Published in final edited form as:
Exp Eye Res. 2012 April ; 97(1): 90–97. doi:10.1016/j.exer.2012.02.001.

Ceruloplasmin alters intracellular iron regulated proteins and pathways: Ferritin, transferrin receptor, glutamate and hypoxia-inducible factor-1α

J. Harned, **J. Ferrell**, **S. Nagar**, **M. Goralska**, **L.N. Fleisher**, and **M.C. McGahan***
Department of Molecular Biomedical Sciences, North Carolina State University, 4700 Hillsborough St., Raleigh, NC 27606, USA

Abstract

Ceruloplasmin (Cp) is a ferroxidase important to the regulation of both systemic and intracellular iron levels. Cp has a critical role in iron metabolism in the brain and retina as shown in patients with aceruloplasminemia and in Cp−/−hep−/y mice where iron accumulates and neural and retinal degeneration ensue. We have previously shown that cultured lens epithelial cells (LEC) secrete Cp. The purpose of the current study was to determine if cultured retinal pigmented epithelial cells (RPE) also secrete Cp. In addition, the effects of exogenously added Cp on iron regulated proteins and pathways, ferritin, transferrin receptor, glutamate secretion and levels of hypoxia-inducible factor-1α in the nucleus were determined. Like LEC, RPE secrete Cp. Cp was found diffusely distributed within both cultured LEC and RPE, but the cell membranes had more intense staining. Exogenously added Cp caused an increase in ferritin levels in both cell types and increased secretion of glutamate. The Cp-induced increase in glutamate secretion was inhibited by both the aconitase inhibitor oxalomalic acid as well as iron chelators. As predicted by the canonical view of the iron regulatory protein (IRP) as the predominant controller of cellular iron status these results indicate that there is an increase in available iron (called the labile iron pool (LIP)) in the cytoplasm. However, both transferrin receptor (TfR) and nuclear levels of HIF-1α were increased and these results point to a decrease in available iron. Such confounding results have been found in other systems and indicate that there is a much more complex regulation of intra-cellularly available iron (LIP) and its downstream effects on cell metabolism. Importantly, the Cp increased production and secretion of the neurotransmitter, glutamate, is a substantive finding of clinical relevance because of the neural and retinal degeneration found in aceruloplasminemia patients. This finding and Cp-induced nuclear translocation of the hypoxia-inducible factor-1 (HIF1) subunit HIF-1α adds novel information to the list of critical pathways impacted by Cp.

Keywords

ceruloplasmin; iron; lens epithelial cells; retinal pigmented epithelial cells glutamate; hypoxia-inducible factor; transferrin receptor; ferritin

1. Introduction

Ceruloplasmin (Cp) is a ferroxidase (Osaki et al., 1966), which exists as both a membrane-anchored protein and a secreted protein. These two forms are mRNA splice variants of the same gene. In some tissues 5 amino acids in the Cp protein are replaced with a 30 amino acid peptide which has a recognition site for the addition of a glycophosphoinositol (GPI)

*Corresponding author. Tel.: +1 919 515 4482; fax: +1 919 513 7301. chris_mcgahan@ncsu.edu (M.C. McGahan).

anchor targeting Cp to the outside of the cell membrane (Patel and David, 1997; Patel et al., 2000). In other tissues, the unmodified Cp is secreted. The relative amount of each protein produced is tissue specific. For example, GPI-Cp is the main form of Cp found in the brain, whereas the liver mainly produces the unmodified, secreted form. Due to its ferroxidase activity, Cp converts ferrous iron to ferric iron, the form that binds to the iron transport protein, transferrin (Tf). Tf can then transport iron to sites of utilization or storage. Tf has a very high affinity for ferric iron with two iron binding sites which are normally unsaturated. The presence of Tf in extracellular fluid will scavenge any unbound iron that may be present. Iron bound to Tf is not capable of catalyzing free radical reactions. Therefore Cp and Tf have antioxidant activities. Indeed, we found that entry of these antioxidant plasma proteins into the intraocular fluids during inflammation increased antioxidant activity in these compartments (McGahan and Fleisher, 1986; McGahan et al., 1989), and that transferrin decreased the inflammatory response (McGahan et al., 1994a). Additionally, Tf protects Müller glial cells from iron-induced toxicity (Picard et al., 2008) and overexpression or intraperitoneal injection of Tf prevents degeneration of photoreceptors in rd10 mice (Picard et al., 2010).

Cp has another important function resulting from its ferroxidase activity. Either present in the membrane or in the extracellular fluid, Cp is a critical component of the iron export system. While this role of Cp was noted over 4 decades ago (Osaki et al., 1971), the molecular mechanisms underlying Cp's effects have only been recently elucidated. These activities include stabilizing the trans-membrane iron export protein, ferroportin, and assisting in export of iron from cells (De Domenico et al., 2006; De Domenico et al., 2007). Additionally, as described above, the ferroxidase activity of Cp ensures that iron removed from the cells is in the ferric form, ready for binding to and transport via Tf.

The importance of Cp to regulation of iron levels in cells is demonstrated in patients with aceruloplasminemia (Harris et al., 1995; Klomp and Gitlin, 1996). This is a human hereditary disease in which absence of Cp activity results in the accumulation of iron in cells. Such accumulation of iron in brain and retina results in degeneration of both tissues (Miyajima et al., 1987; Logan et al., 1994; Texel et al., 2008) The potential role of Cp in numerous other ocular diseases such as glaucoma and age-related macular degeneration (AMD) has recently been the subject of study. In murine and human glaucomatous eyes, Cp was up-regulated in the retina (Stasi et al., 2007). In addition, the double knock-out of Cp and hephaestin in mice results in retinal iron accumulation and pathological changes in the retina which resemble AMD (Hahn et al., 2004). Hephaestin is also a protein with ferroxidase activity similar to Cp, which includes assisting in iron export from cells, especially enterocytes (Vulpe et al., 1999).

The secreted form of Cp is produced by lens epithelial cells (LEC) and released into the overlying culture medium (Harned et al., 2006). Supplementation of Cp to the serum-free medium bathing these cells caused significant changes in intracellular iron metabolism including an increase in the size of the intracellular labile "free" iron pool and increased iron incorporation into ferritin. Importantly, exogenous soluble Cp also increased the export of iron from these cells (Harned et al., 2006).

Along with oxygen, iron is an essential regulator of the levels of hypoxia-inducible factor-1 (HIF1) (Wang and Semenza, 1993), a transcription factor responsible for controlling the synthesis of over 60 proteins (Wang and Semenza, 1993; Semenza, 1999). In normoxic conditions when iron availability is not limiting, HIF1 activity is limited by the degradation of its HIF-1α subunit. However, even in normoxic conditions, decreased iron availability results in inhibition of HIF-1α degradation, its translocation to the nucleus and an increase

in HIF1's transcriptional activation activity. HIF1 controls the transcription of many iron regulatory proteins such as Cp and Tf as well as the iron storage protein ferritin.

Intracellular iron also controls ferritin and transferrin receptor synthesis. Additionally, our recent studies revealed yet another role for iron, which is the regulation of glutamate synthesis through iron's effects on cytosolic aconitase activity (McGahan et al., 2005). In addition to increasing the secretion of the neurotransmitter glutamate in neurons and retinal pigmented epithelial cells (RPE), iron regulated glutamate secretion is coupled to increased cystine uptake and exerts control of glutathione levels in LEC and RPE (Lall et al., 2008).

The importance of iron to cellular metabolism and its competing integral role in catalyzing free radical reactions requires very close regulation of both cellular iron concentration and its movement to sites of storage and utilization. Very little is known about how these processes are regulated. The hypothesis of the current study is that the alteration of intracellular iron metabolism by Cp has significant downstream effects on cellular functions. These include changes in the levels of the iron storage protein ferritin, transferrin receptor, levels of HIF-1α in the nucleus and glutamate secretion.

2. Methods

2.1. Tissue culture

Dogs were obtained from the Johnston County Animal Shelter (NC), after they were euthanized. Primary cultures of lens epithelial and retinal pigmented epithelial cells were prepared as previously described (McGahan et al., 2005) and grown to confluence. They were then plated on 6-well plates for the experiments unless otherwise stated.

2.2. Western blots

2.2.1. Ceruloplasmin—Confluent primary canine LEC and RPE were grown in complete media, rinsed thoroughly and incubated for 24 or 48 h in MEM without serum or glutamine. Aliquots of conditioned medium were collected and the cells were lysed in RIPA buffer (Pierce, Rockford, IL), centrifuged at 14,000 × *g* and supernatants were saved at −20 °C for later analysis. Aliquots of cell conditioned media (CCM) and cell lysates were concentrated on Microcon-50 columns (Millipore, Billerica, MA) and subjected to 7% SDS-PAGE, then moved by semi-dry transfer to Hybond nitrocellulose membranes (GE Healthcare, Munich, Germany). Anti-mouse ceruloplasmin (1:250) and HRP-goat anti-mouse IgG (1:1000) (both from BD Biosciences, Palo Alto, CA) were used for RPE protein immunolabeling, and rabbit anti-dog Cp (1:1000, Alpha Diagnostic, San Antonio, TX) and HRP-goat anti-rabbit IgG (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) for LEC, followed by visualization with ECL reagent (GE Healthcare). Rat testis ceruloplasmin (BD Biosciences) was used as a positive control for both cell types.

2.2.2. Transferrin receptor—Confluent cultures of LEC or RPE were treated for 6 or 24 h in serum-free and glutamine-free MEM supplemented with 55 μg/ml bovine ceruloplasmin (Sigma, St. Louis, MO). Cells were rinsed and lysed in RIPA buffer (Pierce, Rockford, IL), centrifuged at 14,000 × *g* and supernatants were saved at −20 °C for later analysis. The contents of the lysate sample containing 15 μg protein were separated on an 8% TRIS-Tricine gel under reducing and denaturing conditions. Human placental TfR (6 μg, Alpha Diagnostics, San Antonio, TX) was used as positive control. After a 30 min semi-dry transfer to nitrocellulose membrane, TfR was detected using 1:2000 dilution of mouse anti-human TFR (Invitrogen, Carlsbad, CA), followed by a 1:750 dilution of goat anti-mouse IgG, HRP conjugated (BD Bioscience) and visualized by ECL. Blots were then

reprobed with HRP-goat anti-human β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA) which was used as a loading control.

2.3. Determination of total ferritin within the cells

Ferritin concentration in cell lysates was measured by a simple sandwich ELISA with antibodies from Bethyl Labs (Montgomery, TX), as previously described (McGahan et al., 1994b).

2.4. De novo ferritin synthesis

LEC and RPE were pre-treated for 24 h with 55 μg/ml bovine Cp in serum-free and glutamine-free MEM, rinsed and incubated an additional 20 h in methionine-free DMEM with dialyzed 0.1% fetal bovine serum, ceruloplasmin (as above), and 60 μCi Translabel-^{35}S-methionine (MP Biomedicals, Solon, OH). Control wells had no ceruloplasmin. Cells were then lysed, ferritin immunoprecipitated from the lysates and subunits separated as previously described (Goralska et al., 2000). Radioactivity in the ferritin bands was normalized to radioactivity of total cell protein precipitated with trichloroacetic acid.

2.5. Iron uptake

Confluent LEC were incubated in serum-free and glutamine-free MEM with or without 55 μg/ml bovine ceruloplasmin for 24 h, rinsed and incubated for an additional 6 h with 240 μg/well ^{59}Fe-Tf in fresh MEM with or without added Cp. Cells were lysed in distilled water, freeze/thawed and radioactivity of total lysates, as well as membrane and cytosol fractions separated by centrifugation was determined. Radioactivity was measured using a Wallac Wizard Gamma counter (Perkin Elmer, Waltham, MA) and normalized to the amount of protein in the cytosolic fraction determined by the BCA method (Pierce).

2.6. Ceruloplasmin Immunofluorescence

RPE and LEC were cultured in 6-well plates on coverslips, and then fixed in 37 °C 4% formaldehyde in 1× PBS for 15 m. Whole canine lenses and posterior eyecups containing retina and RPE cells were fixed in 4% formaldehyde in 1× PBS and then processed for paraffin sections. In some cases live cells were treated with phosphatidylinositol-specific phospholipase C (PI-PLC) (Sigma) at 37 °C in order to cleave the GPI-linked Cp at the cell surface in an attempt to discriminate it from the secreted form of CP. All immunolabeling steps were performed at room temperature. Cells were permeabilized with 0.1% Triton X-100 in 1× PBS for 10 m. All cells/tissue sections were blocked in 5.0% normal donkey serum in PBS for 1 h, and then incubated for 1 h with goat anti-human ceruloplasmin antibodies (Bethyl Laboratories, A80-124A) at 5 μg/ml in PBS containing 0.1% BSA. Negative controls were incubated with 5 μg/ml non-immune goat IgG instead of anti-ceruloplasmin antibodies. After washing with PBS, cells were incubated with donkey anti-goat Alexa-Fluor® 594 IgG (H + L) secondary antibodies (Invitrogen) at 2.0 μg/ml for 1 h in darkness. After PBS washes, cells/sections were mounted in ProLong® Gold anti-fade reagent containing DAPI (Invitrogen). Cells were then imaged using a Leica DM5000B microscope with conventional epifluorescence. Images were captured with a Retiga 1300 cooled CCD camera and Simple PCI imaging software, and then arranged using Adobe Photoshop CS2.

2.7. Glutamate determination

Confluent cultures in 6-well plates were rinsed in serum-free, L-glutamine-free MEM and after incubation in this medium with or without added treatments (described in the figure legends), glutamate was measured in the CCM using Amplex Red kits (Invitrogen). The

glutamate levels were standardized to the amount of protein (BCA method, Pierce) in the cell lysates.

2.8. Determination of HIF-1α levels in nuclear extracts

Confluent cultures of LEC or RPE were incubated in serum-free and glutamine-free MEM with or without 55 μg/ml bovine Cp (Sigma) for 6 or 24 h. Nuclear extracts were prepared using an NEPER kit (Pierce) and stored at −80 °C. HIF-1α levels were measured in the nuclear extracts by sandwich ELISA with a DuoSet IC Human/Mouse HIF-1α kit (R & D Systems, Minneapolis, MN) according to the manufacturer's provided protocol. The amount of HIF-1α was normalized to the quantity of protein in the corresponding cytosolic extracts and expressed as a percentage of the HIF-1α found in control cells.

2.9. Statistics

The data was analyzed by ANOVA with Tukey's HSD test for multiple comparisons unless otherwise stated.

3. Results

3.1. Cp expression in canine RPE and LEC

Western blot analysis of the medium overlying healthy untreated LEC and RPE (cell conditioned medium; CCM) collected at 24 and 48 h, respectively, revealed that canine RPE secretes Cp (Fig. 2). We have already reported this for LEC (Harned et al., 2006). Much lower levels were found in whole cell lysates from LEC and RPE. These lysates contained cytosol as well as solubilized membranes. There is a much greater amount of Cp in the CCM than in the whole tissue indicating that Cp in CCM is likely secreted, as in other tissues studied, and not the result of cell lysis. This follows the pattern in most tissues where Cp is regarded as a secreted protein.

Immunofluorescent detection of Cp in tissue sections of canine lenses revealed diffuse Cp signals throughout epithelial and fiber cells (Fig. 3B and C). Fiber cells in the outer cortex of the lens showed higher levels of Cp labeling than nuclear layers (Fig. 3B). In tissue sections of the retina, the RPE and the ganglion cell layers showed the highest Cp signals, but other retinal cell layers also had detectable Cp signals (Fig. 3D). The Cp-specific fluorescence in tissue sections is quite diffuse and it is not clear whether Cp is localized in the membranes of any lens or retinal cells. Cultured primary canine RPE (Fig. 3F and G) and LEC (Fig. 3H and I) showed diffuse labeling for Cp throughout the cytoplasm, consistent with the secreted form of Cp. Many cells also revealed stronger labeling of Cp associated with the cell membranes, which suggests these cell types may possess a GPI-anchored form of Cp. However, treatment of these cells with phosphatidylinositol-specific phospholipase C (PI-PLC), which cleaves GPI-linked proteins from the cell surface did not alter the staining patterns in cultured cells, suggesting that Cp in these membranes is not GPI linked. Lack of a specific antibody for GPI-Cp precludes finding a definitive answer.

3.2. Cp effects on intracellular iron regulated processes

Since we had previously demonstrated that Cp increased the size of the intracellular labile iron pool in LEC (Harned et al., 2006), in this study we examined the downstream effects of these changes. Four iron regulated processes were studied, including synthesis and levels of the iron storage protein ferritin, the cytoplasmic aconitase pathway which produces glutamate, expression of the transferrin receptor (TfR) and HIF-1α translocation to the nucleus.

Addition of exogenous Cp to cultured LEC and RPE caused a significant increase in the concentration of the iron storage protein ferritin in both cell types (Fig. 4). Ferritin is a hetero-polymeric protein consisting of 24 subunits of two types, heavy (H-chain; 21 kDa) and light (L-chain; 19 kDa) which are products of two genes. Synthesis of both chains is increased at the transcriptional and translational level by iron and are present in a tissue specific ratio (Arosio and Levi, 2010). We determined that Cp treatment significantly increased de novo synthesis of ferritin L-chain, but not H-chain in cultured LEC (Fig. 5). The increase in ferritin synthesis most likely accounts for the increase in total ferritin levels shown in Fig. 4.

We have previously reported that iron regulates glutamate production and secretion through its activation of a cytosolic aconitase pathway (McGahan et al., 2005). An increase in iron levels increased aconitase activity and glutamate secretion in both LEC and RPE. In the present study Cp treatment caused a significant increase in glutamate secretion into the CCM (Fig. 6). Oxalomalate, an inhibitor of the aconitase pathway, lowered the basal level of glutamate secretion and completely inhibited Cp-induced glutamate secretion into the CCM (Fig. 6). Further evidence that a Cp-induced change in intracellular iron metabolism is responsible for the increase in glutamate secretion comes from the effects of iron chelators pyridoxal isonicotinoyl hydrazone (PIH) and the dipyridyl derivative, Dp44mT. Both chelators decreased glutamate secretion in cells not treated with Cp, and more importantly completely inhibited the effect of Cp on glutamate secretion into the CCM (Fig. 7). These results indicate that Cp increases intracellular iron availability causing an increase in aconitase activity and subsequent glutamate synthesis.

Iron regulates transferrin receptor (TfR) synthesis at the translational level (Hentze et al., 2010). In the presence of low levels of iron, IRP binding to stem loop structures in the 3′UTR of TfR mRNA is increased. This binding stabilizes TfR mRNA and increases its translation. An increase in iron levels decreases IRP binding with the subsequent effect of lowering TfR levels. In the present study, Cp treatment had no effect on TfR levels at 6 h, but confoundingly increased TfR levels at 24 h (Table 1). This was an unexpected response which is in direct contradiction of the findings of Cp's effects on ferritin synthesis and glutamate secretion.

The results of our study on the effect of Cp on HIF-1α also produced conflicting results. The HIF-1α subunit of the transcription factor HIF is regulated by both oxygen and iron. In iron replete cells under normoxic conditions, HIF-1α is degraded and not transferred to the nucleus, while in iron depleted cells HIF-1α is not destroyed but is transferred to the nucleus even in normoxic conditions. In our experiments, Cp induced an increase in HIF-1α levels in the nucleus at 6 h, a result that would be consistent with decreased availability of iron (Table 1). While this is similar to the implications of the TfR results, it is in contrast to that found for Cp's effects on ferritin levels and glutamate secretion.

3.3. Iron movement in cells

In order to determine how Cp affects intracellular iron movement, LEC were incubated with ^{59}Fe-labeled transferrin. All cells acquire iron from transferrin, the main iron transport protein in the extracellular fluids, including the intraocular fluids. And as confirmed in the above studies both RPE and LEC have transferrin receptors. After treating LEC with Cp for 24 h, cells were loaded with ^{59}Fe-Tf (in the presence of Cp) for 6 h and then lysed. Compared to control (non-Cp treated cells) there was an increased amount of ^{59}Fe present in the membrane fraction and a decreased ^{59}Fe content in cytosol of Cp treated cells (Table 2).

4. Discussion

The importance of Cp for maintaining iron homeostasis in the whole organism is dependent on its role in removing iron from cells (Osaki et al., 1966, 1971). We and others have speculated that Cp causes the development of a gradient of iron from intracellular sites to the extracellular fluid which allows for removal of iron from the cells (Harned et al., 2006; De Domenico et al., 2007). Early studies concentrated on Cp's role as an extracellular protein synthesized and secreted into the circulation by the liver. Cp was shown to regulate the movement of iron out of storage tissues and into the circulation when iron levels were low in this extracellular compartment. Recent studies indicate a more complex role for Cp in this process. This evidence includes the finding of a membrane bound form of Cp in the brain which has a GPI linkage (Patel and David, 1997; Patel et al., 2000). The retina has mRNA for GPI-Cp, suggesting that this tissue also makes the membrane bound form, however information about protein expression of GPI-Cp in the retina is still lacking (Chen et al., 2003). The ferroxidase activity of the GPI-linked protein stabilizes the iron export protein ferroportin at the cell membrane and promotes iron efflux (De Domenico et al., 2007). The production of Cp by tissues other than the liver, such as those residing behind blood-barriers, is significant, as is the tissue specific form of Cp produced. For example, the brain produces mainly GPI-linked Cp while the liver produces mainly the secreted form. The lack of availability of antibodies for GPI-linked Cp led us to try to determine its presence by enzymatic cleavage with phosphatidylinositol-specific phospholipase C. Immunolocalization of Cp in cells treated with this enzyme revealed no obvious differences in staining intensity indicating either low amounts or lack of GPI-Cp in these cultured cells. Therefore it is likely that most of the Cp produced by these cells is the secreted form. This is also indicated by the Western blots which show much greater amounts of Cp in the CCM than in the cell extracts. While membrane associated Cp was not easily discernible in tissue there was more intense staining of cell membranes in the cultured cells. These results point to the possibility that GPI-anchored Cp is present in the membrane, but lack of availability of GPI-Cp antibodies does not allow for a definitive answer.

Interestingly, the retina produces both Cp and hephaestin (another ferroxidase with Cp-like activity in promoting iron export from cells (Vulpe et al., 1999)) and only knock-out of both proteins results in iron accumulation and an age-related macular degeneration (AMD)-like pathology in mice (Hahn et al., 2004). The presence of both of these proteins in the retina provides a redundancy of function and implies an essential need to regulate iron levels in this tissue. Indeed, increased iron levels are seen in a number of retinal degenerative diseases and iron chelators can prevent cell death resulting from diverse stimuli, indicating iron's central role in "death pathways" (Lukinova et al., 2009). Importantly, RPE are an integral part of the blood-ocular barrier, which likely plays an important physiological role in regulating the iron content of avascular ocular tissues, like the lens. Iron levels are carefully controlled by the lens (McGahan, 1992) but a breakdown in control systems, such as blood-ocular barrier (BOB) control of intraocular iron levels might result in an increase in lenticular iron levels, a factor associated with cataractogenesis (Truscott, 2005).

While removal of iron from cells and regulation of systemic iron levels appear to be the main roles of Cp in physiology, the consequences of Cp's activity include alteration of intracellular iron levels and effects on iron-dependent processes, both physiological and pathological (Harned et al., 2006). The labile iron pool (LIP) is considered a transit pool from which iron moves between cellular uptake, utilization, storage and efflux. In addition, the size of this pool appears to have regulatory properties of its own and when it becomes too large it is thought to contribute to oxidative damage.

The size of the LIP has been generally assessed by measurement of iron regulated proteins and processes (Fig. 1). For example, an increase in ferritin levels would be expected if there was an increase in the size of the LIP since iron controls the status of the iron regulatory protein (IRP), which in iron depleted conditions binds to ferritin mRNA and prevents ferritin translation (Hentze et al., 2010). This makes sense, since iron storage would not be needed in conditions of iron depletion. On the other hand, high iron levels cause a switch of the IRP to another conformation which contains an iron sulfur cluster and no longer can inhibit ferritin synthesis. Importantly, the IRP then acquires aconitase activity. An increase in cytosolic aconitase activity leads to an increase in glutamate production and secretion as we have previously shown in LEC, RPE and neurons (McGahan et al., 2005).

The results we obtained in the present study indicate Cp causes an increase in the size of the LIP, because both ferritin levels and glutamate secretion are increased. This is a highly significant and novel aspect of Cp's function since glutamate is an important neurotransmitter in the retina and brain, and both iron and glutamate levels are dysregulated in neural and retinal degenerations. The iron-induced increase in glutamate secretion results in increased glutamate/cystine antiporter activity with the resulting increase in the intracellular concentration of the potent antioxidant glutathione, as we demonstrated in our previous study (Lall et al., 2008). Therefore, along with its ferroxidase activity, Cp-induced increase in glutamate secretion may provide Cp another role in cell antioxidant defense because it may also increase intracellular GSH levels.

The effects of Cp on de novo ferritin synthesis indicate that L-chain synthesis is increased, but H-chain is not. These two chains exist in tissue specific ratios. In our studies, the H:L ratio in LEC varied from 1.2 to 2.0 (Goralska et al., 2001) while the H:L ratio in RPE is 0.8–1.0 (unpublished data). It was surprising that Cp only affected the synthesis of one chain, since they both contain the IRE binding site for IRP and should be regulated similarly when intra-cellular iron content is changed. An increase in L:H content of ferritin would increase the long-term iron storage capabilities of this protein, but the mechanism for Cp's effects on the ratio are uncertain.

The IRP also regulates TfR levels in cells (Hentze et al., 2010). An increase in binding of the IRP to TfR mRNA, which occurs in iron depletion, stabilizes the message and increases TfR translation. Therefore an increase in TfR in cells treated with Cp, as shown in this study, would indicate a reduction in the LIP. Because measurement of the TfR is routinely used as an indicator of the size of the LIP, this result was confounding and seemingly contradicted the ferritin and glutamate results which indicated in increase in the size of the LIP.

A decrease in iron levels also causes an increase in HIF-1α translocation to the nucleus and its combination with HIF-1β to form the transcription factor dimer HIF1, which controls the production of dozens of proteins. Therefore, the Cp-induced increase in HIF-1α levels in the nucleus found in this study would, like the TfR results, indicate a decrease in the size of the LIP.

While these results are difficult to explain and appear contradictory, a larger view of the experimental design is worth taking. The time points measured in the current study (6, 24, 48 h) are just snapshots of a dynamic process. An increase in iron efflux by Cp will affect intracellular iron pools in ways that cannot be dynamically tracked with the available technology. The results from our earlier study indicate that Cp increased the LIP at 24 h, but significantly reduced it at 48 h (Harned et al., 2006). Cp treatment, in addition to increasing iron efflux in LEC also increased iron incorporation into ferritin which would most likely decrease the LIP. One can envision that initially Cp facilitates removal of iron from an

available internal LIP which at first lowers iron availability. This is supported by data in Table 2 which indicates a Cp-induced increase in ^{59}Fe content of the membrane fraction of ^{59}Fe-preloaded cells at 6 h. Afterward, there could be an increase in the size of the LIP (perhaps the 24 h point) due to the movement of iron from available intracellular stores. Upon depletion of these stores, the continued presence of Cp could cause a decrease in the LIP that can no longer be repleted. These dynamic changes coupled with the different half-lives of the proteins measured make for a complex story. For example, ferritin is a very long-lived protein, with a half-life greater than 14 h (Goralska et al., 2005), therefore a snap-shot of ferritin levels at 24 or 48 h would not capture the whole picture, and may only reflect an increase in the LIP while masking any period of time when the LIP might have been smaller. Further complicating the picture is the fact that changes in intracellular iron availability also affect the rate of ferritin degradation (Truty et al., 2001).

The increase in glutamate presents a similar problem, since we measured glutamate accumulation after 24 h, so this also may not reflect dynamic changes in the LIP over time. The same reasoning applies to Cp's effects on TfR levels in the membrane and HIF-1α levels in the nucleus. TfR is an even longer-lived protein than ferritin (half-life = 19 h), further complicating this analysis and interpretation of the results. Furthermore, in contrast to ferritin, the rate of degradation of TfR has not been shown to be affected by intracellular iron levels. The time course of HIF1 activation by iron depletion has not been determined, but is likely to also be dynamic, and the end result of these changes could also be hard to predict. Of the 4 predictors of LIP size measured in this study, two point to an increase in the LIP and two point to a decrease in the LIP. Thus, the results of these experiments are not spurious but likely reflect the dynamic and complex nature of iron regulation of cellular processes including protein synthesis and degradation, as well as the regulation of intracellular iron by extracellularly delivered Cp.

Further insight into these apparently discrepant results comes from a study by Robach and colleagues (Robach et al., 2007) in which levels of TfR and ferritin in skeletal muscle from humans exposed to relatively hypoxic conditions at high altitude were measured. Both proteins were found at significantly lower levels than at normoxic conditions. This observation contradicts the canonical view of IRP activity, where the binding of IRP to TfR mRNA increases translation, while binding to ferritin mRNA decreases translation. They conclude that the situation is more complicated than a single control of translation at the IRP level and that HIF1 activity as well as the systemic demand for iron from skeletal muscle for erythropoiesis exerts another layer of control that makes the results more difficult to interpret.

Additionally, there are serious questions about the accuracy of some of the current tools used to measure dynamic changes in the LIP and to determine if there is more than one cytoplasmic pool of intracellular iron (Tenopoulou et al., 2007). Only very recently has there been progress in this area. This includes development of methodology for visualizing iron movement using fluorescent iron sensors (Shvartsman et al., 2010) as well as the discovery of a mammalian siderophore which may move iron between cellular organelles (Devireddy et al., 2010). In an earlier study we found that there were separate source dependent pools of iron (Goralska et al., 1998), however we don't know how these pools are regulated or if the iron in them is targeted for specific utilization or storage in ferritin. A more recent study found that differentiation of oligodendrocytes is dependent upon iron, but only that derived through endocytic uptake, not by passive diffusion (Todorich et al., 2011). Quantification of the LIP and its dynamic regulation is not resolvable with current methods. In fact, the size of the LIP is only indirectly inferred by changes in transferrin receptor or ferritin levels. Our results and those of others indicate that these may not be accurate representations. A determination of total cellular iron is also not helpful in resolving this issue since it is

impossible to tell the exact localization of iron, how much of it is stored, present in iron dependent proteins or in the LIP.

The role of Cp is therefore not limited to controlling systemic iron levels, but has far reaching dynamic effects on important iron-dependent processes in individual cells and the tissues they comprise. These include dynamic changes in the size of the LIP and the regulation of iron dependent enzymes and processes. Future studies that include dynamic measurements of these events in real-time using live-cell imaging will help provide a more complete picture of the complex nature of the regulation of cellular iron metabolism.

Acknowledgments

This work was funded by NIH Grant #EY-04900 and by Funds from the State of North Carolina.

References

Arosio P, Levi S. Cytosolic and mitochondrial ferritins in the regulation of cellular iron homeostasis and oxidative damage. Biochim Biophys Acta. 2010; 1800:783–792. [PubMed: 20176086]

Chen L, Dentchev T, Wong R, Hahn P, Wen R, Bennett J, Dunaief JL. Increased expression of ceruloplasmin in the retina following photic injury. Mol Vis. 2003; 9:151–158. [PubMed: 12724641]

De Domenico I, Vaughn MB, Bagley D, Musci G, Ward DM, Kaplan J. Ferroportin-mediated mobilization of ferritin iron precedes ferritin degradation by the proteasome. EMBO J. 2006; 25:5396–5404. [PubMed: 17082767]

De Domenico I, Ward DM, di Patti MC, Jeong SY, David S, Musci G, Kaplan J. Ferroxidase activity is required for the stability of cell surface ferroportin in cells expressing GPI-ceruloplasmin. EMBO J. 2007; 26:2823–2831. [PubMed: 17541408]

Devireddy LR, Hart DO, Goetz DH, Green MR. A mammalian siderophore synthesised by an enzyme with a bacterial homolog involved in enterobactin production. Cell. 2010; 141:1006–1017. [PubMed: 20550936]

Goralska M, Harned J, Fleisher LN, McGahan MC. The effect of ascorbic acid and ferric ammonium citrate on iron uptake and storage in lens epithelial cells. Exp Eye Res. 1998; 66:687–697. [PubMed: 9657901]

Goralska M, Holley B, McGahan MC. The effects of tempol on ferritin synthesis and Fe metabolism in lens epithelial cells. Biochim Biophys Acta. 2000; 1497:51–60. [PubMed: 10838158]

Goralska M, Holley BL, McGahan MC. Overexpression of H and L ferritin subunits in lens epithelial cells alters Fe metabolism and cellular response to UVB irradiation. Invest Ophthalmol Vis Sci. 2001; 42:1721–1727. [PubMed: 11431434]

Goralska M, Nagar S, Fleisher LN, McGahan MC. Differential degradation of ferritin H- and L-chains: accumulation of L-chain-rich ferritin in lens epithelial cells. Invest Ophthalmol Vis Sci. 2005; 46:3521–3529. [PubMed: 16186329]

Hahn P, Qian Y, Dentchev T, Chen L, Beard J, Harris ZL, Dunaief JL. Disruption of ceruloplasmin and hephaestin in mice causes retinal iron overload and retinal degeneration with features of age-related macular degeneration. Proc Natl Acad Sci U S A. 2004; 101:13850–13855. [PubMed: 15365174]

Harned J, Fleisher LN, McGahan MC. Lens epithelial cells synthesize and secrete ceruloplasmin: effects of ceruloplasmin and transferrin on iron efflux and intracellular iron dynamics. Exp Eye Res. 2006; 83:721–727. [PubMed: 16806171]

Harris ZL, Takahashi Y, Miyajima H. Aceruloplasminemia: molecular characterization of this disorder of iron metabolism. Proc Natl Acad Sci U S A. 1995; 92:2539–2543. [PubMed: 7708681]

Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to Tango: regulation of mammalian iron metabolism. Cell. 2010; 142:24–38. [PubMed: 20603012]

Klomp LW, Gitlin JD. Expression of the ceruloplasmin gene in the human retina and brain: implications for a pathogenic model in aceruloplasminemia. Hum Mol Genet. 1996; 5:1989–1996. [PubMed: 8968753]

Lall MM, Ferrell JB, Nagar S, Fleisher LN, McGahan MC. Iron regulates l-glutamate secretion, $X_{\bar{c}}$ activity and glutathione levels in lens epithelial and retinal pigment epithelial cells by its effect on cytosolic aconitase. Invest Ophthalmol Vis Sci. 2008; 49:310–319. [PubMed: 18172108]

Logan JI, Harveyson KB, Wisdon GB, Hughes AE, Archbold GPR. Hereditary ceruloplasmin deficiency, dementia and diabetes mellitus. Q J Med. 1994; 87:663–670.

Lukinova N, Iacovelli J, Dentchev T, Wolkow N, Hunter A, Amado D, Ying G-S, Sparrow JR, Dunaief JL. Iron Chelation protects the retinal pigment epithelial cell line ARPE-19 against cell death triggered by diverse stimuli. Invest Ophthalmol Vis Sci. 2009; 50:1440–1447. [PubMed: 19182262]

McGahan MC. Does the lens serve as a 'sink' for iron during ocular inflammation? Exp Eye Res. 1992; 54:525–530. [PubMed: 1623939]

McGahan MC, Fleisher LN. Antioxidant activity of aqueous and vitreous humor from the inflamed rabbit eye. Curr Eye Res. 1986; 5:641–645. [PubMed: 3490355]

McGahan MC, Grimes AM, Fleisher LN. Ferroxidase activity increases dramatically in the aqueous humor during the ocular inflammatory response. Ophthal Res. 1989; 21:221–225.

McGahan MC, Grimes AM, Fleisher LN. Transferrin inhibits the ocular inflammatory response. Exp Eye Res. 1994a; 58:509–511. [PubMed: 7925687]

McGahan MC, Harned J, Grimes AM, Fleisher LN. Regulation of ferritin levels in cultured lens epithelial cells. Exp Eye Res. 1994b; 59:551–556. [PubMed: 9492756]

McGahan MC, Harned J, Mukunnemkeril M, Goralska M, Fleisher LN, Ferrell JB. Iron alters glutamate secretion by regulating cytosolic aconitase activity. Am J Physiol Cell Physiol. 2005; 288:C1117–C1124. [PubMed: 15613494]

Miyajima H, Nishimura Y, Mizoguchi K, Sakamoto M, Shimizu T, Honda N. Familial apoceruloplasmin deficiency is associated with blepharospasm and retinal degeneration. Neurology. 1987; 37:761–767. [PubMed: 3574673]

Osaki S, Johnson DA, Frieden E. The possible significance of ferrous oxidase activity of ceruloplasmin in normal human serum. J Biol Chem. 1966; 241:2746–2751. [PubMed: 5912351]

Osaki S, Johnson DA, Frieden E. Mobilization of iron from the perfused mammalian liver by a serum copper enzyme, ferroxidase I. J Biol Chem. 1971; 246:3018–3023. [PubMed: 5554305]

Patel BN, David S. A novel glycosylphosphatidylinositol-anchored form of ceruloplasmin is expressed by mammalian astrocytes. J Biol Chem. 1997; 272:20185–20190. [PubMed: 9242695]

Patel BN, Dunn RJ, David S. Alternative RNA splicing generates a glycosylphosphatidylinositol-anchored form of ceruloplasmin in mammalian brain. J Biol Chem. 2000; 275:4305–4310. [PubMed: 10660599]

Picard E, Fontaine I, Jonet L, Guillou F, Behar-Cohen F, Courtois Y, Jeanny JC. The protective role of transferrin in Muller glial cells after iron-induced toxicity. Mol Vis. 2008; 14:928–941. [PubMed: 18509548]

Picard E, Jonet L, Vesvres MH, Sergeant C, Behar-Cohen F, Courtois Y, Jeanny JC. Overexpressed or intraperitoneally injected human transferrin prevents photoreceptor degeneration in rd10 mice. Mol Vis. 2010; 16:2612–2625. [PubMed: 21179240]

Robach P, Cairo G, Gelfi C, Bernuzzi F, Pilegaard H, Vigano A, Santambrogio P, Cerretelli P, Calbet JAL, Moutereau S, Lundby C. Strong iron demand during hypoxia-induced erythropoiesis with down-regulation of iron-related proteins and myoglobin in human skeletal muscle. Blood. 2007; 109:4724–4731. [PubMed: 17311997]

Semenza GJ. Regulation of mammalian 02 homeostasis by hypoxia-inducible factor 1. Ann Rev Cell Dev Biol. 1999; 15:551–578. [PubMed: 10611972]

Shvartsman M, Fibach E, Cabantchik ZI. Transferrin-iron routing to the cytosol and mitochondria as studied by live and real-time fluorescence. Biochem J. 2010; 429:185–193. [PubMed: 20408812]

Stasi K, Nagel D, Yang X, Ren L, Mittag TW, Danias J. Ceruloplasmin upregulation in retina of murine and human glaucomatous eyes. Invest Ophthalmol Vis Sci. 2007; 48:727–732. [PubMed: 17251471]

Tenopoulou M, Kurz T, Doulias P-T, Calaris D, Brunk UT. Does the calcein-AM method assay the total cellular 'labile iron pool' or only a fraction of it? Biochem J. 2007; 403:261–266. [PubMed: 17233627]

Texel SJ, Xu X, Harris ZL. Ceruloplasmin in neurodegenerative diseases. Biochem Soc Trans. 2008; 36:1277–1281. [PubMed: 19021540]

Todorich B, Zhang X, Connor JR. H-ferritin is the major source of iron for oligodendrocytes. Glia. 2011; 59:927–935. [PubMed: 21446040]

Truscott RJ. Age-related nuclear cataract-oxidation is the key. Exp Eye Res. 2005; 80:709–725. [PubMed: 15862178]

Truty J, Malpe R, Linder MC. Iron prevents ferritin turnover in hepatic cells. J Biol Chem. 2001; 276:48775–48780. [PubMed: 11606570]

Vulpe C, Kuo YM, Libina N, Askwith C, Murphy TLC, Gitschier LJ, Anderson G. Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. Nat Genet. 1999; 21:195–199. [PubMed: 9988272]

Wang GL, Semenza GL. Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. Blood. 1993; 279:714–717.

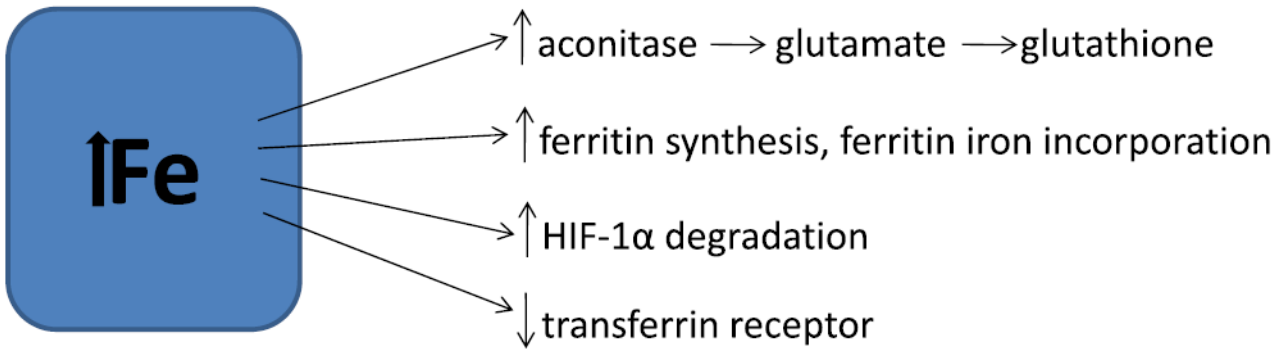

Fig. 1.
The effects of increased intracellular iron on multiple iron dependent pathways. A decrease in iron availability causes opposite effects on these pathways.

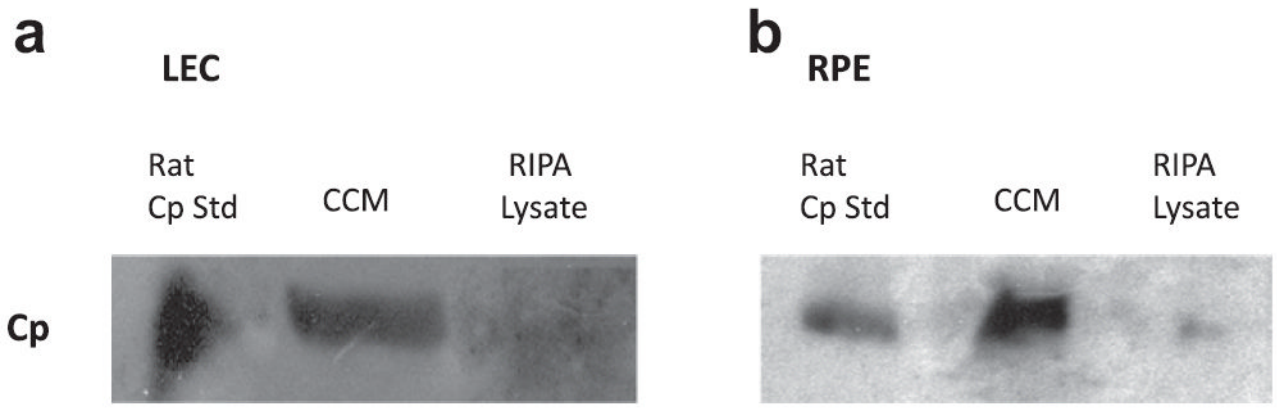

Fig. 2.

Ceruloplasmin is detected in CCM and lysates of LEC and RPE. Confluent cultures of LEC and RPE were incubated in serum-free MEM for 24 h (LEC) and 48 h (RPE). Cell conditioned medium was collected and RIPA lysates were prepared and concentrated on Microcon-50 spin columns and protein concentration determined. Rat testis ceruloplasmin (5 μg) was loaded as a positive control (Rat Cp Std). a. For LEC, 25 μg CCM protein and 50 μg lysate protein were loaded on the gel. b. For RPE, 60 μg CCM protein and 50 μg lysate protein were loaded on the gel. Proteins were separated using 7% SDS-PAGE, followed by transfer to nitrocellulose membranes, immunolabeling and detection by chemiluminesence.

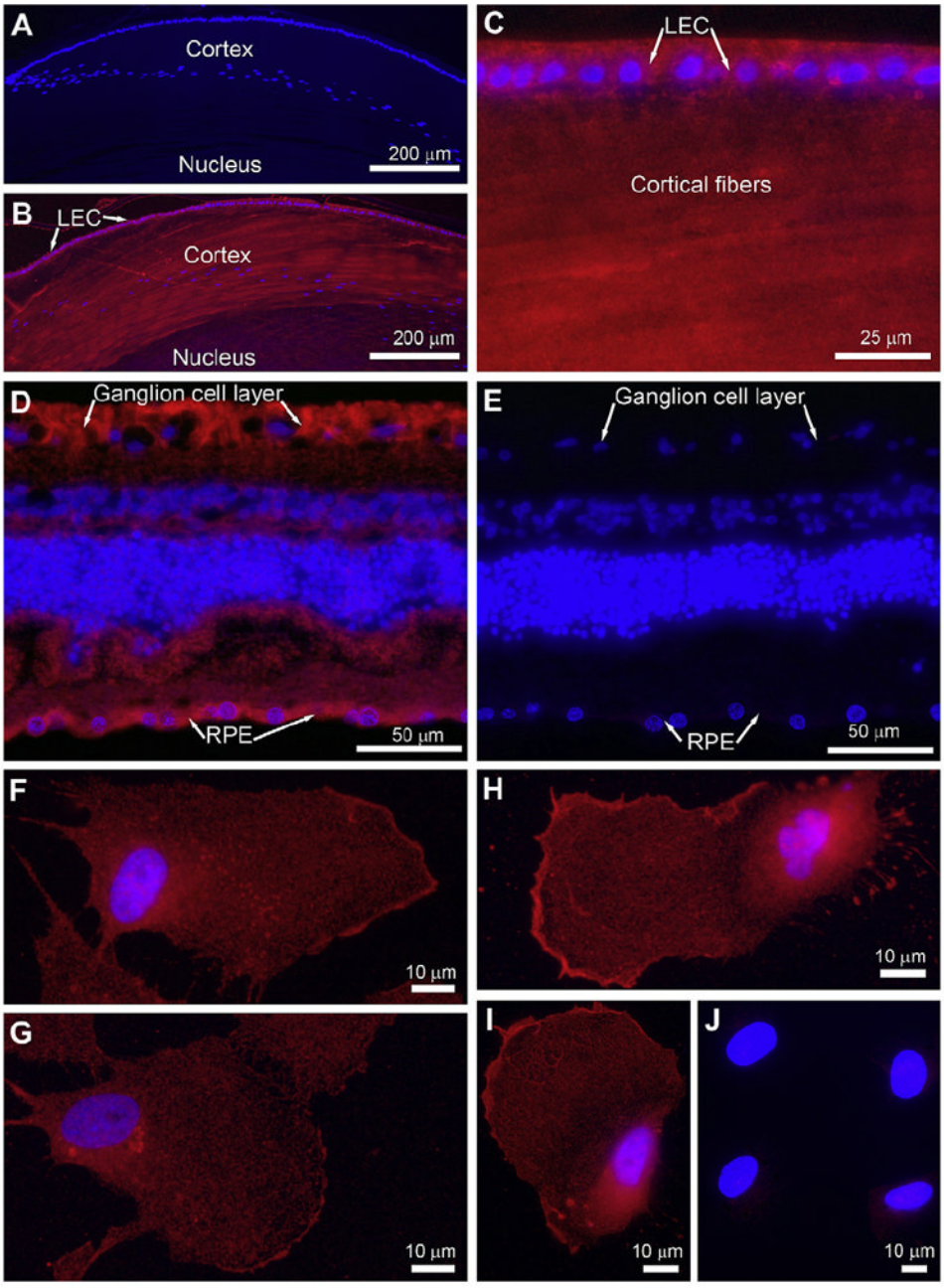

Fig. 3.

Canine lens and retinal epithelial cells express Cp in situ and continue to express Cp in primary culture. Cp antibodies and Alexa-Fluor 594-labeled secondary antibodies (red) were used to localize Cp in paraffin sections of formaldehyde-fixed canine lenses and retinas and primary cultured LEC and RPE. Nuclei were counterstained with DAPI (blue). (A) Lens control section incubated with normal goat IgG in place of anti-Cp antibodies. (B) Lens section incubated with anti-Cp antibodies showing Cp signals in LEC and lens fiber cells. (C) Higher magnification of a lens section showing diffuse Cp signals in LEC and cortical fiber cells. (D) Canine retina section from the tapetal area (containing non-autofluorescent, non-pigmented epithelial cells) showing highest Cp signals in the RPE and the ganglion cell layer. (E) Similar retina section incubated with normal goat IgG in place of anti-Cp

antibodies. (F and G) Primary cultured RPE cells showing diffuse cytoplasmic Cp signals and localized Cp labeling at plasma membranes. (H and I) Primary cultured LEC also showing diffuse cytoplasmic Cp signals and more intense Cp signals in plasma membranes. (J) Primary cultured RPE incubated with normal goat IgG in place of anti-Cp antibodies.

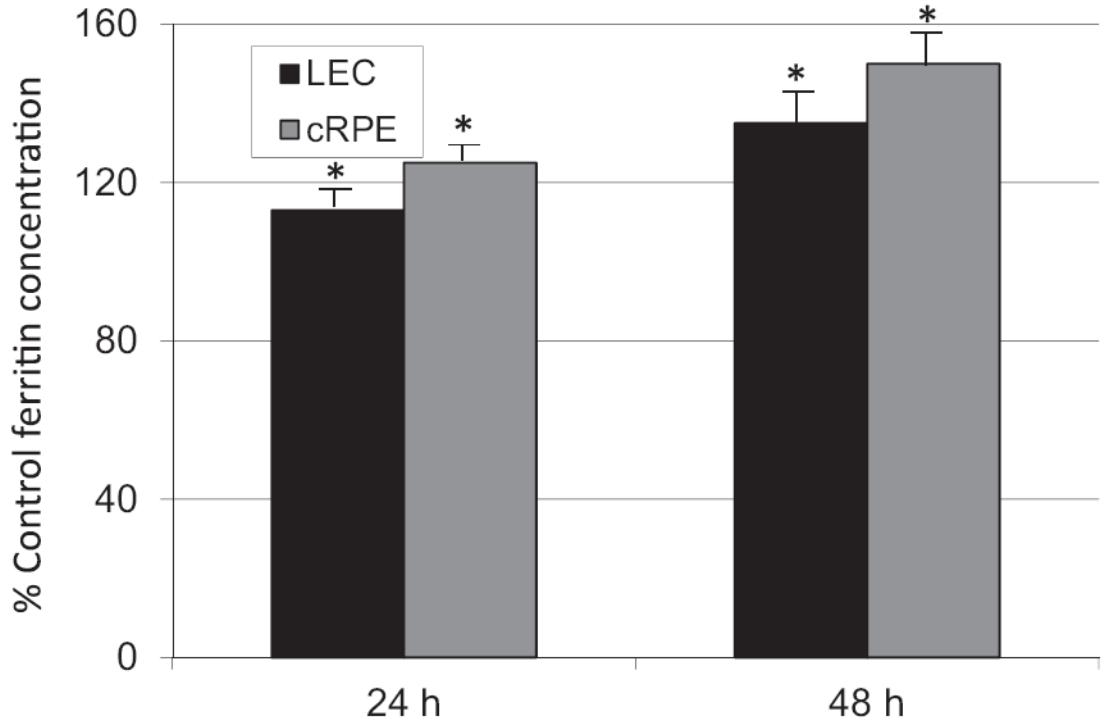

Fig. 4.

Cp increases ferritin levels in LEC and RPE. Cells were incubated with bovine ceruloplasmin (40 μg/ml LEC; 55 μg/ml RPE) in serum-free MEM for 0, 24 or 48 h. Ferritin concentration (ng/mg protein) was determined in cell lysates by a simple sandwich ELISA as previously described (McGahan et al., 1994b). Cp increased ferritin levels in both cell types at 24 h, with an even greater increase at 48 h. Results are presented as a percentage of the ferritin concentration in cells incubated without added Cp (% control), mean ± SEM of at least 3 samples. *Significantly different from control, $p < 0.05$, ANOVA and Tukey's test.

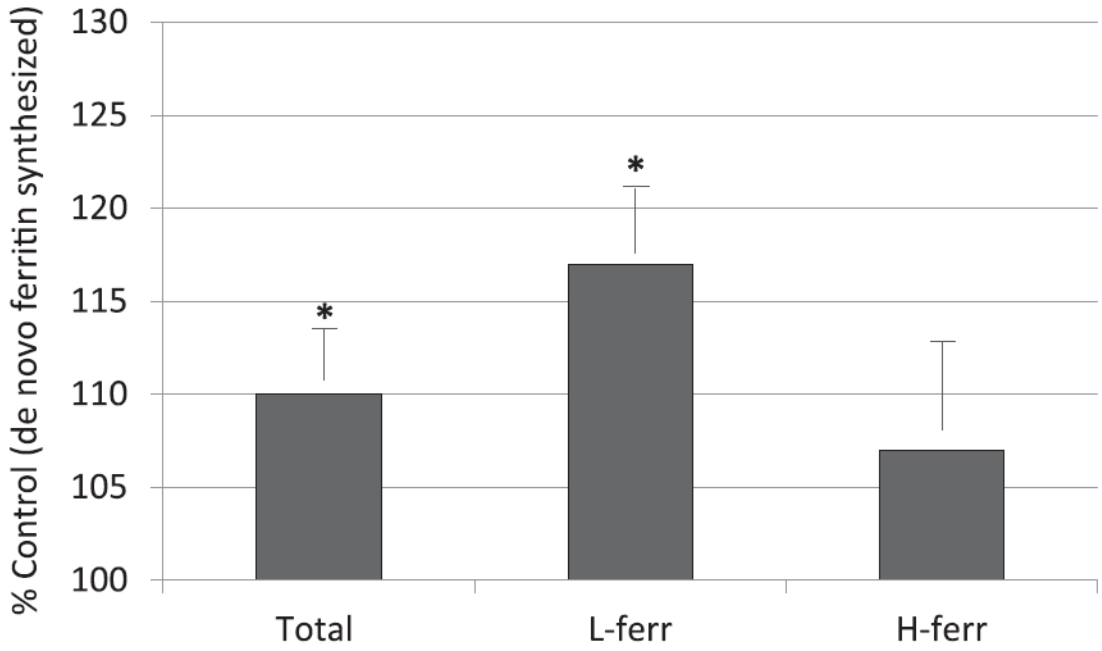

Fig. 5.

Cp increases de novo synthesis of ferritin L-chain. Confluent cultures of LEC were pre-treated for 24 h with bovine Cp (55 μg/ml) in serum-free MEM, followed by metabolic labeling for 20 h in methionine-free DMEM with 0.1% dialyzed fetal bovine serum, ceruloplasmin (as above) and translabel-^{35}S-methionine. Ferritin was immunoprecipitated from the lysates and ferritin chains separated by electrophoresis and analyzed as previously described (Goralska et al., 2000). Radioactivity in the ferritin bands was normalized to the radioactivity incorporated into total cell protein and expressed as % control (cells having no added Cp). Histogram bars represent the mean ± SEM of at least 6 samples. *Significantly different from control, $p \leq 0.01$, ANOVA and Tukey's test.

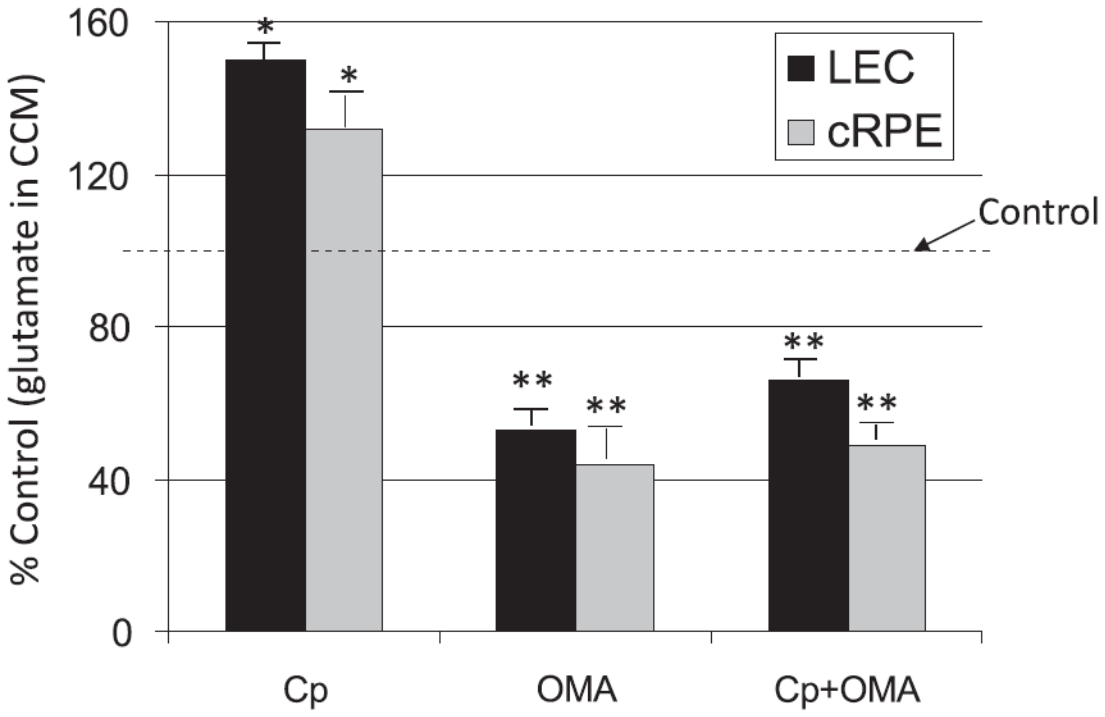

Fig. 6.

Cp increases glutamate production via the iron regulated cytosolic aconitase. Cells were treated for 24 h in serum and glutamine-free MEM with Cp (40 μg/ml) and/or oxalomalate (OMA, 2.5 mM cRPE or 5 mM LEC), an inhibitor of aconitase activity. Glutamate was then measured in the cell conditioned medium and values normalized to protein in the cell lysates. Cp increased glutamate secretion, while OMA decreased it and blocked the Cp effect. Data is expressed as % control (no Cp or OMA treatment), mean ± SEM of at least 4 samples. *Significantly different from control, $p < 0.05$, ANOVA + Tukey's test. **Significantly different from control and Cp, $p < 0.05$, ANOVA + Tukey's test.

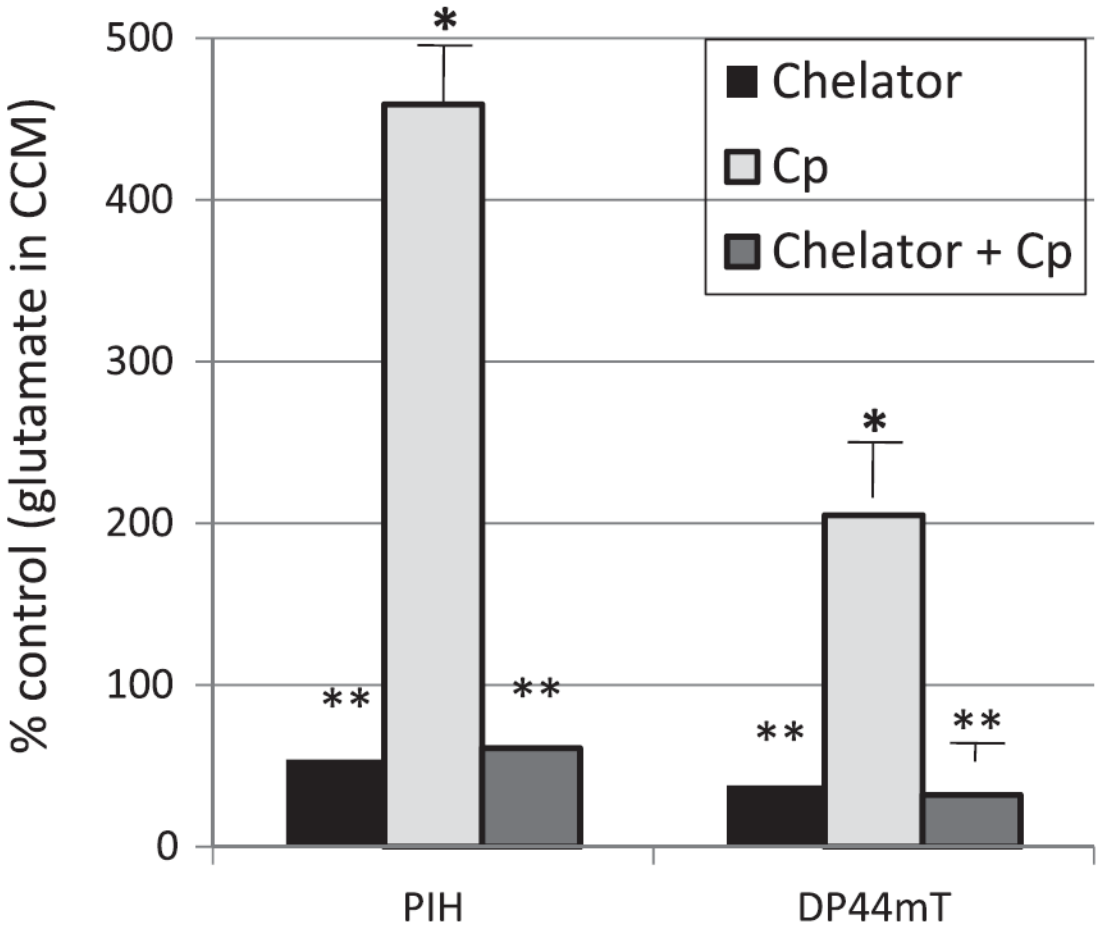

Fig. 7.

Iron chelators reduce Cp-increased glutamate levels in the CCM of RPE. Cells were treated for 24 h in serum-free glutamine-free MEM with either Cp (55 μg/ml), pyridoxal isonicotinoyl hydrazone (PIH, 0.1 mM), DP44mT (0.3 μM) both from (EMD, La Jolla, CA) or a combination of Cp with each chelator. Glutamate levels were then determined in the CCM, normalized to protein in the cell lysates, and expressed as % control (untreated cells). Histogram bars represent the mean ± SEM of 6 samples. *Significantly different from control and **Significantly different from control and Cp, $p < 0.05$, ANOVA and Tukey's test.

Table 1

The effect of Cp on transferrin receptor and on HIF-1α translocation to the nucleus. LEC or RPE cells were treated for 6 or 24 h with or without bovine ceruloplasmin (55 μg/ml) in serum-free MEM. For TfR analysis, RIPA lysates were prepared and aliquots containing 15 μg protein underwent electrophoresis and Western blot analysis. Blots were also probed for β-actin as a loading control. Data shown are the results of densitometric analysis of the bands (TfR/Actin) from Cp treated cells and are expressed as a percentage of that of the non-Cp treated control cells. Values are means ± SEM of at least 4 experiments.

	LEC (% Control)	RPE (% Control)
TfR (6 h)	101 ± 3.1 (n = 7)	93 ± 4.0 (n = 4)
TfR (24 h)	125 ± 10.5 * (n = 9)	119 ± 4.4 * (n = 6)
HIF-1α (6 h)	131 ± 13.2 * (n = 8)	129 ± 7.5 * (n = 10)

* Significantly different from control, $p < 0.05$, ANOVA and Tukey's test. For HIF-1α analysis, cytosolic and nuclear extracts of the treated cells were prepared and HIF-1α quantified by sandwich ELISA. The amount of HIF-1α in the nuclear extract was normalized to the amount of protein in the corresponding cytosolic extract and expressed here as a percentage of the HIF-1α found in nuclear extracts from control (no Cp) cells. Values are the mean ± SEM of at least 8 samples from 2 separate experiments. *Significantly different from control, $p < 0.05$, ANOVA and Tukey's test.

Table 2

Cp changes the distribution of iron in LEC. Cells were treated with bovine ceruloplasmin (55 μg/ml) in serum-free MEM for 24 h and then incubated for 6 h with 240 μg/ml ^{59}Fe-transferrin, also with Cp. Radioactivity was measured in the total lysates as well as in the membrane and cytosol fractions after separation by centrifugation. Data is expressed as the percentage of ^{59}Fe (CPM/mg protein) from the total lysate that was found in the corresponding membrane or cytosol fraction in control (no Cp treatment) versus Cp treated cells. Values shown represent the mean ± SEM ($n \geq 5$).

	Membrane fraction	Cytosol
CTL	15.8% ± 0.83	83.2% ± 1.6
Cp	26.3% ± 2.9 *	73.7% ± 1.4 *

* Significantly different from control, $p < 0.05$, Student's unpaired t-test.