Iron Regulates L-Cystine Uptake and Glutathione Levels in Lens Epithelial and Retinal Pigment Epithelial Cells by Its Effect on Cytosolic Aconitase

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PURPOSE. The authors previously published the novel finding that iron regulates L-glutamate synthesis and accumulation in the cell-conditioned medium (CCM) by increasing cytosolic aconitase activity in cultured lens epithelial cells (LECs), retinal pigment epithelial (RPE) cells, and neurons. The present study was designed to determine whether iron-induced L-glutamate accumulation in the CCM regulates L-cystine uptake and glutathione (GSH) levels through the aconitase pathway in LECs and RPE cells.

METHODS. The presence of xCT, the light chain of Xc Metadata, a glutamate/cysteine antipporter, was analyzed by RT-PCR, immunoblotting, and immunocytochemistry. Uptake of L-[35S]cysteine and L-[3H]glutamate was measured in the presence or absence of transporter inhibitors. L-cystine uptake and intracellular GSH concentration were measured in the presence or absence of iron-saturated transferrin, the iron chelator dipyridyl (DP), or oxalomalic acid (OMA), an aconitase inhibitor.

RESULTS. LECs and RPE cells express xCT, as evidenced by RT-PCR analysis and immunoblotting. xCT was localized by immunocytochemistry. The authors found that the iron-induced increase in L-glutamate availability increased L-cystine uptake, with subsequent increases in GSH levels. In addition, L-glutamate production, L-cystine uptake, and GSH concentration were inhibited by OMA and DP, indicating a central role for iron-regulated aconitase activity in GSH synthesis in LECs and RPE cells.

CONCLUSIONS. These results demonstrate for the first time that iron regulates L-cystine uptake and the downstream production of GSH in two mammalian cell types. It is possible that the increase in intracellular antioxidant concentration induced by iron serves as a protective mechanism against the well-established capacity of iron to induce oxidative damage. (Invest Ophthalmol Vis Sci. 2008;49:310–319) DOI:10.1167/iovs.07-1041

Cytosolic aconitase (c-aconitase) is a dual-function protein found in all mammalian cell types studied. When iron is scarce, c-aconitase functions as an iron regulatory protein (IRP) controlling the translation of numerous proteins. However, when iron is abundant, the IRP attains aconitase activity. The function of c-aconitase was unknown until we demonstrated that it regulates L-glutamate production. We found that cultured lens epithelial cells (LECs), retinal pigment epithelial (RPE) cells, and neurons synthesize and secrete L-glutamate and that this process is regulated by iron by way of its effect on c-aconitase (Fig. 1). This established for the first time a metabolic link between iron and the neurotransmitter L-glutamate, each of which is dysregulated in a number of neurologic and retinal degenerations.

It was the purpose of this study to define the localization of the cystine/glutamate antipporter Xc Metadata and its activity in the lens and retinal pigment epithelium. In addition, it was critical to determine whether iron regulates L-cystine uptake and the downstream production of glutathione (GSH) by stimulation of L-glutamate synthesis through the aconitase pathway. GSH is one of the most important intracellular antioxidants, and iron is known to catalyze oxidative damage. Therefore, one of the critical roles of iron may be to participate in cellular defense against its own potential oxidative damage.

The ubiquitous tripeptide GSH (L-glutamyl-L-cysteinyl-glycine) is essential to many physiological processes. As a potent antioxidant, GSH maintains enzymes and protein thiois in their reduced state and scavenges free radicals and other reactive oxygen species. This reducing power is particularly important in the lens, which is continuously exposed to free radicals and reactive oxygen species generated from the aqueous humor, lens mitochondria, NADPH oxidase, peroxisomes, and inflammatory cytokines and by exposure to ultraviolet light, ionizing radiation, and chemotherapeutic agents.

Of all the retinal cell types, RPE cells are the most susceptible to oxidative damage because of their proximity to the choriocapillaries. The epithelium is also the site of melanin synthesis and phagocytosis of polyunsaturated fatty acids from shed photoreceptor cell membranes; hence, the need for reducing power is great given that these processes result in the formation of free radicals.

GSH synthesis is dependent on its amino acid precursors L-glutamate, L-glycine, and L-cysteine. Of the three, L-cysteine has been identified as rate limiting in GSH synthesis in primary human RPE cells, primary rat astrocytes, retinal ganglion cells, human lens epithelial cells (LECs), and retinal Müller cells (Lou MF, et al. IOVS 1995;36:ARVO Abstract 4063). Potentially, the rate-limiting substrate is system Xc Metadata. The antipporter has been identified in many cell types, including human RPE, human fibroblasts, rat alveolar type II cells, rat LECs, human macrophages, and fetal rat brain cells. L-cysteine enters the cell, it is likely reduced to two L-cysteine residues that are available for incorporation into protein or GSH synthesis.

In the present study, we attempted to determine why epithelial cells secrete L-glutamate and whether this process is
regulated by iron. We hypothesized that L-glutamate is transported by \( \text{X}_{\text{c}} \) in exchange for L-cystine. Elevated levels of intracellular L-glutamate, resulting from iron-induced aconitase activity, may stimulate antipporter activity because of the increased intracellular L-glutamate available for exchange with extracellular L-cystine. As a result, GSH production may be stimulated because of the increased availability of L-cysteine.

**Materials and Methods**

**Tissue Culture**

Dogs were obtained from Johnston County Animal Shelter in North Carolina after they were euthanized. Eyes were removed from the orbits within 3 hours of death and were cut 7 to 8 mm posterior to the limbus. Lenses were dissected free from the anterior portion of the globe. The anterior lens capsule with adherent epithelial cells was removed from the lens and placed in 10-cm tissue culture plates containing Dulbecco’s modified Eagle medium (Invitrogen, Rockville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% antibiotic antimycotic solution (Mediatech, Herndon, VA) at 37°C in a humidified environment containing 5% CO₂ and 95% humidity. Generally, cells from each eye filled a 10-cm plate. Cultures were used for all experiments unless otherwise noted.

To culture canine RPE cells, the posterior section of the globe was placed in a cup-shaped dissection dish to maintain the normal shape of the globe. After removal of the vitreous and the retina, Hanks’ balanced salt solution without calcium or magnesium (catalog no. 21-022-CV; Mediatech) but supplemented with 0.05% Trypsin and 0.53 mM EDTA (catalog no. 21-022-CV; Mediatech) was added to the exposed RPE cells. After incubation for 10 minutes at 37°C, patches of retinal pigment epithelium were lifted and transferred to a centrifuge tube containing Ham’s F12 - Dulbecco’s modified Eagle medium (1:1; Invitrogen) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT) and 1% antibiotic antimycotic solution (Mediatech), which was then incubated at 37°C in 95% air, 5% CO₂, and 95% humidity. Generally, cells from each eye filled a 10-cm plate. Cultures became confluent after 6 to 10 days. At that point, the cells were trypsinized and plated in six-well clusters for experiments. Confluent cultures were used for all experiments unless otherwise noted.

**Reverse Transcription–PCR for Identification of xCT mRNA**

Total RNA was isolated from primary cultured canine LECs and RPE cells and ARPE-19 cells (human RPE) using the RNeasy Mini Kit (Qiagen, Valencia, CA). Mouse brain, mouse spinal cord, and human retina total RNA were purchased from BD Biosciences Clontech (Mountain View, CA). Five micrograms total RNA from canine LECs, canine RPE cells, and ARPE-19 cells and 1 μg total RNA from mouse brain, mouse spinal cord, and human retina were used for RT-PCR (Ready-to-Go RT-PCR beads; Amersham Biosciences) in a 50-μL reaction. PCR amplification of the cDNA was performed with 40 cycles of 1-minute denaturation at 95°C, 1-minute annealing at 58°C, and 2-minute extension at 72°C. PCR products were electrophoresed on 2.5% agarose gel in the presence of ethidium bromide and were visualized under ultraviolet light. Primers for xCT amplification were 5'-CTTCGGCATTTGGAGCCTACAT-3' and 5'-TCTGAATTTCTGAGCTTGA-3'. Expected product size was 182 bp.
Immunoblotting for Identification of xCT Protein

Membrane fractions were prepared from primary cultured canine LECs, RPE cells, and ARPE-19 cells using SDS solubilization buffer consisting of 2% SDS, 1% diethiothreitol, and 1% 2-(N-cyclohexylamino) ethanesulfonic acid. Cells were washed in PBS and homogenized in SDS solubilization buffer containing 10 μL/mL phenylmethylsulfonyl fluoride (57 mM) and 6 μL/mL protease inhibitor cocktail (Sigma). The homogenate was spun for 1 hour at 200,000g at 20°C. The resultant supernatant containing the membrane fraction was analyzed by immunoblotting. Mouse brain tissue extract was used as a positive control (Stressgen Biotechnologies, Victoria, Canada). Twenty micrograms total protein from mouse brain tissue extract and primary cultured canine LECs, canine RPE cells, and ARPE-19 cells were subjected to 10% SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk powder in Tris-buffered saline containing Tween 20 (TBST) and then probed with antibodies against xCT (1:500; TransGenic Inc., Kumamoto, Japan). After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibodies (1:5000; Alpha Diagnostic, San Antonio, TX). Proteins were visualized using the enhanced chemiluminescence detection system (Amersham) according to the manufacturer’s protocol.

Immunolocalization of xCT in Lens and RPE

Anterior segments of eyes containing intact lenses and posterior eye cups from dogs 6 months to 8 years old were fixed in 4% formaldehyde in 1× calcium- and magnesium-free Dulbecco phosphate-buffered saline (DPBS) at room temperature for several hours and then stored at 4°C until sectioned. Lenses were removed from the anterior portions, rinsed in DPBS, and cut into 40-μm sections (Vibratome; TPI, St. Louis, MO) to yield sections parallel and perpendicular to the visual axis. Sections were collected in Netwell (Corning, Inc., Corning, NY) membrane inserts containing 1× PBS and stored at 4°C until use. For isolated fiber cells, lenses from 4-month-old dogs were fixed in 4% formaldehyde in 1× DPBS for 2 to 4 hours at room temperature and then stored in 1% formaldehyde in 1× DPBS at 4°C. Cortical fiber cells were dissected in larger clumps using a stereomicroscope and forceps, gently teased apart into individual fibers, and processed for xCT labeling in the same manner as lens sections. For retinal/RPE sections, tissue pieces of sclera, chorioid, retinal pigment epithelium, and retina were processed for standard paraffin sections. These sections and isolated fibers were permeabilized with 0.2% Triton X-100 in PBS for 25 minutes, blocked in 5% normal goat serum in PBS for 1 hour, and incubated with rabbit polyclonal antibodies to xCT (2.5 μg/mL; TransGenic, Inc.) diluted in PBS containing 0.1% IgG-free bovine serum albumin (BSA; Jackson ImmunoResearch Laboratories, West Grove, PA) at 4°C for 18 hours. Negative controls included normal rabbit IgG (Sigma) in place of xCT antibodies at the same protein concentration. For RPE labeling, mouse antibodies to the retinal pigment epithelium-specific marker RPE65 (2.2 μg/mL; Novus Biologicals, Littleton, CO) were applied to the retina/RPE sections along with xCT antibodies. As a negative control, serial sections were incubated with mouse IgG at the same concentration. After PBS washes, the sections were incubated for 2 hours with fluorescent dye-conjugated anti-rabbit secondary antibodies (5 μg/mL; Alexa Fluor 568; Invitrogen, Carlsbad, CA) diluted in PBS. For RPE65 detection, fluorescent dye-conjugated anti-mouse secondary antibodies (5 μg/mL; Alexa Fluor 488) were included. After PBS washes, sections were mounted on slides in antifade reagent containing DAPI (Prolong Gold; Invitrogen). Sections were then imaged using a microscope (DM5000B; Leica, Wetzlar, Germany) with conventional epifluorescence and DIC optics. Images were captured with a cooled CCD camera (Retiga 1300; QImaging, Surrey, BC, Canada) and imaging software (Simple PCI; Compix, Inc., Sewickley, PA) and then arranged (Photoshop CS2; Adobe, San Jose, CA).

L-[35S]Cystine and L-[3H]Glutamate Uptake

L-[35S]Cystine and L-[3H]Glutamate uptake experiments were performed in either Na+-dependent or Na+-independent uptake buffer. Under Na+-dependent conditions, LECs or RPE cells were incubated in uptake buffer, as previously described by Rimaniol et al.17 For Na+-independent studies, an equimolar concentration of choline chloride was substituted for NaCl. Cells were labeled with 5 μL/L [35S]cystine or 2 μL/L [3H]glutamate in the presence or absence of inhibitors and incubated for 2 minutes at 37°C in 5% CO2. Uptake was terminated by removing the uptake buffer and washing the cells three times with ice-cold DPBS. Cells were harvested using 10 mM Tris buffer containing 10 μL/mL phenylmethylsulfonyl fluoride and 6 μL/mL protease inhibitor cocktail. After 20-minute incubation on ice, cells were centrifuged for 5 minutes at 4°C at 14,700g. Ten microliters of the resultant supernatant was transferred to a vial containing 10 mL scintillation fluid (National Diagnostics, Atlanta, GA), and radioactivity was measured using a liquid scintillation counter (1409 Wallac; Perkin Elmer, Wellesley, MA). Incorporation of radioactivity into protein was determined by trichloroacetic acid precipitation.

Statistical Analysis

Results are presented as the mean ± SEM. Statistical analysis of data was performed (Systat, version 9.01; San Jose, CA). Unpaired t-test was used to determine the statistical significance between two group means. When analysis involved more than two means, one-way ANOVA was conducted, and the significance of differences between the means was determined using Tukey’s test. The difference in means was considered significant when P < 0.05.

RESULTS

Molecular Evidence for the Expression of System Xc− in Primary Cultured Canine LECs and RPE Cells, Canine Whole Lenses, and Human Retinal Pigment Epithelium

The xct transcript was detected by RT-PCR in cultured canine LECs and RPE cells and in the positive controls, mouse brain, mouse spinal cord, human retina, and ARPE-19 cells at the expected size of 182 bp (Fig. 2A). Western blot analysis confirmed the presence of xct at the protein level (Fig. 2B). A
FIGURE 2. Molecular expression of xCT, light chain of system X_c^−, in canine LECs and canine and human RPE cells. (A) RT-PCR products in canine LECs, mouse brain, mouse spinal cord, human retina, ARPE-19 cells (human RPE cells), and canine RPE cells using specific primers for xCT. Expected size of product was 182 bp. (B) Western blot analysis of xCT obtained from the membrane fraction of mouse brain, canine LECs and RPE cells, and ARPE-19 cells probed with antibody specific for xCT (55 kDa).

Single band (approximately 55 kDa MWt) was detected in cultured canine LECs and RPE cells and in the positive controls, mouse brain, and ARPE-19.

We investigated the distribution of xCT in different cell types and regions of the whole canine lens and retina. Fluorescent immunocolocalization studies were performed using lenses from dogs aged 3 months to 8 years. Each lens was sectioned in one of two different orientations, either parallel to the visual axis (longitudinal sections; Fig. 3A–3G) or perpendicularly to the visual axis (transverse sections; Fig. 3J–3N). In general, xCT-specific labeling was detected in epithelial and outer cortical fiber cells of the lens. Lenses from all age groups showed the strongest xCT signals in the fiber cells of the bow region and, to a lesser degree, at the posterior cortical regions of the lens. Interestingly, the intensity of xCT signals in epithelial cells showed age-related differences. In lenses from 3- to 6-month-old dogs, xCT signals were barely detectable in epithelial cells in the quiescent zone (Fig. 3A). The anterior cortical region fiber cells generally had lower levels of xCT than the other regions, in all lenses examined. xCT was not detected in the inner nuclear region of the lens.

In the long sides of the fiber cells membranes (Fig. 3N), but higher levels of xCT were observed in older lens cortical epithelial cells (similar to Fig. 3E). In older cortical fiber cells that lose their characteristic hexagonal shape and metabolic activity as they mature and are compacted into interior layers of the lens, xCT signals were observed to persist primarily at the long sides of the fiber cell membranes (Fig. 3N). These results indicate that there are striking differences in the distribution of xCT across different cell types and regions of the canine lens and in lenses of different ages.

To localize xCT in canine RPE cells, sections were incubated with both antibodies to the retinal pigment epithelium-specific marker RPE65 (green signal) to clearly identify the RPE layer (Fig. 3O) and xCT antibodies (red signal). Uniform xCT signals were detected in the cytoplasm of RPE cells (Fig. 3P) and in other retina cell layers (data not shown). The overlay of RPE65 and xCT signals (greenish-yellow signal) confirmed the colocalization of both proteins in RPE cells (Fig. 3Q). Serial sections incubated with normal rabbit serum and mouse IgG as negative controls for xCT and RPE65 antibodies showed no xCT or RPE65 labeling (Fig. 3R).

Functional Evidence for System X_c^− in Primary Cultured LECs

X_c^− has been characterized as mediating the Na^+-independent exchange of extracellular L-cystine for intracellular L-glutamate in many cell types. Thus, the uptake of L-cystine in the presence or absence of sodium was measured to determine whether uptake is dependent on sodium. Under Na^+-independent conditions, NaCl was replaced by an equimolar concentration of choline chloride. Our results indicate that L-cystine uptake in cultured LECs is not dependent on sodium because uptake in the absence of sodium was not significantly different from that in the control (84% of control; Fig. 4A).

Competition experiments were performed to determine the substrate specificity of the transporters mediating L-cystine uptake in cultured LECs. L-cystine uptake was measured in the presence or absence of various unlabeled amino acids. Excess unlabeled L-glutamate and L-cystine inhibited L-cystine uptake in LECs to 55% and 60% of control, respectively (Fig. 4A). L- and D-aspartate, substrates of the Na^+-dependent systems X_A^− and excitatory amino acid transporter (EAAT) inhibitors, failed to significantly inhibit L-cystine uptake (Fig. 4A). In addition, L-arginine and L-alanine, substrates of the Na^+-independent B^0^ system and the Na^+-dependent B^0^ system, and L-serine, substrate for the Na^+-dependent system alanine-serine-cysteine (ASC), had no significant effect on L-cystine uptake (Fig. 4A). These results indicate that systems X_A^−, EAAT1–5, B^0^, B^0^, and ASC are not primarily involved in L-cystine uptake in cultured LECs.
FIGURE 3. The glutamate/cystine antiporter \(X_c^-\) is localized to LECs, lens fiber cell membranes, and RPE cells. xCT antibodies and fluorescent secondary antibodies were used to visualize the xCT subunit of the antiporter (red). DAPI staining shows nuclei of epithelial cells and young fiber cells (blue). Vibratome sections were cut in parallel to the visual axis (longitudinal sections) and are from 6-month-old dogs (A, B, D, F-G) and
Iron Regulates L-Cystine Uptake and GSH Levels

Next, the effect of various transporter inhibitors on L-cystine uptake was evaluated. $X^\text{−}$ competitive inhibitors, Quis, CPG, and HCA significantly inhibited L-cystine uptake to approximately 70% of control (Fig. 4B). DHK, a nontransportable inhibitor of EAAT2 (GLT-1), and trans-PDC and THA, nontransportable inhibitors of EAAT5, failed to significantly inhibit uptake (Fig. 4B). Collectively, these results demonstrate that system $X^\text{−}$ is primarily responsible for the uptake of L-cystine in cultured LECs.

In the presence of high extracellular levels of L-glutamate, $X^\text{−}$ can function in reverse; therefore, L-glutamate uptake in the absence of sodium was investigated. The $Na^\text{+}$-independent uptake of L-glutamate exhibited the same characteristics as L-cystine uptake. Uptake of L-glutamate occurred in the absence of sodium and was inhibited by excess unlabeled L-cystine and L-glutamate by 35% and 47%, respectively (Fig. 5). Quis decreased the $Na^\text{+}$-independent uptake of L-glutamate to 46% of control (Fig. 5). The EAAT inhibitors DHK, THA, and trans-PDC failed to significantly inhibit L-glutamate uptake, indicating that EAAT1–5 and XAG are not involved in the $Na^\text{+}$-independent uptake of L-glutamate in cultured LECs (Fig. 5).

an 8-year-old dog (C, E). Fiber cells (H, I) were isolated from fixed lenses. Some images (H–N) contain differential interference contrast (DIC) overlay information to provide additional structural information. Sections (J–N) were cut perpendicularly to the visual axis at the equator (transverse equatorial sections) and are all from a 6-month-old dog. The retina/RPE sections (O–R) are from a 5-year-old dog and was simultaneously labeled for xCT (red), as described, and with RPE65 antibodies followed by AlexaFluor 488 antibodies (green). (A) Representative longitudinal lens section showing the different regions of the lens and the pronounced cortical band of xCT-specific signal. (B) Section from the quiescent epithelial zone and anterior cortex of a 6-month-old dog. (C) Section from similar anterior region as in (B) but from an 8-year-old dog. (D) Section from the epithelial differentiation/division and cortical regions of a 6-month-old dog. (E) Section from similar region as in (D) but from an 8-year-old dog. (F) Serial section from 8-year-old dog similar to the one shown in (E) but incubated with normal rabbit IgG in place of xCT antibodies. (G) Nucleated fiber cells underlying the epithelium in the bow region of a 6-month-old lens. (H) Two nonlabeled cortical fiber cells teased apart; DIC image to show detail of the interlocking lateral interdigitations (arrows) between cells. (I) Cluster of fiber cells similar to those in (H) but labeled for xCT (arrows). (J) Transverse equatorial control section incubated with normal rabbit IgG in place of xCT antibodies. (K) Serial section similar to (J) but labeled for xCT. (L) Higher magnification of an area similar to that outlined (1) in (K), showing epithelial and adjacent cortical fiber cells. (M) Higher magnification of an area similar to that outlined (2) in (K), further toward the center of the cortex. (N) Higher magnification of an area similar to that outlined (3) in (K), corresponding to deeper cortical cells. (O) Retina/RPE tissue section labeled for the RPE-specific marker RPE65 (green). (P) Same section as that of (O) showing xCT signal (red). (Q) Overlay of red and green channels showing colocalization (yellow) of RPE65 and xCT in RPE cells. (R) Control retina/RPE tissue section overlay similar to (Q) but incubated with normal mouse IgG and normal rabbit IgG in place of RPE65 and xCT antibodies, respectively. e, epithelial cell; cf., cortical fiber cell; n, cell nucleus; RPE, retinal pigment epithelium.
exchange with L-cystine via Xc^- activity, which is consistent with c-aconitase-derived L-glutamate. L-glutamate is involved in cystine/glutamate exchange. After 1-hour incubation at 37°C for 1 hour or 6 hours in serum-free, L-glutamine-free MEM in the presence or absence of OMA (5 mM). The effect on L-glutamate accumulation in CCM (1 hour), L-cystine uptake (1 hour), and GSH synthesis (6 hours) were measured. Each histogram bar represents mean ± SE of at least six samples. *P < 0.05, **P < 0.001, significantly different from control. Statistical tests for all data included ANOVA and Tukey’s test. L-glu, L-glutamate; GSH, glutathione; OMA, oxalomalic acid.

C-Aconitase–Regulated L-Glutamate Accumulation in the CCM via Xc^- in Cultured LECs and RPE Cells

We have previously shown that the c-aconitase inhibitor OMA decreases L-glutamate accumulation in the CCM of cultured LECs and RPE cells after 24 hours. In the present study, OMA decreased L-glutamate accumulation in the CCM by 38% and 27% in cultured LECs and RPE cells, respectively, after 1 hour (Fig. 6). We next examined whether c-aconitase-driven L-glutamate is involved in cystine/glutamate exchange. After 1-hour exposure to OMA, L-cystine uptake was significantly reduced by 28% and 37% in cultured LECs and RPE cells, respectively, which is consistent with c-aconitase-derived L-glutamate exchanging with L-cystine via Xc^- (Fig. 6).

Iron-Regulated L-Glutamate Accumulation in the CCM via c-Aconitase Controls GSH Levels in Cultured LECs and RPE Cells

Given that c-aconitase-induced L-glutamate production regulates L-cystine uptake, we investigated its effects on intracellular GSH levels because L-cystine (L-cysteine) is a rate-limiting substrate in GSH production in many cell types. After 1 hour, OMA significantly reduced intracellular GSH levels in cultured RPE cells to 58% of control (data not shown). However, no change in GSH levels was observed in cultured LECs at this time point (data not shown). After 6 hours, OMA significantly reduced intracellular GSH levels in cultured LECs and RPE cells to 81% and 56% of control, respectively (Fig. 6). These data indicate that the regulatory effect of iron on c-aconitase activity affects not only the downstream production and accumulation of L-glutamate in the CCM and L-cystine uptake but also intracellular GSH levels in both cell types.

Next, the effect of the iron chelator, DP, on L-glutamate synthesis and accumulation in CCM, L-cystine uptake and intracellular GSH levels was investigated. Exposure to the iron chelator for 1 hour significantly reduced L-glutamate accumulation in the CCM of cultured LECs (90% of control; Fig. 7). However, DP did not affect L-glutamate accumulation in cultured RPE cells at 1 hour (102% of control; data not shown) or at 6 hours (91% of control; Fig. 7). Interestingly, L-cystine uptake was significantly decreased to 71% of control in cultured LECs after 1-hour treatment with DP (Fig. 7). Exposure to DP did not produce a significant decrease in L-cystine uptake in cultured RPE cells until 6 hours, reducing uptake to 74% of control (Fig. 7). GSH concentration in both cell types also decreased significantly after treatment with the iron chelator. DP reduced the intracellular GSH levels in cultured LECs to 75% of control after 1 hour and 68% of control in cultured RPE cells after 6 hours (Fig. 7).

As we reported previously, iron, in the form of ferric ammonium citrate (FAC), increases c-aconitase activity, which results in an increase in L-glutamate synthesis and accumulation in CCM from cultured LECs, RPE cells, and neurons (Fig. 1). L-glutamate accumulation in the CCM and L-cystine uptake were also evaluated after cultured LECs and RPE cells were exposed to a more physiological source of iron. Iron-saturated transferrin (holoTf) failed to significantly increase L-glutamate accumulation in the CCM of either cell type after 1 hour (Fig. 8A). However, the intracellular L-glutamate concentration increased significantly by 51% and 30% in cultured LECs and RPE cells, respectively, after exposure for 1 hour (Fig. 8A). After 30-minute exposure, L-cystine uptake increased significantly by 48% and 32% compared with control in cultured LECs and RPE cells, respectively (Fig. 8A). Quis significantly reduced the holoTf-driven increase in L-cystine uptake by 44% in cultured LECs, demonstrating that iron increased L-cystine uptake by stimulating Xc^- activity (Fig. 8B). Interestingly, L-cystine uptake in the presence of holoTf and Quis is significantly different from Quis treatment alone, indicating that holoTf may have an effect that is partially independent of Xc^- activity (Fig. 8B). Intracellular GSH levels increased significantly to 149% and 115% of control in cultured LECs and RPE cells, respectively, after 1-hour exposure to holoTf, probably because of the increase in L-glutamate and L-cystine availability (Fig. 8C). As noted, OMA treatment alone for 1 hour in cultured LECs did not decrease the intracellular GSH concentration but did block the holoTf-induced increase (not significantly different from control; Fig. 8C). In addition, the holoTf-driven increase in intracellular GSH in cultured RPE was completely blocked by OMA (Fig. 8C). This finding clearly demonstrated that iron-driven production of GSH is a function of the effect of iron on c-aconitase activity.

FIGURE 6. The effect of the c-aconitase inhibitor, OMA, on L-glutamate accumulation in the CCM, L-cystine uptake, and intracellular glutathione concentration in cultured LECs and RPE cells. Cells were incubated at 37°C for 1 hour or 6 hours in serum-free, L-glutamine-free MEM in the presence or absence of OMA (5 mM). The effect on L-glutamate accumulation in CCM (1 hour), L-cystine uptake (1 hour), and GSH synthesis (6 hours) were measured. Each histogram bar represents mean ± SE of at least six samples. *P < 0.05, **P < 0.001, significantly different from control. Statistical tests for all data included ANOVA and Tukey’s test. L-glu, L-glutamate; GSH, glutathione; OMA, oxalomalic acid.

FIGURE 7. The effect of the iron chelator, DP, on L-glutamate accumulation in the CCM, L-cystine uptake, and intracellular GSH concentration in cultured LECs and RPE cells. LECs were incubated at 37°C for 1 hour, and RPE cells were incubated at 37°C for 6 hours in serum-free, L-glutamine-free MEM in the presence or absence of DP (300 μM). The effect on L-glutamate accumulation in CCM, L-cystine uptake, and GSH synthesis were measured. Each histogram bar represents mean ± SE of at least six samples. **P < 0.001, significantly different from control. Statistical tests for all data included ANOVA and Tukey’s test. DP, dipyridyl; L-glu, L-glutamate; GSH, glutathione.
**DISCUSSION**

Examination of the distribution of xCT in the whole lens illustrates that L-cystine and L-glutamate can likely be transported across various regions of the lens fiber mass, thereby making substrates available for GSH synthesis. The overall highest xCT signals were observed in cortical fiber cells, which is consistent with reported high levels of GSH in the lens cortex.²³,²⁴ xCT was not detected in the nuclear region of the lens. This finding is in contrast to that of Lim et al.,¹⁶ who localized xCT to the nuclear region of lenses from 21-day-old rats. Furthermore, Lim et al.¹⁶ found Xc⁻⁻⁻ labeling predominantly in the cytoplasm of peripheral fiber cells, whereas we found Xc⁻⁻⁻ primarily in the membranes of these cells. This may reflect species differences because dog lenses were used in our study.

Staining for xCT in lenses of all ages was most intense in fiber cells at the equatorial region of the lens, where epithelial cells divide and differentiate into fiber cells. Interestingly, Sweeney et al.²⁵ demonstrated that L-[³⁵S]cysteine moves into cells divide and differentiate into fiber cells. Interestingly, Sweeney et al.²⁵ demonstrated that L-[³⁵S]cysteine moves into the lens almost exclusively at the germinative region near the equator. The presence of high levels of xCT in this area suggests that high levels of antioxidants may be needed to protect actively dividing and differentiating cells from oxidative damage.

The observed higher levels of xCT in LECs from older dogs suggest the need for increased Xc⁻⁻⁻-mediated L-cystine uptake. Indeed, GSH levels in the lens decrease with age,²⁶–²⁸ and more L-cystine would be required to maintain adequate GSH levels. Accordingly, younger lenses generally have higher levels of GSH, and their LECs may not require high levels of Xc⁻⁻⁻ to maximize L-cystine uptake. However, newly formed fiber cells of young and old lenses, which are more isolated from the amino acid-enriched aqueous humor than LECs, have high xCT levels that may facilitate amino acid transport and GSH production within the lens cortex. The presence of higher levels of xCT in the posterior region of the lens compared with the anterior region may also reflect the lower levels of amino acids in the vitreous humor compared with the amino acid-rich aqueous humor²⁹,³⁰ and may indicate a requirement for higher Xc⁻⁻⁻ capacity in this region. Interestingly, strong xCT labeling was found on the lateral interdigitations of fiber cells, which are thought to contain gap junctions.³¹ The close association of gap junctions and amino acid transporters may also promote efficient cell-to-cell amino acid and GSH transport through the lens cortex. Although we found more distinct areas of xCT localization in lens fiber cells, such as at lateral interdigitations and along the cell membranes of fibers, xCT labeling in LECs and RPE cells appeared to be uniform.

The present study demonstrates that primary cultured LECs use an L-cystine uptake mechanism previously described in rat retinal Müller cells and human RPE cells and shown to be present in rat lenses.¹⁶,¹⁹,²² The uptake of L-cystine in the absence of sodium, along with results of competition studies and use of specific transporter inhibitors, indicates that L-cystine is taken up primarily by system Xc⁻⁻⁻ and not Xc⁻⁻⁻⁻⁻⁻⁻⁻. The presence of high levels of xCT suggests the need for increased xCT-mediated L-cystine uptake.

![Figure 8](image-url)

**Figure 8.** Iron increases L-glutamate synthesis and accumulation in the CCM, L-cystine uptake, and intracellular GSH concentration. (A) Effect of holoTf on L-glutamate accumulation in CCM, and lysate and L-cystine uptake in cultured LECs and RPE cells. Cells were incubated at 37°C in serum-free, L-glutamine-free MEM in the presence or absence of holoTf for 30 minutes (L-cystine uptake) or 1 hour (L-glutamate concentration in CCM and lysate). (B) Effect of holoTf and Quis on L-cystine uptake in cultured LECs. Cells were incubated at 37°C in serum-free, L-glutamine-free MEM in the presence or absence of holoTf and Quis for 30 minutes. (C) Effect of holoTf and OMA on intracellular GSH concentration in cultured LECs and RPE cells. Cells were incubated at 37°C in serum-free, L-glutamine-free MEM in the presence or absence of holoTf and OMA for 1 hour. HoloTf concentration was 720 μg/mL. Quis concentration was 100 μM. OMA concentration was 5 mM. Each histogram bar represents mean ± SE of at least six samples. (A) *P < 0.05, **P < 0.001, significantly different from control. (B) *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from all other groups. (C) **P < 0.05, significantly different from control; ***P < 0.05, significantly different from all other groups. Statistical tests for all data included ANOVA and Tukey’s test. L-glu, L-glutamate; holoTf, holotransferrin; GSH, glutathione; OMA, oxalomallic acid; Quis, quisqualic acid.
dent uptake of L-glutamate. Currently, $X_c^-$ is the only known system that transports L-glutamate under Na$^+$-independent conditions. Collectively, these results clearly support the functionality of system $X_c^-$, which is used by both L-cystine and L-glutamate, in cultured LECs. As shown in our earlier study, cultured LECs and RPE cells synthesize and secrete L-glutamate by way of a c-aconitase-regulated pathway (Fig. 1). In the present study, we investigated why epithelial cells secrete L-glutamate and whether this process is regulated by iron. Inhibition of c-aconitase by OMA reduced L-glutamate accumulation in the CCM and L-cystine uptake in cultured LECs and RPE cells, probably because of the decreased levels of L-glutamate available for exchange with L-cystine through $X_c^-$. Because L-cysteine (L-cysteine) is the rate-limiting substrate for GSH synthesis, reduction of L-glutamate availability by OMA would limit L-cystine exchange and likely affect GSH levels. Indeed, OMA significantly decreased intracellular GSH levels in both cell types. These findings are consistent with GSH production in cultured LECs and RPE cells being dependent, at least in part, on iron-driven and L-cystine-mediated L-cysteine exchange for intracellular L-glutamate. OMA may inhibit mitochondrial aconitase and c-aconitase and, therefore, make some contribution to L-glutamate secretion and glutathione levels. However, mitochondrial aconitase is a separate gene product and is not actually regulated by iron.

The bidentate iron chelator DP, shown to bind iron as it partitions through the cell membrane and to chelate iron as it is released from transferrin, was used to determine what effect reduced c-aconitase activity would have on the synthesis and accumulation in CCM of L-glutamate, uptake of L-cystine, and concentration of GSH. Exposure to DP for 1 hour decreased L-glutamate accumulation in the CCM of cultured LECs, with no significant effect observed in cultured RPE cells (Fig. 7). However, a significant reduction in L-glutamate synthesis and accumulation in CCM of cultured RPE cells were observed after a 4-hour exposure to DP, as shown in our previous study (26 ± 3.0 to 22 ± 2.6 μM; P < 0.003, paired t-test). In addition, the uptake of L-cystine and levels of GSH were decreased in both cell types after DP exposure in the present study. It is difficult to explain how the uptake of L-cystine and the concentration of GSH would decrease with no significant reduction in L-glutamate accumulation in the CCM. It is possible that not all the glutamate that appeared in the CCM was related to a decrease in the production of GSH and to subsequent oxidative damage. Based on these findings, we evaluated whether the addition of iron would result in increased L-glutamate synthesis, CCM accumulation, L-cystine uptake, and GSH levels. In our previous study we used iron in the form of FAC, which resulted in increased c-aconitase activity, synthesis, and accumulation in CCM of L-glutamate in cultured LECs, RPE cells, and neurons. In the present study, we investigated the effects of holotf, a more physiological source of iron. Exposure to holotf did not increase L-glutamate accumulation in the CCM of cultured LECs or RPE cells; however, the concentration of L-glutamate in the cell lysates of both cell types was significantly higher than in the controls. This finding suggests that there may be a protective mechanism involved in preventing the accumulation of L-glutamate in the extracellular space. Lim et al. demonstrated that $X_c^-$ and the Na$^+$-dependent L-glutamate transporters EAATs colocalize in the outer cortex of the rat lens. Other studies have demonstrated that L-glutamate transporters and system $X_c^-$ work cooperatively in human fibroblasts, HT22 cells, LRM55 glioma cells, retinal glial cells, and human macrophages, resulting in the recycling of L-glutamate.

As intracellular L-glutamate is exchanged for extracellular L-cystine by $X_c^-$, L-glutamate transporters would facilitate the reuptake of L-glutamate into the cell. In fact, our previous study demonstrated that L-glutamate accumulates in the CCM of cultured LECs and RPE cells after treatment with trans-PDC, an inhibitor of Na$^+$-dependent L-glutamate transporters, indicating the possibility that this cooperative system exists in these cell types. L-glutamate transporters working in conjunction with $X_c^-$ would explain why a significantly higher concentration of L-glutamate was measured in the cell lysates and not in the CCM, along with an increase in L-cysteine uptake after holotf treatment.

OMA inhibition of holotf effect on GSH levels in cultured RPE cells indicates that regulation of c-aconitase activity by iron not only drives the synthesis and accumulation in CCM of L-glutamate and the uptake of L-cystine by $X_c^-$ but also the downstream production of GSH.

Clearly, our novel findings indicate that the relationships among iron, L-glutamate synthesis, accumulation in CCM, and GSH levels warrant further study. For example, at which concentration or form of iron will the upregulation of $X_c^-$ activity result in an accumulation of extracellular L-glutamate? In the present study, iron bound to transferrin increased L-glutamate accumulation in the cell lysates, whereas in our previous study FAC increased L-glutamate accumulation in the CCM. In addition, it will be important to know whether iron chelators can reduce extracellular L-glutamate accumulation without depleting intracellular GSH levels. Another important question is whether $X_c^-$ and the EAATs can work together to prevent the accumulation of extracellular L-glutamate while maintaining adequate levels of GSH in the lens. This information could prove beneficial in developing new therapies and treatments for many conditions in which iron, L-glutamate secretion, and metabolism of GSH levels are dysregulated.

In conclusion, it makes physiological sense that iron, which has an enormous potential for catalyzing damaging oxidative reactions, would catalyze a cascade of reactions resulting in the production of the potent antioxidant GSH. The fact that deferoxamine, an iron chelator, causes retinal damage may be related to a decrease in the production of GSH and to subsequent oxidative damage.

References
