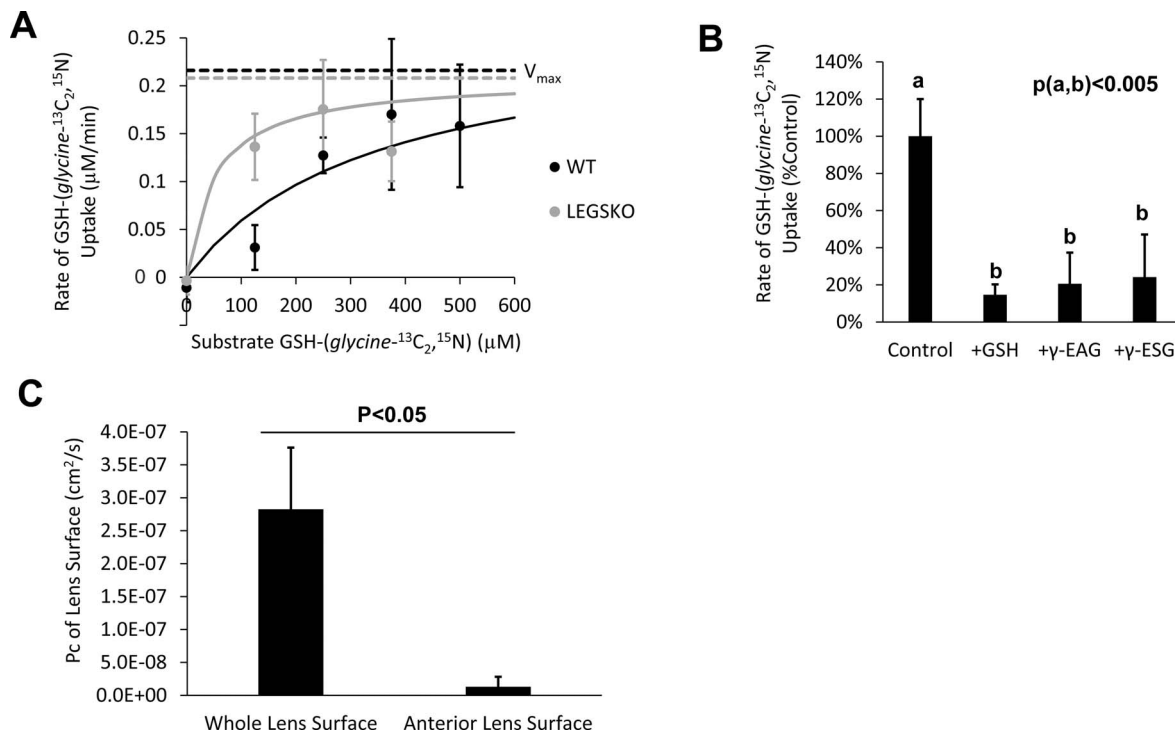


FIGURE 4. Characterization of lens GSH uptake from the anterior chamber. (A) Uptake buffer containing various concentrations of GSH- $(\text{glycine-}^{13}\text{C}_2,^{15}\text{N})$ was heated to 37°C and injected into the anterior chambers of WT and LEGSKO mouse eyes after aqueous humor was removed. Lenses were taken for analysis after 30 minutes of incubation. Curves are best fits to the Michaelis-Menten equation. Both of the groups had approximately the same V_{max} of $0.21 \mu\text{M}/\text{min}$, but LEGSKO mice had a lower K_m value of $\sim 50 \mu\text{M}$ compared to $\sim 250 \mu\text{M}$ for WT lenses. Values are means \pm SEM; $n = 4$. (B) Uptake buffer containing $125 \mu\text{M}$ GSH- $(\text{glycine-}^{13}\text{C}_2,^{15}\text{N})$ and $500 \mu\text{M}$ GSH, γ -EAG, γ -EAG or none of these was injected into the anterior chambers of WT mouse eyes after removal of the aqueous humor. Lenses were taken for analysis after 30 minutes of incubation. All three compounds significantly inhibited lens uptake of GSH- $(\text{glycine-}^{13}\text{C}_2,^{15}\text{N})$ ($\sim 75\%$; $P < 0.005$). There were no significant differences in degree of inhibition among the compounds. Values are means \pm SD; $n = 4$. (C) Permeability of lens surfaces was tested by administering $100 \mu\text{M}$ lucifer yellow in uptake buffer to cultured lenses or injecting it into the anterior chambers of WT mouse eyes. Cultured lenses showed a significant increase in Pc ($P < 0.05$) compared to that in lenses treated with lucifer yellow only at the anterior surface. Values are means \pm SD; $n = 3$.

located intracellularly and not in the extracellular fluid surrounding lens fiber cells.

In order for GSH uptake to be a passive process, endogenous GSH should efflux from the lens at the same rate at comparable concentrations. This was tested by culturing lenses in GSH-free buffer and measuring the amount of GSH released into the medium by using LC-MS/MS (Fig. 2H). Under these conditions, GSH was released from lenses but at a significantly ($P < 0.05$) slower rate than anticipated based on the rate of uptake at GSH concentrations similar to those found in the lens. One possibility is that this was due to the activity of the lens microcirculation, which drives solute flow into the center of the lens.²³ However, ouabain (an inhibitor of $\text{Na}^+\text{-K}^+$ -ATPase pumps) and Na^+ -free buffer had no effect on the GSH efflux rate, indicating that the microcirculation does not play a significant role in this system. However, when GSH- $(\text{glycine-}^{13}\text{C}_2,^{15}\text{N})$ was present in buffer at a concentration higher than the lenticular GSH concentrations, efflux rate increased significantly ($P < 0.05$) and matched the expected rate. Furthermore, the connexin inhibitors 18β -glycyrrhetic acid and octanol reduced the efflux rate back to that of the control ($P < 0.05$), indicating that passive GSH exchange in lenses may be at least partially mediated by connexin hemichannels that are gated and influenced by relative GSH concentrations.

Localization of GSH uptake was assessed by incubating lenses with a fluorescent GS-B conjugate. Although this conjugate may have slightly different properties than GSH, it was determined that GS-B was taken up by lenses at the same rate as GSH- $(\text{glycine-}^{13}\text{C}_2,^{15}\text{N})$ (Fig. 3B) and is thus likely taken

up by the same mechanism. A time course of GS-B uptake revealed that uptake was visible at both the anterior and posterior lens within 5 minutes of incubation, but uptake along the lens posterior and equator appeared to occur much more rapidly than at the anterior, accounting for the majority of uptake seen at 15 and 30 minutes (Fig. 3C). GS-B entering from the posterior lens appeared to progressively accumulate in cortical fiber cells and diffuse toward the anterior lens surface. Penetration into the lens core was low, and GS-B only appeared significantly in the region after 30 minutes of incubation. This is not consistent with the lens microcirculation model of solute delivery and further confirms that this system is unlikely to be responsible for GSH uptake in cultured lenses.

Lens GSH Uptake From Aqueous Humor Is Carrier-Mediated

Glutathione uptake at the anterior lens was characterized by replacing endogenous aqueous humor with uptake buffer containing various concentrations of GSH- $(\text{glycine-}^{13}\text{C}_2,^{15}\text{N})$ (Fig. 4A). Anterior lens GSH uptake in both WT and LEGSKO appeared to be saturable and fit well to the Michaelis-Menten equation, with an apparent V_{max} of $\sim 0.21 \mu\text{M}/\text{min}$. LEGSKO lens uptake showed an enhanced affinity for GSH with an apparent K_m of $\sim 50 \mu\text{M}$ compared to K_m of $\sim 250 \mu\text{M}$ in WT.

Glutathione uptake at the anterior lens could be significantly ($P < 0.005$) inhibited by including an excess of unlabeled GSH or the closely analogous peptide γ -EAG or γ -ESG in the uptake buffer, indicating a carrier-mediated

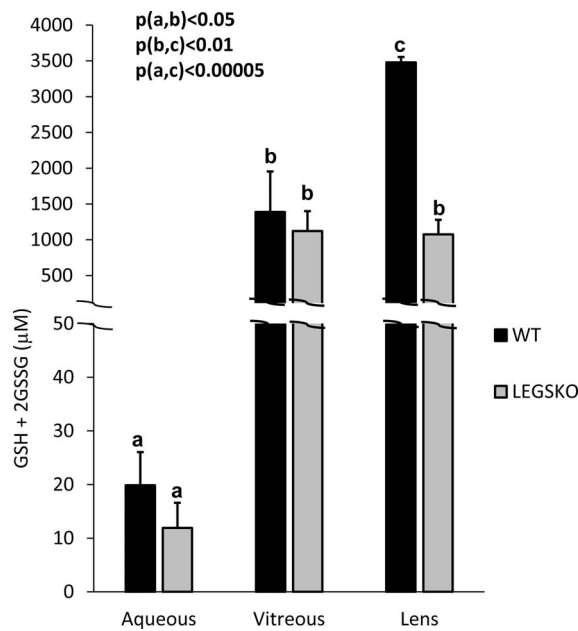


FIGURE 5. Comparison of concentrations of aqueous, vitreous, and lens GSH in LEGSKO and WT eyes. Aqueous, vitreous, and lenses were dissected from WT and LEGSKO mice and analyzed for total glutathione content. Wild-type and LEGSKO aqueous and vitreous had similar glutathione content, but LEGSKO lenses showed a significant loss of glutathione ($P < 0.01$). LEGSKO lens glutathione concentration matches LEGSKO vitreous glutathione concentration. Values are means \pm SD; $n = 4$.

mechanism (Fig. 4B). Because the serine and alanine analogs of GSH could competitively inhibit the uptake to the same degree as GSH, it appears that this uptake is not highly selective for GSH and could be a more general peptide transport mechanism.

The permeability of lens cell barriers was tested at the anterior lens and the whole cultured lens, using lucifer yellow (Fig. 4C). Whole, cultured lenses had a >20 -fold higher Pc ($P < 0.05$) than the anterior lens surface. This indicates that the anterior lens surface is much less permeable than the posterior lens, which may account for the differences between GSH uptake mechanisms in cultured lenses and that in the anterior lens.

Mouse Vitreous Contains High Levels of GSH

Glutathione content in aqueous, vitreous, and lenses from WT and LEGSKO mice was analyzed by LC-MS/MS (Fig. 5). These data revealed surprisingly high (>1 mM) vitreous GSH content and very low (<20 μ M) aqueous GSH content in both LEGSKO and WT lenses. LEGSKO vitreous and lens GSH concentrations were robustly the same, strongly implying an equilibration between the tissues.

Vitreous but Not Aqueous Contributes Highly to Lens GSH In Vivo

The ability of vitreous and aqueous GSH pools to contribute to the lens GSH pool in vivo was tested at physiologically relevant concentrations by injecting WT and LEGSKO mice with GSH-($^{13}\text{C}_2,^{15}\text{N}$) to final concentrations of approximately 20 μ M and 1 mM in aqueous and vitreous, respectively. Figure 6A shows that the lens can take up, in vivo, a significant ($P < 0.05$) amount of GSH from the available pool in the vitreous but not in the aqueous and that LEGSKO and WT uptake rates are the same. As in ex vivo lens culture experiments, excess unlabeled

GSH could not inhibit the uptake of GSH-($^{13}\text{C}_2,^{15}\text{N}$) from the vitreous (Fig. 6B).

To determine the relative rates at which circulating GSH moved into these compartments, mouse eyes were perfused by catheterization of the common carotid artery, which was pumped with fluid containing GSH-($^{13}\text{C}_2,^{15}\text{N}$) (Fig. 6C). GSH-($^{13}\text{C}_2,^{15}\text{N}$) accumulated within the vitreous more rapidly than the lens or aqueous ($P < 0.05$). Furthermore, the vitreous and the lens both accumulated a significant ($P < 0.05$) amount of GSH-($^{13}\text{C}_2,^{15}\text{N}$) before any was detectable in the aqueous.

In order to further examine the relationship between systemic, vitreous, and lenticular GSH, circulating GSH was depleted in LEGSKO mice by replacing drinking water with water containing various concentrations of BSO. This treatment had little to no effect on aqueous humor GSH but significantly lowered lens and vitreous GSH contents ($P < 0.05$) (Fig. 6D). At all concentrations of BSO given, the concentrations of GSH in the lens and vitreous of these mice were approximately equal, supporting the hypothesis that the vitreous provides GSH to the lens through a passive process.

High Vitreous GSH Content Is Unique to Small Animals

Finally, we investigated whether the above observation of high vitreous GSH in mice was applicable to other species by determining glutathione content of ocular tissue compartments from WT human, rat, cow, pig, *Xenopus* frogs, minnows, and mice (Fig. 7A). All small animals had ~ 1 mM vitreous GSH, whereas large mammals, including humans, pigs, and cows, had much lower levels (<100 μ M). All species had low levels (<100 μ M) of GSH in aqueous humor. Intriguingly, fish and frogs had significantly ($P < 0.05$) lower lens GSH than other species, which was at approximately the same level as their vitreous humor. This could indicate that vitreous humor is a major source of lens GSH in these species.

Unlike the vitreous humor in rodents and other small animals, the vitreous humor of large mammals is large in volume and can be dissected into distinct regions, including the base (near the lens), cortex (near the retina), and core. These regions were isolated and analyzed for GSH content in human and porcine eyes (Fig. 7B). Base vitreous was found to have significantly ($P < 0.005$) higher GSH than that in the core or cortex in humans and significantly ($P < 0.01$) higher GSH than that in the core but not the cortex in porcine eyes.

DISCUSSION

Evidence of Vitreous GSH Transport From Other Studies

This paper represents, to our knowledge, the first report of significant vitreous-to-lens GSH transport. To date, researchers studying lens uptake of GSH have focused their attention on the aqueous humor, which is conventionally considered the source of lens nutrition. However, several other studies have demonstrated that vitreous GSH is dynamic and that circulating GSH readily enters the vitreous. Measurement of GSH in rabbit ocular tissues by the HPLC method revealed that vitreous total glutathione nearly doubled 3 hours after treatment with *N*-acetylcysteine.²⁴ Even without treatment, vitreous GSH concentration was found to be very high, just under 20% of that found in lenses. Another study directly measured the production and movement of ^{35}S -GSH in ocular tissues after injection of ^{35}S -GSSG into the anterior chamber or vitreous body of rabbit eyes.²⁵ Although anterior chamber injections led

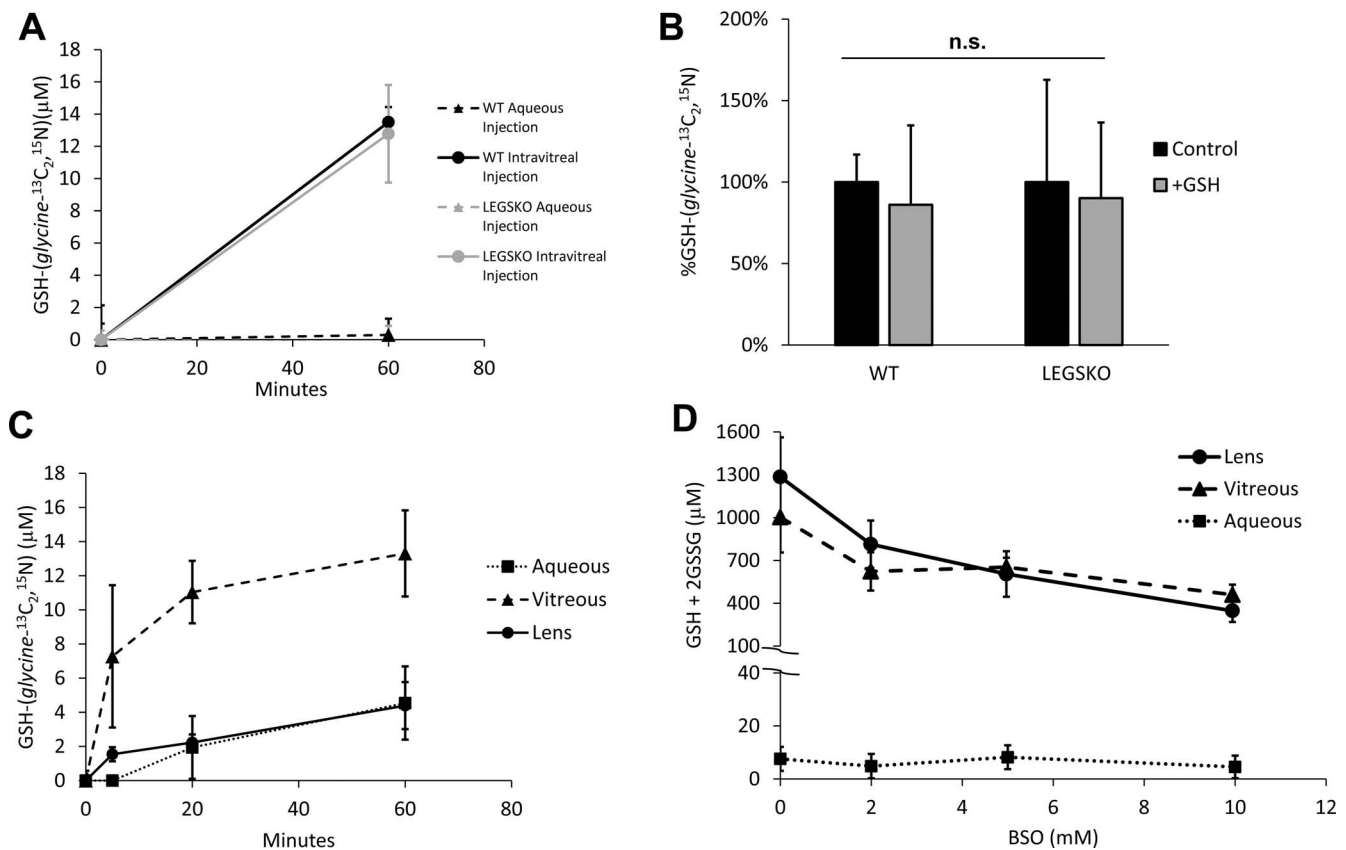


FIGURE 6. Comparison of in vivo GSH transport and homeostasis in mouse eyes. (A) Wild-type and LEGSKO mice were anesthetized, and GSH-(glycine-¹³C₂, ¹⁵N) in physiological (pH 7.4) solution was injected to a final concentration of 1 mM in the vitreous body or 20 μM in the anterior chamber of eyes in order to mimic physiological conditions. After 1 hour, mice were euthanized, and lenses were analyzed along with control lenses for GSH-(glycine-¹³C₂, ¹⁵N) content. Anterior chamber injections did not lead to an increase in lens GSH-(glycine-¹³C₂, ¹⁵N), but intravitreal injections did lead to a significant accumulation of the compound in lenses ($P < 0.05$). LEGSKO and WT lenses showed no differences in rates of GSH-(glycine-¹³C₂, ¹⁵N) accumulation. (B) Mice were injected with GSH-(glycine-¹³C₂, ¹⁵N) intravitreally as in A, but one group was also injected with unlabeled GSH at a 10-fold excess. In both the WT and the LEGSKO mice, this excess unlabeled GSH had no effect on the lens uptake of GSH-(glycine-¹³C₂, ¹⁵N). (C) Wild-type mouse carotid arteries were catheterized and pumped with bicarbonate-buffered physiological solution containing 200 μM GSH-(glycine-¹³C₂, ¹⁵N) at a rate of 1 mL/min. Mice were anesthetized and kept warm throughout. Ocular tissue was isolated and analyzed for GSH-(glycine-¹³C₂, ¹⁵N) content. GSH-(glycine-¹³C₂, ¹⁵N) was detectable in lens and vitreous samples before any accumulated in the aqueous ($P < 0.05$) and accumulated in vitreous humor to a significantly higher degree than in lens or aqueous humor ($P < 0.005$). (D) LEGSKO mice were given BSO-containing water of various concentrations for 2 months. Total glutathione contents of lens, vitreous, and aqueous were determined. A concentration of 10 mM BSO was sufficient to significantly lower ($P < 0.05$) lens and vitreous glutathione contents but had no effect on aqueous glutathione levels. For each BSO treatment, there were no significant differences between the lens and vitreous glutathione concentrations. Values are means \pm SEM for line graphs and \pm SD for bar graphs. (A–C) $n = 4$; (D) $n = 6$.

to an accumulation of ³⁵S-GSH in corneal cells, the lens did not appear to take up any significant amount of ³⁵S-GSH. Conversely, the lens did uptake a significant amount of ³⁵S-GSH from intravitreal injections, which increased steadily in the lens cortex and then slowly into the lens nucleus. In a study by Stewart-DeHaan et al.,¹⁰ rats were injected intraperitoneally with ³H- or ³⁵S-GSH, and the uptake of these compounds into ocular tissues was measured by HPLC. From these measurements, it was found that the lens could obtain more than 12% of its total GSH from circulating GSH in just 4 hours. To determine the source of GSH transported into the lens, the researchers compared the concentration of ³H-GSH and unlabeled GSH in the aqueous, vitreous, and lens after injection. Although the ³H-GSH-to-GSH ratio was much higher in the aqueous than in the vitreous, this was due only to the large difference in endogenous GSH content of these fluids, because the actual levels of ³H-GSH and ³H-GSSG found in the vitreous were >200-fold greater than that in the aqueous. Additionally, the concentration of ³H-GSH in the vitreous increased from 30 minutes to 4 hours, whereas aqueous ³H-

GSH did not. Thus, despite the authors' statement that aqueous humor must have been the source of lens ³H-GSH during that experiment, reevaluation of the data shows that the data appears to robustly support our mouse eye perfusion results.

Uptake From Vitreous but Not Aqueous Is Sufficient to Maintain Steady State GSH Concentration in LEGSKO Lenses

While it is clear that some uptake of GSH can and does occur at the anterior lens, it cannot account for the levels of GSH found in the whole LEGSKO lens. Our measurements show that the rate of GSH transport into the LEGSKO lens from the aqueous humor at physiologically relevant concentrations is approximately 35 nM/min (Fig. 3A). Glutathione turnover in rodent lenses has been measured in the range of 0.014%⁻¹hr to 0.018%⁻¹hr.²⁶ In mice, this equates to a GSH turnover rate of approximately 1 μM/min. Based on this, it is simply not possible for lenses to maintain a millimolar concentration of GSH by transporting GSH from the aqueous, although it may be an

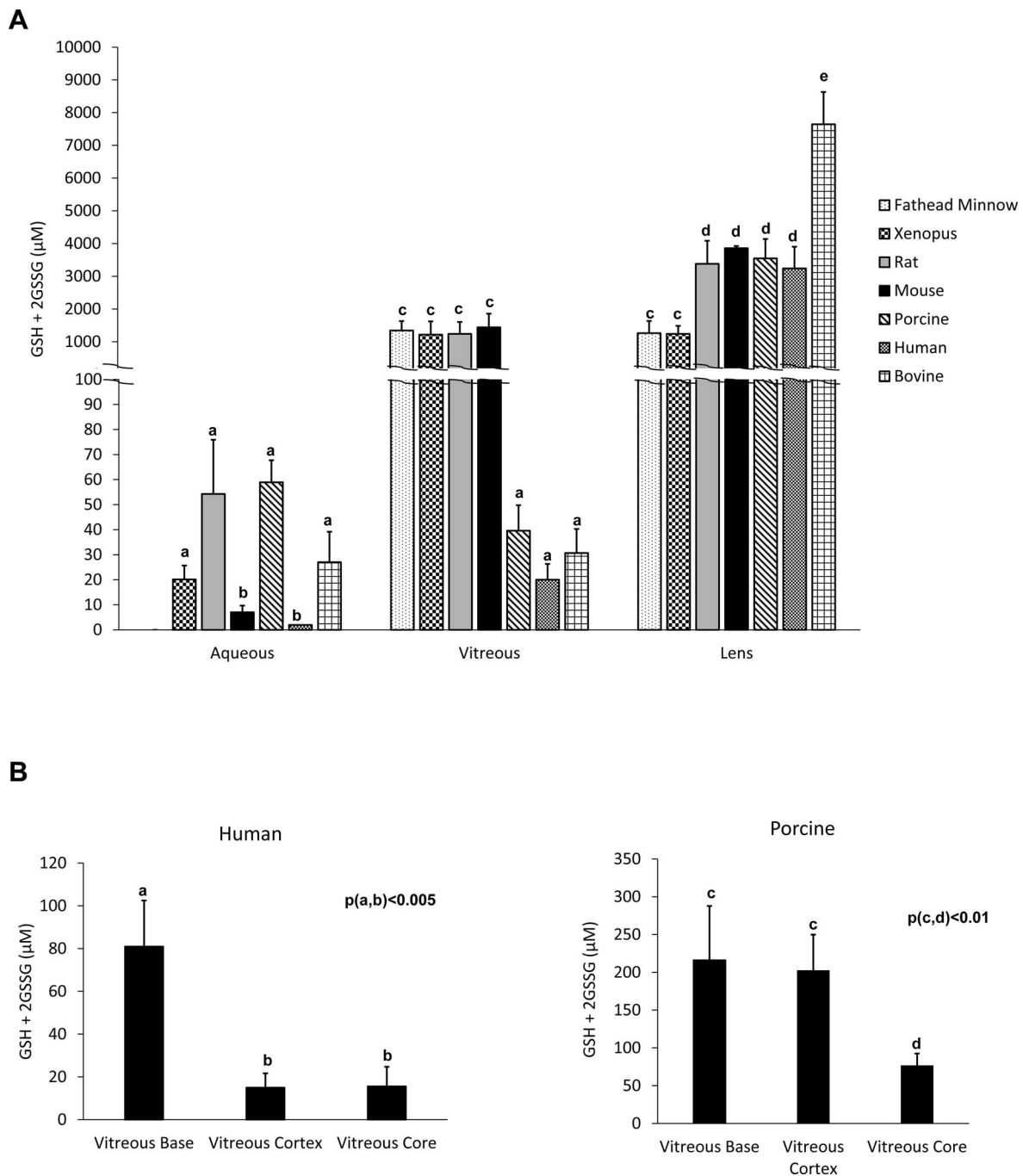


FIGURE 7. Comparative ocular distribution of glutathione in aqueous, vitreous, and lens in eyes of various species. **(A)** Fresh tissue was taken from members of each species. Bars with different letters show a statistically significant difference ($P < 0.05$). Rat, mouse, minnow, and frog vitreous glutathione contents were significantly ($P < 0.05$) higher than those of human, bovine, or porcine vitreous. Lens glutathione contents were similar for human, rat, mouse, and porcine eyes but significantly elevated ($P < 0.05$) in bovine eyes and significantly lower in minnow and frog eyes ($P < 0.05$). Values are means \pm SD. Bovine, mouse, and porcine $n = 4$; rat and human $n = 3$. **(B)** Regional distribution of GSH within human and porcine vitreous. Vitreous was sampled at the base (near lens), core, and cortex (near retina) regions. Human base vitreous had significantly ($P < 0.005$) higher GSH content than that in core and cortex, which had similarly low levels of GSH. Porcine base vitreous had significantly higher ($P < 0.01$) GSH content than the core but not the cortex. Values are means \pm SD. Porcine $n = 4$; human $n = 3$.

important mechanism for protecting the epithelium from oxidative stress. Conversely, measurements of lens GSH uptake at concentrations found within the vitreous show a rate of $\sim 1 \mu\text{M}/\text{min}$ (Fig. 2C). This shows that vitreous GSH content is sufficient to maintain a high steady state concentration in lenses if it is continuously supplied. Our perfusion data showed circulating GSH entering the vitreous humor at an initial rate of

~ 1 to $2 \mu\text{M}/\text{min}$ (Fig. 6C). Thus, vitreous GSH that is lost to the lens appears to be readily replaced by circulating GSH.

Vitreous GSH Flows Into Lenses but Not Vice Versa

Interestingly, while our ex vivo and in vivo data are in agreement that lens GSH uptake from the vitreous is not an

energy-dependent process, transport appears to occur significantly in only one direction. LEGSKO and WT vitreous GSH contents are robustly the same, indicating that the lens does not supply the vitreous with its high GSH content (Fig. 5). If the lens and vitreous truly do exchange GSH through a passive process, why do WT lens and vitreous GSH pools not reach an equilibrium? One potential explanation is that lens microcirculation causes an inward flux of solutes along the posterior lens and that lenticular GSH cannot move against this current. However, we have determined that inhibiting the microcirculation has no effect on lens GSH release (Fig. 2H), and imaging of GS-B indicates that microcirculation is not the delivery method for GSH. More likely, lens-vitreous GSH exchange is mediated by hemichannels, and the opening of these channels is gated. We have demonstrated that GSH release from lenses appears to be at least partially mediated by extralenticular GSH levels and that GSH release can be partially inhibited by connexin inhibitors (Fig. 2H). This is consistent with the findings that gated hemichannels, which have a known permeability to GSH,²⁷ have been found on fiber cell membranes along the posterior lens.²⁸ However, the methods used here were nonspecific, and further research will need to be performed to conclusively determine the exact mechanism.

Based on these data, it appears that mice have adapted a system wherein the vitreous can serve as a pool of GSH that will flow into the lens when lens GSH content is the same or lower than vitreous GSH content, but under normal conditions, lens GSH will not flow into the vitreous to a significant degree. This allows WT lenses to maintain a high lens-to-vitreous GSH gradient while also permitting LEGSKO lenses to equilibrate with the vitreous. However, vitreous GSH is not a significant source of lens GSH in mouse lenses with active synthesis.

Vitreous GSH Varies Greatly Among Species

We found striking differences in the vitreous humor GSH content of small animals, including rodents and large mammals (Fig. 7A). Very similar results were reported by others when comparing rat, rabbit, and bovine vitreous GSH.²⁹ The differences we report on vitreous GSH content of various species show no correlation with lens GSH content or temporal (i.e., diurnal/nocturnal) activity of the species. The most obvious explanation for the difference in vitreous GSH content is that it results from morphologic differences in eyes based on species size. In large mammals, the vitreous humor typically makes up >80% of the eye volume, whereas in the eyes of smaller species, the lens makes up the major portion of the eye volume, and the vitreous humor volume is relatively small. In large-mammal eyes, GSH entering the vitreous from the retina or ciliary body, whether by diffusion or active transport, has a comparatively large volume to diffuse through relative to the surface area of cells secreting it. Thus, it is expected that GSH could not easily reach a high concentration in the vitreous of these eyes. Conversely, in mouse, rat, fish, and frog eyes, the volume of the vitreous is very small and GSH entering from the retina or the ciliary body, or both, could reach a much higher concentration. Rabbits form an interesting compromise in that they are a larger rodent with an eye volume of ~60% vitreous humor. As would be expected, rabbit vitreous GSH content falls between that found in mice and rats and larger mammals, with ~650 μ M reported.²⁹ Based on these findings, it is possible that vitreous GSH content is not highly regulated and may just be a result of the high (~3 to 5 mM) GSH content in surrounding tissues, such as the retina and ciliary body. Further studies of GSH transport into and throughout the vitreous and an assessment of vitreous

oxidative compounds in various species will be required to fully understand the role and regulation of vitreous GSH.

Role of Vitreous GSH in Health and Disease

Due to the striking differences in GSH contents of human and mouse vitreous, it is difficult to conclude the role of the vitreous GSH pool in human lens homeostasis. However, the elevated levels of GSH found in the vitreous base (Fig. 7B) indicate that the vitreous GSH pool may be important for protecting lenses or providing constituent amino acids because these levels are nearly 50-fold higher than those found in the human aqueous. This may help explain why removal of the vitreous is tightly linked to the formation of cataracts in humans. Of patients who undergo vitrectomy surgery, 60% to 98% develop cataract within 2 years according to various reports.³⁰⁻³² A loss of vitreous humor as a source of nutrients and protective buffer against oxygen and oxidative species could greatly predispose lenses to cataract.

Additionally, it has been shown that human lens GSH synthesis is approximately 16-fold decreased over an 83 year timespan, even though lens GSH content only decreases ~50% during this time.³³ This high residual GSH cannot be accounted for by passive diffusion, as in LEGSKO, and implies that human lenses have a more robust lenticular GSH transport system than rodents. Such a difference would not be unprecedented because human lenses show a 10-fold greater propensity for the uptake of ascorbic acid, another important lenticular antioxidant, than rodents.³⁴ Given the much greater levels of GSH in the vitreous surrounding the lens compared to the aqueous, it would appear to be the more likely source of transported GSH. The relationship between the lens and vitreous in humans requires further investigation and could be of critical importance for maintaining human lenses in a healthy reduced state.

CONCLUSIONS

We have shown, for the first time, that the vitreous humor can supply high levels of GSH to the mouse lens in the absence of its synthesis. Transport of GSH into the lens from this compartment is a passive process, in contrast to the anterior lens, where we show that an active carrier-mediated uptake of GSH occurs. Further study is needed to determine the mechanism by which rodent vitreous obtains such high concentrations of GSH. Our findings bring into question the concepts that lenses receive nutrients solely from the aqueous humor and that the vitreous is not a highly dynamic fluid, and indicate that the vitreous may be an essential component in the maintenance of healthy lenses.

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