Regulation of L-Cystine Transport and Intracellular GSH Level by a Nitric Oxide Donor in Primary Cultured Rabbit Conjunctival Epithelial Cell Layers

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PURPOSE. Metabolism and transport of cysteine are critical for maintenance of the intracellular glutathione (GSH) level. In this study, transport mechanisms of l-cystine and regulation of GSH biosynthesis in the absence or presence of NO-induced oxidant stress were investigated in primary cultured rabbit conjunctival epithelial cells (RCECs).

METHODS. RCECs were grown in membrane filters to exhibit tight barrier properties. Uptake and transepithelial transport of l-cystine were determined in the presence or absence of extracellular Na+. Uptake was determined at 10 minutes after 14C-L-cystine instillation into apical (a) or basolateral (b) bathing fluid. The effect of nitric oxide (NO) on l-cystine uptake, cellular GSH level, and expression level of two subunits of the rate-limiting enzyme γ-glutamylcysteine synthetase (GCS) was examined after a 24-hour incubation of primary cultured RCECs with an NO donor, S-nitroso-N-acetylpenicillamine (SNAP, Nacetyl-3-(nitrosothio)-o-valine).

RESULTS. Cellular uptake of l-cystine by RCECs occurred through both Na+-dependent and -independent mechanisms. Uptake from apical fluid was higher than that from basolateral fluid, except for the highest concentration of L-cystine tested (200 μM). Transepithelial permeability (Psuper) of l-cystine (at 2.5 μM) was three times higher in the a-to-b direction than in the b-to-a direction in the presence of Na+, whereas the reverse was true in the absence of Na+. Na+-dependent l-cystine uptake from apical fluid was significantly elevated in primary cultured RCECs treated for 24 hours with various concentrations (0.1–2.0 mM) of SNAP, with maximum uptake observed at 1 mM. A similar pattern of SNAP-induced increase of Na+-independent l-cystine uptake from apical fluid was observed, whereas no significant difference was observed for basolateral uptake. Concomitantly, a significant elevation of intracellular GSH (up to fivefold versus the control) was recorded, with the highest increase occurring at 0.1 to 0.25 mM SNAP. A parallel increase in the expression levels of both catalytic and regulatory subunits of GCS was observed by Western blot analysis of lysates from RCECs treated with 0.25 mM SNAP for 24 hours.

CONCLUSIONS. L-Cystine is transported by both Na+-dependent and -independent amino acid transport systems in RCECs. At low substrate concentrations, l-cystine uptake was higher from apical than basolateral fluid. Permeability studies indicated net absorption of l-cystine across RCECs. SNAP caused significant increases in both l-cystine uptake and intracellular GSH level, which occurred concomitantly with elevation of both catalytic and regulatory subunits of GCS. Understanding sulfur amino acid precursor-dependent cellular mechanisms of GSH homeostasis would be of value in devising GSH-based treatment for conjunctival or other ocular disorders. (Invest Ophthalmol Vis Sci. 2003;44:1202–1210) DOI:10.1167/iovs.02-0409

Glutathione (GSH) is an endogenous, thiol (SH) containing, anionic tripeptide synthesized in all cells from the precursors: cysteine, glycine, and glutamic acid.1,2 Cells synthesize GSH through a pathway involving the rate-limiting enzyme γ-glutamylcysteine synthetase (GCS) and GSH synthetase.2 GSH serves vital functions including detoxifying electrophiles, maintaining the essential thiol status of proteins by preventing oxidation of SH groups in cellular proteins or by reducing disulfide bonds induced by oxidant stress, scavenging free radicals, providing an intracellular reservoir for cysteine, and modulating critical cellular processes such as DNA synthesis, microtubule-involving processes, and immune function.2 Information on the metabolism, transport, or biosynthesis of GSH in normal and abnormal conjunctiva is scarce. It has been shown by Nucci et al.3 that GSH supplementation attenuates keratitis and conjunctivitis in a rabbit model of corneal injury. The same group also provided evidence for antiviral effects of GSH in other cell types, including VERO cells.4 Using primary rabbit conjunctival epithelial cells (RCECs) as a model in the first part of this study, also published in this Journal,5 we showed net secretion of intact GSH across RCEC layers under physiological conditions and ascertained the expression of key enzymes responsible for GSH synthesis and degradation.

It is well established that the sulfur amino acid precursor l-cystine is critical for maintenance of the intracellular GSH level. Intracellular concentrations of glutamate and glycine are relatively high, in that an adequate intracellular level of cysteine is a prerequisite for GSH biosynthesis. An anionic amino acid transport system highly specific for l-cystine and glutamate, operating in an Na+-independent manner, has been described in various cells, including cultured human fibroblasts,6 rat hepatoma cells,7 rat hepatocytes,8 mouse peritoneal macrophages,9 alveolar type II cells,10,11 and human retinal pigment epithelial cells.12 This l-cystine-glutamate transport system, termed Xc− system, is an exchange route, in which l-cystine is taken up in an anionic form in exchange for intracellular glutamate. The Xc− system has been identified in blood-brain...
barrier and ocular tissues.\textsuperscript{15,14} X_c^- is a heterodimer, consisting of \textit{Xc}2h as the heavy chain and \textit{xCT} (an \textit{Xc}^- transporter) as the light chain.\textsuperscript{15} The \textit{X_c}^- system is widely distributed, but other known transporters (\textit{b}0,+\textit{X}_{AG}) for \textit{l}-cysteine are expressed predominantly in the kidney, intestine, and lung.\textsuperscript{14} Transport mechanisms for \textit{l}-cysteine in ocular tissues including conjunctiva have not been systematically investigated to date.\textsuperscript{21,22}

Our recent evidence showing the expression of \textit{γ}-glutamyl transpeptidase (GGT) in freshly excised conjunctival tissue and primary RCECs suggests that breakdown and resynthesis of GSH entails important components of overall GSH metabolism in this tissue.\textsuperscript{5} Uptake of the sulfur amino acid precursor cysteine in the biosynthetic pathway of GSH is likely to play an important role in GSH homeostasis in conjunctiva.\textsuperscript{20,21}

\textit{l}-Cysteine transport activity is involved in defense against oxidant stress in endothelial cells.\textsuperscript{17} Exposure of endothelial cells,\textsuperscript{17} \textit{v7} cells,\textsuperscript{18} conditionally immortalized rat retinal capillary endothelial cells,\textsuperscript{18} and macrophages\textsuperscript{18} to agents that cause oxidant stress (e.g., \textit{H}2\textit{O}2, arsenite, diethyl maleate [DEM], hyperoxia, and cadmium) lead to increased activity of the \textit{Xc}^- system, but not the ASC uptake system. Moreover, in immortalized human retinal pigmented epithelial cells, NO has been shown to cause adaptive induction of the \textit{Xc}^- amino acid transport system and to increase \textit{l}-cysteine uptake, elevating intracellular GSH levels.\textsuperscript{12} The present study describes the transport properties of \textit{l}-cysteine in a primary culture model of rabbit conjunctival epithelial cell layers grown on permeable filters (12 mm diameter, 0.4 \textmu m pore size; Clearwell; Corning-Costar, Cambridge, MA) that were precoated with a mixture of 25 \textmu M/cm² rat tail collagen (types I and III, Collaborative Biomedical Products, Bedford, MA) and cultured in a humidified atmosphere of 5% \textit{CO}2 and 95% air at 37°C. The cell density of cultured RCEC layers on day 7 was 0.65 \times 10^6 cells/cm².

\textbf{METHODS}

\textbf{Primary Culture of RCEC Layers}

Research using rabbits described in this report conformed to the Guiding Principles in the Care and Use of Animals of the National Institutes of Health and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male, Dutch belted pigmented rabbits, weighing 2.0 to 2.5 kg, were used for isolation of conjunctival epithelial cells, as described in detail in our previous publications.\textsuperscript{20,21} Briefly, rabbits were killed with an injection of 85 mg/kg \textit{Na}+ pentobarbital solution into a marginal ear vein. Conjunctival tissues were excised and incubated in 0.2% protease type XIV (Sigma, St. Louis, MO) at 37°C for 30 minutes. Epithelial cells were scraped off using a sterile scalpel blade, suspended in a minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and 0.5 mg/mL D/Nase I, and centrifuged at 200 x \textit{g}.

The volumes of the apical (a) and basolateral (b) sides, respectively. During \textit{Na}+-free uptake studies the choline chloride buffer contained 2.5 \mu M unlabeled \textit{l}-cysteine and 2.5 \mu M \textit{14C}-cysteine for 10 minutes on day-7 RCEC layers. Choline chloride buffer had the same composition as the \textit{Na}+ buffer, except that 157 mM choline chloride and 8 mM choline bicarbonate replaced 137 mM \textit{Na}Cl and 8 mM \textit{Na}2\textit{HPO}4, respectively. During \textit{Na}+-free uptake studies the choline chloride buffer was present on both sides of the RCEC layers. The rate of \textit{l}-cysteine uptake (in picomoles per minute per milligram protein) was plotted against the concentration of \textit{l}-cysteine in the uptake buffer and analyzed using a Michaelis-Menten plot. The kinetic parameters were estimated on a computer (Origin software, ver.6.0; Microcal Software, Inc., Northampton, MA).

\textbf{Concentration- and Na+ -Dependency of \textit{l}-Cysteine Uptake.} To determine whether \textit{l}-cysteine entry into RCEC layers is concentration-dependent, apical or basolateral uptake of 1, 2.5, 50, and 200 \mu M unlabeled \textit{l}-cysteine in either \textit{Na}+ or choline chloride buffer containing 2.5 \mu M unlabeled \textit{l}-cysteine were performed for 10 minutes on day-7 RCEC layers. Choline chloride buffer had the same composition as the \textit{Na}+ buffer, except that 157 mM choline chloride and 8 mM choline bicarbonate replaced 137 mM \textit{Na}Cl and 8 mM \textit{Na}2\textit{HPO}4, respectively. During \textit{Na}+-free uptake studies the choline chloride buffer was present on both sides of the RCEC layers. The rate of \textit{l}-cysteine uptake (in picomoles per minute per milligram protein) was plotted against the concentration of \textit{l}-cysteine in the uptake buffer and analyzed using a Michaelis-Menten plot. The kinetic parameters were estimated on a computer (Origin software, ver.6.0; Microcal Software, Inc., Northampton, MA).

\textbf{Transepithelial \textit{l}-Cysteine Fluxes}

Transepithelial permeability of \textit{l}-cysteine across primary cultured RCECs on day 7 was measured by using 2.5 \mu Ci/mL \textit{14C}-cysteine and 2.5 \mu M unlabeled \textit{l}-cysteine, at 37°C, in \textit{Na}+ or choline chloride buffers. During \textit{Na}+-free transepithelial transport studies the choline chloride buffer was present on both sides of RCEC layers. \textit{l}-Cysteine fluxes were measured in both the apical-to-basolateral (a-to-b) and basolateral-to-apical (b-to-a) directions. Sample aliquots were taken from the fluid contralateral to the radioactivity-dosed fluid at 30-minute intervals for...
up to 3 hours and were analyzed for the accumulated radioactivity. Removed aliquots were immediately replaced with an equal amount of respective fresh buffer pre-equilibrated at 37°C. Unidirectional l-cystine flux (dQ/dt, mol/sec) is obtained from the steady state slope of cumulative amount of l-cystine transported versus time plot. The apparent permeability coefficient (P_app, in centimeters per second) is estimated from the relation, P_app = (dQ/dt)/C_o ∙ A, where C_o is the initial dose concentration (in moles per milliliter) of l-cystine, and A is the nominal surface area (~1.13 cm²) of the RCEC layers.

Effect of S-Nitroso-N-Acetylpenicillamine on l-Cystine Uptake

To determine the effects of oxidative stress on l-cystine uptake in primary cultured RCEC layers, cells were treated on both apical and basolateral sides with 0.10, 0.25, 0.50, 1, or 2 mM S-nitroso-N-acetylpenicillamine (SNAP; N-acetyl-3-[nitrosothio]-o-valine), an NO donor, at 37°C for 24 hours starting on culture day 6. Cells treated the same way in the absence of SNAP served as the control. After the treatment, cells were rinsed and l-cystine uptake was determined, as was de-}

RESULTS

Time Course of l-Cystine Uptake in RCECs

Uptake of l-cystine by RCECs from the apical and basolateral fluids of Na⁺-containing buffer was studied as a function of time for up to 10 minutes at 37°C. Figure 1 shows uptake of radiolabeled l-cystine. Apical and basolateral uptakes were both linear up to 10 minutes. All subsequent uptake experiments were thus performed for 10 minutes. Uptake from the apical fluid was significantly greater than that from the basolateral fluid for the entire 10-minute period (Fig. 1), where apical uptake rate (0.670 pmol/min per milligram of protein) was twice the basolateral uptake rate (0.334 pmol/min per milligram protein). l-cystine uptake from either apical or basolateral fluid in the absence of Na⁺ in the incubation buffer

<table>
<thead>
<tr>
<th>l-Cystine (µM)</th>
<th>+Na⁺</th>
<th>−Na⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.3</td>
<td>0.25 ± 0.03</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>Basolateral</td>
<td>0.09 ± 0.02</td>
<td>0.09 ± 0.001</td>
</tr>
<tr>
<td>10.8</td>
<td>0.45 ± 0.13</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>Basolateral</td>
<td>0.28 ± 0.01</td>
<td>0.20 ± 0.001</td>
</tr>
<tr>
<td>58.3</td>
<td>7.25 ± 0.71</td>
<td>5.67 ± 0.27</td>
</tr>
<tr>
<td>Basolateral</td>
<td>5.84 ± 0.16</td>
<td>2.32 ± 0.83</td>
</tr>
<tr>
<td>208.3</td>
<td>11.3 ± 1.11</td>
<td>14.2 ± 1.22</td>
</tr>
<tr>
<td>Basolateral</td>
<td>6.18 ± 1.34</td>
<td>8.58 ± 1.19</td>
</tr>
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</table>

Concentrations (µM) refer to the sum of the mass of labeled and unlabeled l-cystine. The amount of apical 14C-l-cystine was 2.5 nmol (2.5 µCi/mL in 0.3 mL buffer) and 8.3 nmol for basolateral (2.5 µCi/mL in 1 mL buffer) studies, respectively. Uptake of l-cystine was measured at 37°C for 10 minutes. RCECs were grown on membrane filters for 7 days, with TEER reaching 1000 Ω × cm². Data are mean ± SEM expressed in picomoles per minute per milligram protein (n = 4). Significantly greater than:

* Basolateral uptake rate under +Na⁺ condition at the same [l-cystine].
† Basolateral uptake rate under −Na⁺ condition at the same [l-cystine].

All comparisons were made by one-way ANOVA with post hoc tests based on a modified Fisher least-squares difference approach.
CONCENTRATION AND Na⁺ DEPENDENCY OF L-CYSTINE UPTAKE

Uptake rates, expressed as picomoles per minute per milligram protein, increased with increasing L-cystine concentrations in either apical or basolateral fluid in the presence or absence of Na⁺. The rate of uptake was nearly two times higher from the apical than basolateral fluid for all concentrations studied (Table 1). At 1, 2.5, and 50 μM unlabeled l-cystine, removal of Na⁺ caused a significant decrease in 14C-l-cystine uptake from apical fluid, suggesting the presence of a high-affinity Na⁺-dependent uptake process or processes (Fig. 2). Uptake from apical fluid was predominantly Na⁺-independent at 200 μM unlabeled l-cystine. Kₘ for uptake of l-cystine from apical fluid was 4.7 and 203 ± 10.5 μM in the presence and absence of Na⁺, respectively. Kₘ for uptake of l-cystine from basolateral fluid was 52 ± 3.6 and 1305 ± 285.6 μM in the presence and absence of Na⁺, respectively. Corresponding Vₘₐₓ for Na⁺-dependent l-cystine uptake was 14 ± 0.4 and 7.8 ± 0.2 pmol/min per milligram protein, whereas Vₘₐₓ for Na⁺-independent l-cystine uptake was 29 ± 0.8 and 65.5 ± 10.2 pmol/min-per milligram protein from apical and basolateral fluids, respectively. By contrast, basolateral uptake measured at 1 and 200 μM l-cystine did not exhibit significant Na⁺ dependency. TEERs observed before (1000 ± 150 Ω × cm²) and after (950 ± 125 Ω × cm²) the 10-minute uptake studies in the presence of Na⁺ were not altered. Similar observations were noted for uptake studies performed in the absence of Na⁺ (950 ± 100 vs. 900 ± 100 Ω × cm²).

TRANSEPIHELIAL PERMEABILITY OF L-CYSTINE

In the presence of Na⁺, the a-to-b permeability of l-cystine was nearly two times higher than the b-to-a permeability (Table 2). In contrast, the permeability in the b-to-a direction was twice that in the a-to-b direction in the absence of Na⁺. All l-cystine Pₐₐₜ was about three orders of magnitude greater than that for mannitol, a paracellular transport marker (Table 2). As would be expected, mannitol did not exhibit asymmetry in the presence or absence of Na⁺. Both Jₐₐₜ (unidirectional flux in the a-to-b direction) and Jₐₐₜ (unidirectional flux in the b-to-a direction) of l-cystine in the presence of physiological concentrations of Na⁺ (~135 mM) were 247 pmol (h × cm²) and 96 pmol (h × cm²), respectively, at approximately 11 μM (l-cystine concentration in treated fluid, Cₛₜ, due to combined masses of labeled and unlabeled l-cystine) in either apical or basolateral donor fluid, yielding net flux (Jₐₜ − Jₐₜ) of l-cystine of 151 pmol (h × cm²) in the a-to-b direction. These data suggest the polarized presence of several carrier-mediated l-cystine transport systems in the apical and basolateral membranes of primary cultured RCEC layers. The integrity of the epithelial barrier was maintained between 800 and 1000 Ω × cm² during the flux experiments.

EFFECT OF SNAP TREATMENT ON INTRACELLULAR GSH LEVELS AND GCS EXPRESSION IN RCECs

The effect of 24 hours incubation of various concentrations of SNAP on intracellular GSH levels expressed as the percentage increase over the untreated condition is shown in Figure 3. Total

CHOLINE CHLORIDE BUFFER was also linear up to 10 minutes (data not shown).

CONCENTRATION AND Na⁺ DEPENDENCY OF L-CYSTINE UPTAKE

Uptake rate, expressed as picomoles per minute per milligram protein, increased with increasing L-cystine concentrations in either apical or basolateral fluid in the presence or absence of Na⁺. The rate of uptake was nearly two times higher from the apical than basolateral fluid for all concentrations studied (Table 1). At 1, 2.5, and 50 μM unlabeled l-cystine, removal of Na⁺ caused a significant decrease in 14C-l-cystine uptake from apical fluid, suggesting the presence of a high-affinity Na⁺-dependent uptake process or processes (Fig. 2). Uptake from apical fluid was primarily Na⁺-independent at 200 μM unlabeled l-cystine. Kₘ for uptake of l-cystine from apical fluid was 48 ±

| Table 2. Transcellular Permeability of L-Cystine across RCECs |
|------------------|------------------|------------------|
|                  | Apical-to-Basolateral | Basolateral-to-Apical |
| Na⁺              | (Pₐₜ × 10⁻⁷ cm/sec) | (Pₐₜ × 10⁻⁷ cm/sec) |
| Cystine          | 274 ± 28*†§       | 107 ± 8          |
|                  | 193 ± 10          | 381 ± 19§       |
| Mannitol         | 2.10 ± 0.15       | 2.49 ± 0.11      |

Transport of l-cystine (2.5 μCi/ml 14C-l-cystine and 2.5 μM unlabeled l-cystine) was studied across day 7 RCEC layers with TEER ≈ 1000 ± 100 Ω × cm². *, measured in the NaCl buffer; †, measured in the choline chloride buffer; §, measured in both NaCl and choline chloride buffers. Data are mean ± SEM. (n = 4).

Significantly greater than:

* Apical-to-basolateral permeability under Na⁺ condition.
† Basolateral-to-apical permeability under Na⁺ condition.
§ Apical-to-basolateral permeability under Na⁺ condition.
|| Mannitol permeability observed in either direction.

All comparisons were made by one-way ANOVA with post hoc tests based on a modified Fisher least-squares difference approach.
intracellular GSH level (GSH/oxidized GSH [GSSG]) in control primary cultured RCECs was 21 nmol/mg protein. GSH levels in RCECs increased to 270% of control after treatment with 0.10 mM SNAP for 24 hours. The maximum increase occurred at 0.25 to 0.5 mM SNAP (850% increase in GSH), whereas the GSH level decreased after 1 mM treatment with SNAP to the level observed for 0.1 mM SNAP (Fig. 3). Soluble thiols from control and SNAP-treated RCEC layers were processed as previously described. HPLC analysis revealed that the increases were specifically for reduced GSH, whereas the levels of the oxidized form GSSG remained unaltered (at ~0.75% of total GSH) with treatment of SNAP at all concentrations (data not shown).

The expression of HS and LS of GCS was determined by Western blot analysis of cell lysate proteins obtained from control cells and RCECs treated for 24 hours with 0.25 mM SNAP, the concentration that caused the highest increase in intracellular GSH levels (Fig. 3). The expression of both GCS subunits was increased significantly after treatment with 0.25 mM SNAP (Fig. 4, right). Quantitation by computer (Scion Image for Windows; Scion Corp., Frederick, MD) showed that both heavy and light subunits showed similar increases in expression, reaching approximately 2.5-fold above control levels (Fig. 4), after treatment with 0.25 mM SNAP for 24 hours.

Effect of SNAP on l-Cystine Uptake

We also studied the effect of 24 hours of SNAP treatment on Na+-dependent and -independent uptake of l-cystine from the apical and basolateral fluid of RCECs. These experiments were conducted in an NaCl or choline chloride buffer after treating the cells with 0.1, 0.25, 0.5, 1.0, and 2.0 mM SNAP for 24 hours. Apical uptake of l-cystine was not significantly changed with 0.1 mM SNAP, whereas it increased significantly (>6 fold) with 1 mM SNAP in both apical and basolateral fluids. By contrast, the increase of apical l-cystine uptake was only approximately twofold after treatment with 2 mM SNAP for 24 hours (Fig. 5A). The pattern of stimulation of basolateral l-cystine uptake was similar to that of apical uptake (Fig. 5B). For the treatment with 2 mM SNAP for 24 hours, the increase in basolateral uptake remained higher than fivefold. Treatment of RCECs with either a protein synthesis inhibitor (CHX) or RNA synthesis inhibitor (AD) caused the uptake rate for l-cystine after 1 mM SNAP (Fig. 6A) to decline to that of the untreated control. Basolateral uptake of l-cystine was also increased by SNAP, although the SNAP sensitivity was different from that observed for apical l-cystine uptake, in that a significant increase in basolateral l-cystine uptake was observed even at 0.1 mM SNAP (not shown), and a nearly fivefold increase occurred with 1 mM SNAP (Fig. 6B). SNAP-induced stimulation of basolateral l-cystine uptake was blocked similarly by either CHX or AD. The pattern of SNAP-induced increase in the uptake rate of l-cystine from apical fluid observed in the absence of Na⁺ (Fig. 6C) was almost identical with that observed in the presence of...
apical fluid was induced by treatment with an NO donor, SNAP. Only the Na\(^+\)-dependent component of basolateral L-cystine uptake was significantly stimulated with SNAP. The NO donor-mediated stimulation of L-cystine uptake resulted in increases in both intracellular GSH level and expression of GCS-HS and -LS proteins.

Biosynthesis of GSH from cysteine is the rate-limiting step, because the intracellular concentrations of the other two precursors, glycine and glutamate, are in several millimolar ranges. Cysteine is cytotoxic, whereas its disulfide form of L-cystine is not.\(^{28}\) Moreover, cystine is found inside or outside cells as the more abundant molecular species of the two.\(^{29}\) Availability and uptake of L-cystine are essential for de novo biosynthesis of GSH. Several transport systems have been reported as carriers of L-cystine in mammalian cells,\(^{6,27}\) which may play important roles in GSH metabolism. As mentioned in the introduction, rabbit conjunctival epithelial cells contain millimolar concentrations of GSH and possess the enzymatic machinery to biosynthesize GSH from cysteine,\(^{8,9}\) although information on regulation of L-cystine transport in conjunctival epithelial cells was not available to date.

Our findings show that L-cystine is transported by both Na\(^+\)-dependent and -independent processes in the rabbit conjunctiva. Concentration-dependent uptake of L-cystine from both apical and basolateral fluids was also found. Our data suggest the presence of a high-affinity Na\(^+\)-dependent uptake system for L-cystine (\(K_m\) of 48 ± 4.7 \(\mu\)M and a \(V_{max}\) of 14 ± 0.41 pmol/min per milligram protein) only on the apical cell membranes of primary cultured RCEC layers. Transepithelial flux measurements indicated net absorption of L-cystine. Greater apical uptake of L-cystine occurred in the presence and absence of Na\(^+\) at the concentration (≈11 \(\mu\)M) used in transepithelial flux measurements. In the absence of Na\(^+\), transepithelial flux measurements indicated a net secretion of L-cystine. The mechanism of this Na\(^+\)-dependent asymmetry in unidirectional L-cystine flux is uncertain. Essentially, the net absorption component should prevail under physiological situations. The Na\(^+\)-independent b-to-a permeability of L-cystine becomes greater, because Na\(^+\)-dependent reuptake on the apical side was reduced greatly at 11 \(\mu\)M L-cystine. Molecular characterization of various L-cystine transporters expressed at apical and basolateral membranes of primary cultured RCEC layers should be performed. Such studies are likely to substantiate our present findings. Our results on kinetic parameters and net absorption of L-cystine are similar to those reported for the L-cystine transport processes in a mouse brain endothelial cell line,\(^{30}\) luminal membrane of jejunal epithelial cells,\(^{31}\) and renal tubular cells.\(^{32}\)

Apically, L-cystine may be formed from cysteine produced from the hydrolysis of GSH released from cells by the ectoenzyme GGT. Subsequently, L-cystine is taken up by RCECs to be incorporated into GSH, completing the GSH cycle.\(^{1,2}\) The human tear film GSH and L-cysteine concentrations are between 76 and 107 \(\mu\)M and 13 and 49 \(\mu\)M, respectively, when measured in basal tears collected by Schirmer strips.\(^{33}\) In this context, recent work from other laboratories suggests that GGT may also play a role in oxidant stress, because the expression of this ectoenzyme was upregulated in certain pathologic conditions including glutathionuria, glutathionemia, mental retardation, and oxidant-induced cell death.\(^{34,35}\) We have recently verified the baseline expression of GGT in freshly excised rabbit conjunctival tissue and in primary cultured RCECs.\(^{3}\) The exact role of GGT in conjunctival epithelial cells remains to be characterized.

Studies in other cells and tissues with several amino acids and analogues that are known substrates or specific inhibitors of amino acid transport systems revealed that L-cystine is car-

### DISCUSSION

For the first time, we have obtained data on transport of L-cystine, regulation of its uptake, and the resultant GSH biosynthesis in primary cultured rabbit conjunctival epithelial cells. Our data indicate that L-cystine was transported by multiple carrier proteins in the conjunctiva as in other cells. Net absorption of L-cystine in the a-to-b direction was observed under physiological conditions. A significant stimulation of both Na\(^+\)-dependent and -independent L-cystine uptake from apical fluid was induced by treatment with an NO donor, SNAP. Only the Na\(^+\)-dependent component of basolateral L-cystine uptake was significantly stimulated with SNAP. The NO donor-mediated stimulation of L-cystine uptake resulted in increases in both intracellular GSH level and expression of GCS-HS and -LS proteins.

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Apically, L-cystine may be formed from cysteine produced from the hydrolysis of GSH released from cells by the ectoenzyme GGT. Subsequently, L-cystine is taken up by RCECs to be incorporated into GSH, completing the GSH cycle.\(^{1,2}\) The human tear film GSH and L-cysteine concentrations are between 76 and 107 \(\mu\)M and 13 and 49 \(\mu\)M, respectively, when measured in basal tears collected by Schirmer strips.\(^{33}\) In this context, recent work from other laboratories suggests that GGT may also play a role in oxidant stress, because the expression of this ectoenzyme was upregulated in certain pathologic conditions including glutathionuria, glutathionemia, mental retardation, and oxidant-induced cell death.\(^{34,35}\) We have recently verified the baseline expression of GGT in freshly excised rabbit conjunctival tissue and in primary cultured RCECs.\(^{3}\) The exact role of GGT in conjunctival epithelial cells remains to be characterized.

Studies in other cells and tissues with several amino acids and analogues that are known substrates or specific inhibitors of amino acid transport systems revealed that L-cystine is car-
ried by more than one transport process. L-Cystine is not thought to be transported by A, ASC, and L amino acid transporters.\textsuperscript{36} L-Arginine, a substrate for B\textsubscript{0},H\textsubscript{1}001 and b0\textsubscript{1},H\textsubscript{1}001, has been shown to inhibit L-cystine uptake significantly,\textsuperscript{37} suggesting that these two amino acids may share some common transport mechanisms. Furthermore, the half maximal concentrations for L-arginine transport (i.e., high- and low-affinity processes) in conjunctiva are in a similar range (\(K_{m}\) of 50 M and 1 mM, respectively) to those for L-cystine transport reported herein.

Oxidation-based modulation of biochemical parameters is being widely examined. Among the compounds that release NO, Na\textsuperscript{+} nitroprusside (SNP), SNAP, and other S-nitrosothiols have received great attention. \textsuperscript{38} S-nitrosothiols are thermodynamically and photolytically unstable compounds. \textsuperscript{39} It has been shown that nitric oxide formation from SNAP is high compared with that induced by other S-nitrosothiols (100 \(\mu\)M of SNAP releases approximately 1.4 \(\mu\)M NO/min at 37°C), and is linear over a wide concentration range. \textsuperscript{39} Our finding that intracellular GSH level of RCECs increased with 24 hours of SNAP treatment is consistent with similar studies in vascular smooth muscle cells and human retinal pigmented epithelial cells.\textsuperscript{12} The increase in cellular GSH level after oxidant stress may be a compensatory mechanism for scavenging nitrogen-based free radicals\textsuperscript{12}; we demonstrated that the expression of GCS, the key enzyme of GSH biosynthesis, was significantly elevated in SNAP-treated RCECs compared with the control. Whether transcriptional and/or translational regulation of other GSH-related enzymes (such as GSH peroxidase, GSH transferase, and GSSG reductase) in RCECs accompanies changes in GCS under conditions of oxidant stress, remains unknown.

Treatment of RCECs with either a protein synthesis inhibitor (CHX) or an RNA synthesis inhibitor (AD) caused the rate of L-cystine uptake to decline to that of the untreated control. The exact mechanisms (e.g., transcriptional and/or translational regulation) for GCS expression remain to be determined. One feature of the SNAP-induced changes in L-cystine uptake and intracellular GSH level is that the concentration of SNAP needed to produce a maximal increase in L-cystine uptake was higher than that needed for a maximal increase in intracellular GSH level. In addition, the stimulation of L-cystine uptake rate and increase in GSH levels were both less effective at the highest concentration of SNAP used (2 mM). The mechanism of this phenomenon is not clear, although Lander et al.\textsuperscript{40} found that treatment of human peripheral blood mononuclear cells with a wide range of SNAP concentrations leads to similar responses in glucose transport. SNAP has been found to be less effective at en-
hancing glucose uptake at higher concentrations (≥1 mM) than at its lower concentrations. These investigators ascertained by trypan blue exclusion studies that the lesser enhancement of glucose uptake at higher [SNAP] is not due to increased cytotoxicity rendered by SNAP. We found that both apical and basolateral l-cystine uptake increased after SNAP treatment of RCECs. In the presence of Na⁺ in the incubation buffer, this increase occurred from both apical and basolateral fluids. In contrast, in the absence of Na⁺ only the apical, but not basolateral, rate of l-cystine uptake was stimulated in a pattern similar to those observed in the presence of Na⁺. This latter finding suggests that various Na⁺-dependent and -independent l-cystine transporters (XAG, B₀, b⁰,⁰, X₄₋) may all be involved in the regulation of l-cystine uptake. We speculate that X₄₋ and/or b⁰,⁰ (Na⁺-independent processes), in addition to XAG and/or B₀ (Na⁺-dependent processes) are upregulated for the apical l-cystine uptake. Basolaterally, by contrast, XAG and/or B₀,⁰,⁰ perhaps are upregulated. Further studies are needed to determine the relative contributions of these transport systems to the elevation of cellular GSH due to oxidant stress.

In conclusion, we have obtained evidence for Na⁺-dependent and -independent processes for l-cystine uptake in conjunctival epithelial cells and net absorption of l-cystine across the primary conjunctival epithelial barrier. l-Cystine uptake was stimulated by SNAP-induced oxidant stress, yielding increased cellular GSH levels. This response is probably one of the underlying adaptive cellular defense mechanisms perhaps common to several pathologic conditions of conjunctiva and other ocular diseases involving oxidant injury and stress.

Acknowledgments

The authors thank Bin Ouyang for skillful technical assistance and Terrence Cavanagh (University of Washington, Seattle, WA) for kindly providing the rabbit polyclonal antibodies for GCS-LS and -HS.

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