Glutathione in Calf Trabecular Meshwork and its Relation to Aqueous Humor Outflow Facility

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Previous studies have shown that sulfhydryl reagents can alter the facility of aqueous humor outflow but little is known about the sulfhydryl constituents of the aqueous outflow system or the effect of oxidants upon outflow facility. In the present study the concentration of glutathione (GSH) was measured in excised calf trabecular meshwork (TM) and found to be 0.40 μ mol/g wet wt (0.027 μ mol/mg protein). Oxidized glutathione was not detectable in the tissue. TM was found to have significant hexose monophosphate shunt activity as determined by measurement of the oxidation of 14C-1 and 14C-6-labeled glucose in tissue homogenates. The concentration of GSH in TM of enucleated calf eyes could be totally depleted by infusion of medium containing both diamide, which is an oxidant of GSH, and 1,3bis(2-chlorethyl)-1-nitrosourea (BCNU), which is an inhibitor of the enzyme glutathione reductase. The depletion of GSH was found to have no effect on the facility of aqueous outflow. Experiments were also done in which normal and TM GSH-depleted eyes were perfused with medium containing H202. Exposure to H202 produced no effect on outflow facility in the normal eyes but caused a 33% decrease in facility in eyes with the GSH-depleted TM. The results indicate that GSH may not participate directly in regulating aqueous humor outflow but is able to protect TM against H202-induced oxidative damage that would otherwise lead to a decrease in outflow facility. Invest Ophthalmol Vis Sci 24:1283–1287, 1983

Anterior chamber perfusion studies have shown that the presence of sulfhydryl (-SH) reagents can alter the resistance to aqueous humor outflow,[1-3] which suggests that thiols present in the trabecular meshwork (TM) may be involved in modulating aqueous humor outflow resistance. One thiol that may be involved in this process is glutathione (GSH), which has been found to be present in a number of ocular tissues[4-6] but whose concentration has not been determined in TM. The present study was designed to determine the level of GSH present in calf TM and to investigate its function. In addition, since the hexose monophosphate shunt (HMPS) is an integral part of GSH metabolism,[7] the presence of this pathway in TM was also investigated.

Since our studies to be reported demonstrated the presence of GSH in TM, it was of interest to deplete the level of the tripeptide in TM of perfused calf eyes and study the effect on aqueous outflow facility. For this purpose advantage was taken of diamide that has been shown to be a relatively specific oxidant of GSH[8] and of 1,3bis(2-chloroethyl)-1-nitrosourea (BCNU), which is a specific inhibitor of the enzyme glutathione reductase.[9]

Of special importance was the possible interaction of GSH in TM with H202, an oxidant that has been found to be a normal constituent of aqueous humor in at least three species including man.[10-12] Since aqueous humor is drained via the TM it is conceivable that oxidant stresses that exceed the capacity of TM for detoxification could result in cellular damage and subsequent alteration of aqueous humor outflow resistance.

We report here on changes that occur in the facility of aqueous outflow in calf eyes when GSH-depleted TM is exposed to H202.

Materials and Methods

Calf eyes were obtained from Joseph T. Trelegan and Co. (Cambridge, MA). Shortly after the animals were killed by exsanguination the eyes were enucleated and placed in iced saline solution for transportation and use within 24 hrs. Lenses used in the study were from albino rabbits weighing between 1.8 and 2.2 kg.

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Table 1. The concentration of GSH in various ocular tissues of the calf

	GSH Concentration	
	μmol/g wet wt	*μmol/mg protein*
Trabecular meshwork*	0.40 ± 0.03	.027 ± .003
Lens†	9.4	0.031
Cornea:		
Epithelium‡	3.6	0.018
Endothelium‡	2.0	0.010

* GSH in trabecular meshwork was determined with the use of an amino acid analyzer. Each analysis required 100–150 mg wet wt of tissue excised from 20–30 calf eyes. Results are expressed as the means ± SD for five experiments. The tissue was approximately 1.5% soluble protein.
† Data from Merola and Kinoshita[5] assuming 30% protein.
‡ Data from Riley and Yates[6] assuming 20% protein.

Glucose-1-14C and glucose-6-14C (2–10 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA). Diamide and H202 were obtained from Sigma Chemical Co. (St. Louis, MO) and Mallinckrodt, Inc. (St. Louis, MO), respectively. BCNU (NSC-409962-NC) was kindly supplied by Dr. Ven Narayanan of the National Cancer Institute, Bethesda, MD.

Excision of TM from perfused and nonperfused calf eyes was carried out as previously described.[13] Following excision the tissue was blotted on Whatman No. 1 filter paper and placed into tared vials for storage at −80 C. Precautions were taken to ensure that blood was not associated with the TM (the absence of hemoglobin was taken as an indicator of lack of contamination with blood).

Concentrations of GSH, oxidized GSH (GSSG) and free amino acids were determined in both perfused and nonperfused TM. Each analysis required 100–150 mg wet wt of tissue obtained from 20–30 calf eyes. The tissue was removed in Boston, stored at −80 C, shipped to Michigan in dry ice, and stored again at −80 C until use within 1 month after excision. The transport and storage of the tissue did not result in the formation of a detectable level of GSSG. The TM was homogenized in six volumes of cold 0.05 M EDTA in a glass homogenizer and centrifuged for 10 min at 10,000 × g. The supernatant was made 10% in trichloroacetic acid and centrifuged again. Analysis was carried out with use of an automatic amino acid analyzer that was sensitive to 1 nmol of GSH.[14] Protein concentration was assayed by means of Bio-Rad protein assay solution using gamma globulin as a standard.

Measurements of the oxidation of glucose-1-14C and glucose-6-14C were made using fresh rabbit lenses or calf TM that had been stored at −80 C. The tissue was homogenized in eight volumes of cold 0.01 M tris-sulfate buffer, pH 7.4. Centrifugation was done at 1000 × g for 10 min. Lens supernatants were diluted to a protein concentration of 2.4 mg/ml to approximate that of the TM supernatant. Equal volumes (0.5 ml) of supernatant and substrate medium were added to a capped tube and incubated for 2 hrs at 37 C. The specific activities for the labeled glucose present in the substrate media were 12,000 cpm/μmol for glucose-1-14C and 67,000 cpm/μmol for glucose-6-14C. Details concerning the composition of the substrate medium and the procedure for measuring the release of 14C02 from the incubation mixture have been described previously.[15] Tissue homogenate was omitted from the blanks.

Quantitative anterior chamber perfusion studies were performed using a constant-pressure perfusion technique for whole eyes that has been described previously.[2,16] Paired calf eyes were used. All perfusions were carried out at room temperature at a pressure of 15 mmHg. The medium employed was Dulbecco's phosphate-buffered salt solution (PBS) (Grand Island Biological Co., Grand Island, NY) to which was added 5.5 mM glucose. The media were passed through a 0.22 micron Nuclepore filter immediately prior to use. In certain studies the medium also contained either H202 or a combination of BCNU and diamide (when BCNU was used, normal saline was substituted for PBS). This was added to the anterior chamber in an open system with the corneal fitting removed,[2] which results in an effectively lower concentration actually reaching the trabecular meshwork. In this system compounds added to the anterior chamber are first exposed to the lens, iris, and corneal endothelium before passage through to the trabecular meshwork. Following subsequent perfusion with normal medium for 1 hr, we estimate that added compounds are diluted in the anterior chamber to approximately 5% of the original concentration.[2]

Results

The concentration of GSH was determined in calf TM and compared to previously reported values for calf lens and cornea (Table 1). On a per g wet wt basis, the level of GSH was found to be from 5 to 24 times lower in TM than in corneal endothelium or lens, respectively. However, on a per mg protein basis the value for TM was similar to that for lens, corneal epithelium and corneal endothelium. GSSG was not detectable in calf TM.

Since GSH was found to be present in TM, experiments were done to determine whether HMPS activity also existed in the tissue. The oxidation of glucose-1-14C and glucose-6-14C was measured in supernatants of calf TM and rabbit lens that were incubated for 2 hrs in the presence of glucose, ATP,

Table 2. The oxidation of glucose-1-^{14}C and glucose-6-^{14}C in supernatants of calf trabecular meshwork (TM) and rabbit lens*

	μmol CO_2/g protein/2 hr	
	glucose-1-^{14}C	*glucose-6-^{14}C*
Calf TM	80 ± 2	not detectable
Rabbit lens	101 ± 8	1.1 ± 0.2

* Incubation of the supernatants was carried out in the presence of glucose (20mM), ATP and other substrates (see "Materials and Methods"). Each determination for TM required approximately 150 mg wet wt of tissue obtained from 30 calf eyes. Protein concentrations in the incubation mixtures were approximately 1.2 mg/ml. Results are expressed as the means ± SD for four experiments.

and other substrates (see Materials and Methods). A significant level of oxidation of glucose-1-14C was observed in the TM supernatant and, on a per g protein basis, was comparable to that in the lens supernatant (Table 2). Under the conditions employed, oxidation of glucose-6-14C was not detectable in the TM supernatant.

Preliminary experiments were conducted to determine the extent of oxidation of GSH occurring when calf eyes were perfused with medium containing both 1.0 mM BCNU and 100 mM diamide. Experimental calf eyes were perfused for 1 hr with normal medium, for a second hour with medium containing BCNU/diamide and for a third hour with normal medium. This treatment was found to result in complete depletion of GSH in the TM tissue (Table 3). Despite the loss of GSH relatively little GSSG was found in the tissue. The concentration of GSH in control TM, which was perfused for 3 hrs with normal medium, remained at a value comparable to that of TM from nonperfused eyes. Exposure to BCNU/diamide was found not to affect the concentrations of most of the free amino acids which were analyzed. Exceptions included the concentrations of glycine, proline and aspartate which were 40 to 70% lower than in the controls (Table 3).

The effect of oxidation of TM GSH on aqueous outflow was investigated by perfusing calf eyes with BCNU/diamide as described above and measuring induced changes in outflow facility. In preliminary experiments neither 1 mM BCNU alone nor diamide alone, in concentrations ranging from 1 to 100 mM, significantly altered outflow facility. The results showed that depletion of GSH in TM by combined BCNU/diamide treatment had no significant effect on measured outflow facility during a four hour period (Table 4).

Experiments were conducted to learn whether exposure of GSH-depleted TM to H202 would alter aqueous humor outflow facility. Eyes were exposed

Table 3. The effect of perfusion of calf eyes with medium containing diamide and BCNU on the concentrations of GSH, GSSG and free amino acids in the trabecular meshwork (TM)*

	Concentration (μmol/g wet wt)		
	Nonperfused	*Control*	*Experimental*
GSH	0.40 ± 0.03	0.58	not detectable
GSSG	not detectable	0.03	0.03
ASP	0.29 ± 0.08	0.35	0.11
THR	0.15 ± 0.02	0.12	0.09
SER	1.14 ± 0.18	1.18	0.97
GLU	0.93 ± 0.24	1.24	1.12
PRO	0.20 ± 0.03	0.28	0.15
GLY	0.56 ± 0.06	0.67	0.41
ALA	0.60 ± 0.08	0.54	0.55
VAL	0.29 ± 0.05	0.16	0.17
MET	0.05 ± 0.03	0.03	0.03
ILEU	0.09 ± 0.02	0.04	0.04
LEU	0.19 ± 0.03	0.10	0.07
TYR	0.08 ± 0.01	0.04	0.04
PHE	0.09 ± 0.01	0.04	0.04

* Analyses were performed on TM from perfused and non-perfused eyes. Control eyes were perfused for three hours with Dulbecco's phosphate medium containing 5.5 mM glucose. Experimental eyes were also perfused for three hours: the first hour with normal medium, the second hour with medium containing 100 mM diamide and 1 mM BCNU and the third hour with normal medium (see "Materials and Methods"). Results for TM from non-perfused eyes are expressed as the mean ± SD for five experiments. Results for the perfusion experiments represent single analyses done on TM pooled from 24 control or experimental paired eyes.

to BCNU and diamide to deplete GSH and then perfused with 25 mM H202 for 1 hr followed by perfusion with normal medium for 4 hrs. Control eyes were also perfused with H202 for 1 hr but the pretreatment did not include exposure to BCNU/diamide. It was found that outflow facility in the control eyes was not affected by the H202 exposure (Table 5). However, in the BCNU/diamide-treated eyes, H202 produced a reduction in outflow facility that was not observed until at least 2 hrs after the peroxide had been removed. During the 4th hr of perfusion following treatment with H202, a 33% decrease in

Table 4. The effect of perfusion with BCNU and diamide on the outflow facility of calf eyes*

Perfusion Time (hr)	*Conditions*		*Facility (μl/min/mmHg)*	
	Control	*Exptl.*	*Control*	*Exptl.*
0–1	normal	normal	1.93 ± 0.27	1.73 ± 0.17
1–2	"	BCNU/Diamide	—	—
2–3	"	normal	2.07 ± 0.41	1.98 ± 0.22
3–4	"	"	2.04 ± 0.49	2.03 ± 0.25
4–5	"	"	1.97 ± 0.55	1.97 ± 0.22
5–6	"	"	1.85 ± 0.46	1.89 ± 0.17

* Paired calf eyes were perfused with Dulbecco's phosphate medium containing 5.5 mM glucose in either the absence (control) or presence of 100 mM diamide and 1 mM BCNU (see "Materials and Methods"). Results for outflow facility are expressed as the mean ± SEM for five experiments.

Table 5. The effect of perfusion with BCNU and diamide and subsequent exposure to H_2O_2 on outflow facility in calf eyes*

Perfusion Time (hr)	*Conditions*		*Facility (μl/min/mmHg)*	
	Control	*Exptl.*	*Control*	*Exptl.*
0–1	normal	normal	1.79 ± 0.09	1.93 ± 0.18
1–2	"	BCNU/ Diamide	—	—
2–3	"	normal	2.20 ± 0.08	2.22 ± 0.20
3–4	H_2O_2	H_2O_2	—	—
4–5	normal	normal	2.06 ± 0.07	1.99 ± 0.22
5–6	"	"	2.14 ± 0.10	1.88 ± 0.21
6–7	"	"	2.21 ± 0.14	1.70 ± 0.21
7–8	"	"	2.22 ± 0.14	1.48 ± 0.21

* Perfusion of paired calf eyes was carried out with three types of media: Dulbecco's phosphate medium containing 5.5 mM glucose (normal), normal medium containing 100 mM diamide and 1 mM BCNU and normal medium containing 25 mM H_2O_2 (see "Materials and Methods"). Results for outflow facility are expressed as the mean ± SEM for nine experiments.

facility was observed in the TM GSH-depleted eyes. Control eyes demonstrated the expected slight increase in facility following prolonged anterior chamber perfusion- the "washout" effect.[1,17]

Discussion

This study has shown that GSH but not GSSG is present in calf TM at a significant concentration. In addition, activity of the HMPS was also demonstrated to be present in the tissue. These findings suggest that TM, like many other tissues, has an active mechanism for maintaining GSH in the reduced state. The observed evidence for HMPS activity is supported by the results of a previous study that indicated that the enzyme glucose-6-phosphate dehydrogenase is active in calf TM.[13] The inability to detect oxidation of glucose-6-14C in 1,000 g supernatants of TM may have been caused in part by damage to mitochondria occurring when the tissue, which is tough and collagenous, was homogenized. Previous investigations have indicated that respiration does take place in TM.[13]

The use of both diamide and BCNU in the experimental perfusion medium of this study allowed GSH to be depleted in TM without the need for an absence of glucose in the medium. Other investigators who employed diamide as a GSH-oxidant in lens[18] and cornea[19] omitted glucose in order to prevent the regeneration of GSH from GSSG via glutathione reductase, NADPH, and the HMPS. In the present study it was considered important to include glucose in order to maintain the viability of the TM cells. The finding that only low levels of GSSG were present in TM after BCNU/diamide exposure must be attributed to leakage of GSSG from TM cells or possible combination of GSSG with cellular proteins to form mixed disulfides.

The results of this study indicate that depletion of GSH in TM alone is not sufficient to produce an alteration in outflow facility (at least during a 4-hr period). In addition, there seemed to be little effect on cellular permeability since concentrations of most of the free amino acids in the TM tissue remained normal after BCNU/diamide treatment. It appears that an additional cellular insult such as that provided by H202 is required before detrimental effects on aqueous humor outflow are observed. Studies involving other ocular tissues have shown that oxidation of GSH by diamide can produce damaging effects without the need for additional oxidative stress. These effects have included corneal swelling,[19] inhibition of cation transport in lens[18] and alterations of light induced activity in retina.[20] The reason for this unique lack of response of TM to diamide is not clear, but it is possible that the role of GSH in TM is not the same as that in other ocular tissues. In many cells GSH appears to be important in maintaining normal cation transport; for example, in the lens, it has been shown that a 60% decrease in the concentration of GSH in the epithelium produces significant inhibition of the active transport of cations.[21] However in TM, active transport is not believed to be involved in aqueous humor outflow.[2,22]

One possible role of GSH in TM may be the detoxification of H202, which is continuously flowing through the trabecular meshwork as a constituent of aqueous humor.[10–12] The origin of H202 in aqueous is not well established but it may be produced from the reaction of oxygen with ascorbate.[23] It would seem to be important for ocular tissues that are exposed continuously to aqueous humor to have adequate means of detoxifying peroxide. In the lens the metabolism of GSH, including activity of the HMPS, has been shown to contribute significantly to the removal of H202.[24,25] The present finding that exposure of normal eyes to H202 had no effect on the outflow facility suggests that TM may have a similar protective capability.

We thus hypothesize that GSH may have an indirect role in maintaining normal aqueous humor outflow facility. It is possible that GSH is involved in protecting the membrane -SH groups of TM against oxidative insult from the aqueous humor. It is noteworthy that reagents such as iodoacetamide and N-ethyl maleimide, which can react directly with membrane -SH groups, produce a marked alteration in the facility of aqueous outflow.[2,3] However, the effects of these sulfhydryl reagents are opposite to that of H202 in that they cause an increase rather than a decrease in outflow facility. The reason for this difference is probably the fact that

iodoacetamide and N-ethyl maleimide are able to block membrane -SH groups but, unlike H202, do not produce membrane disulfide bonds. It is possible that the oxidative action of H202 may have produced damage and swelling of TM endothelial cells and thus caused blockage of the aqueous humor outflow channels. The delay that was observed in the effect of H202 on outflow facility would be expected if it were required that cells swell to a certain critical dimension before obstruction of flow occurred. Morphologic studies are obviously needed to understand fully these effects of H202 on GSH-depleted TM.

In summary, it has been shown that calf TM contains a significant concentration of GSH as well as a mechanism for maintaining the tripeptide in the reduced state. It appears that GSH does not participate directly in regulating aqueous humor outflow but is able to protect TM against oxidative damage from H202 which would otherwise lead to a decrease in outflow facility.

Key words: glutathione, hydrogen peroxide, trabecular meshwork, -SH groups, aqueous humor outflow facility, hexose monophosphate shunt, diamide, BCNU, oxidant damage

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