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Iron metabolism in the eye, a review

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Introduction

The key roles of iron in cellular metabolism have been studied for over a century and more recently the involvement of iron in oxidative damage has become clear. Reactive oxygen species (ROS) play a fundamental role in the pathophysiology of dozens of diseases. Iron-catalyzed formation of ROS is a major player in these processes (Gutteridge et al. 1981). The oxygen radical superoxide is produced in cells from oxidized reactions in the mitochondria and other redox reactions in cells. Superoxide is detoxified by superoxide dismutase with the resulting formation of hydrogen peroxide, which in the presence of ferrous iron can form the highly reactive and damaging hydroxyl radical. Clearly, careful control of iron availability is central to maintenance of normal cell function. Specifically in the eye, ROS participate in tissue damage which contributes to many diseases (which are covered in the referenced reviews) including cataractogenesis (Lou 2003; Spector 1995; Spector 2000; Truscott 2005b), diseases of the cornea (Shoham et al. 2008), retinal degeneration (He et al. 2007), diabetic retinopathy (Feng et al. 2007), glaucoma (Aslan et al. 2008), photoreceptor damage in uveitis (Saraswathy and Rao 2008), light-induced retinopathy (Siu et al. 2008), and age-related macular degeneration (Beatty et al. 2000; Dunaief 2006; Dunaief et al. 2005).

The story of iron metabolism continues to evolve with new discoveries including iron regulation of glutamate production and secretion, glutathione (GSH) synthesis and the activity of hypoxia-inducible factor-1 (HIF-1) which will be described below. Despite the importance of iron in these varied roles, our understanding of the control of intracellular iron metabolism has only moved forward conspicuously in the last 10 years. During this time rapid developments in our understanding of iron metabolism were made possible by the discovery of several key iron regulatory proteins. Our current knowledge of iron metabolism in ocular fluids and tissues, especially the lens, cornea, retina and retinal pigmented epithelial cells is the subject of this review and it is clear that it has not kept pace with this rapidly developing field of study of iron physiology in other, non-ocular tissues.

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1. Iron content of the eye

1.1. Intraocular fluids (IOFs)

The iron content of the fluids and tissues of the eye of numerous species has been studied since the 1940s (Tauber and Krause 1943). We and others found very low levels of iron in the normal aqueous and vitreous humors of the eye (McGahan and Fleisher 1986b). This level was a small percentage (less than 1%) of the iron content of plasma likely reflecting the ability of the blood-ocular barrier to prevent the entrance of the plasma iron transport protein, transferrin, into the eye. Inflammation, which causes a breakdown of this barrier, induced a large increase in the iron concentration in both the aqueous and vitreous humors (McGahan and Fleisher 1988). In the aqueous humor much of this iron was likely bound to transferrin during the course of the inflammatory response due to the availability of iron-binding capacity in this fluid. However, there was some hemorrhage in the posterior segment which resulted in iron concentrations exceeding the transferrin-iron-binding-capacity of the vitreous. Such non-transferrin bound, redox-active iron could cause tissue damage due to free radical formation.

1.2. Lens

The iron content of the lens has been determined in numerous species over the years by a variety of methods (Agarwal et al. 1976; Lakomaa and Eklund 1978; McGahan 1992; Oksala 1954; Yamaguchi et al. 1980). Iron concentration in these lenses has been reported to be between 0.18 and 9.6 $\mu\text{g/g}$ wet weight. There is a wealth of evidence linking oxidation of proteins to cataract formation (Spector 2000; Truscott 2005b). Iron has a central role in catalyzing free radical reactions leading to oxidative damage. Iron-catalyzed reactions have been linked to changes in lens crystallins (Garland 1990; McDermott et al. 1988), lens DNA damage (Kleiman et al. 1990) and cataract formation (Garland 1990; Levi et al. 1998; Truscott 2005a). Therefore, it was important to determine if iron levels and reactivity change in the lens during cataractogenesis. A number of studies found increases in lens iron content with cataract formation (Dawczynski et al. 2002; Garner et al. 2000b). Significantly, redox-active iron (not bound in the iron storage protein ferritin) was found at higher levels in cataractous versus non-cataractous lenses (Garner et al. 2000a; Garner et al. 1999).

The lens has remarkable control over its iron content. During inflammation, the lens accumulates iron most likely by taking it up from the increased plasma transferrin and non-transferrin bound iron present in the IOFs after breakdown of the blood-ocular barriers (McGahan 1992). Importantly, the iron concentration of the lens declined to control levels upon resolution of the inflammatory episode. Therefore, the lens may not only provide a buffer for removal of potentially damaging intraocular iron, but must have carefully controlled mechanisms for release of iron in order to maintain iron levels within a narrow range. These mechanisms may change with age and such changes contribute to the accumulation of iron and to oxidative damage seen in cataractogenesis. Regulation of systems responsible for iron metabolism in the lens is the subject of active investigation in our laboratory.

1.3. Retina and retinal pigmented epithelium (RPE)

It is well known that intraocular foreign bodies containing iron cause retinal degeneration (He et al. 2007). However, the normal functions and regulation of iron in the retina have only recently come under study. The largest amounts of iron in the normal retina, as determined by proton induced X-ray emission, were in the RPE, choroid and inner segments of photoreceptors. Iron stored in ferritin was detected on the disc membranes of intact photoreceptors as well as in RPE phagosomes (Yefimova et al. 2000).

Retinal iron levels increase with age in humans (Hahn et al. 2006) and rodents (Chen et al. 2008) and with disease (Dunaief 2006; He et al. 2007). In addition, iron deposits were found in the macula of humans with age-related macular degeneration (AMD) (Dentchev et al. 2005). In a model of retinal degeneration, the Royal College of Surgeons (RCS) rat, non-heme iron deposits accumulated with time in a debris field resulting from the pathological manifestation of this genetic model, the inability to phagocytose photoreceptor outer segments. Neither the iron binding protein transferrin, nor the iron storage protein, ferritin, was found associated with this accumulated iron. It was hypothesized that the accumulation of debris disrupted normal movement of iron from the RPE to the retina. Since this iron was apparently not bound to proteins that normally block its ability to catalyze oxidative damage (ferritin and transferrin), it was hypothesized that iron accumulation could contribute to retinal degeneration in the RCS model (Yefimova et al. 2002). Finally, alterations in the levels of proteins involved in iron metabolism, i.e. decreased transferrin and increased transferrin receptor, ferritin, ferroportin, and ceruloplasmin in the neural retina of aging rodents, suggest that dysregulation of iron metabolism and the resulting in accumulation of iron, could be a causative factor in age-related retinal degenerations (Chen et al. 2008).

2. Mechanisms for maintaining iron homeostasis in cells

2.1. Iron regulatory proteins (IRPs)

2.1.1 IRP-1 and IRP-2—Two IRPs have been described, IRP-1 and IRP-2, which bind stem loop structures, called iron responsive elements (IREs), in the 3' and 5' untranslated regions of mRNAs of several proteins involved in iron storage and metabolism (Muckenthaler et al. 2008). While they perform similar tasks, their activities are regulated in different ways. IRP-1 can exist in two different conformations. When iron is abundant IRP-1 assumes aconitase activity (cytosolic aconitase, or c-aconitase) and cannot bind to mRNA (Fig. 1). IRP-2 is degraded by the ubiquitin-proteasomal system in an iron-dependent manner. Abundance of iron also decreases the ability of the IRPs to bind to IRE allowing for translation of ferritin and the divalent metal transporter as well as the destabilization of transferrin receptor mRNA. When iron is scarce, IRP-1 and 2 have the ability to bind to the various mRNAs, preventing the translation of ferritin and stabilizing mRNA for transferrin receptor and ferroportin. It has recently been hypothesized that IRP-2 is the major iron sensor in most cells and that under physiological conditions IRP-1 functions mainly as a c-aconitase (Rouault 2006). This is clearly an elegant system for controlling iron levels and storage within cells.

2.1.2. The dual role of IRP-1—IRP-1/c-aconitase regulates glutamate production and glutathione (GSH) levels in lens epithelial cells (LEC) and RPE. The function of a c-aconitase (the non-mRNA binding form of IRP-1), has been a matter of investigation. While mitochondrial aconitase clearly plays an important role in the mitochondrial citric acid cycle, the cytosolic form has no known function. In one study, it was determined that the conversion of citrate to isocitrate by c-aconitase, with the subsequent metabolism of isocitrate to α -ketoglutarate resulted in the production of the important antioxidant, NADPH. These investigators hypothesized that NADPH was formed as a protective mechanism in response to an increase in iron load (Narahari et al. 2000). However, we were unable to show that there was increased NADPH formed as a result of an increase in iron levels. In our studies in LEC, RPE and neurons, we found that NADPH, formed in response to of increased iron levels, was used in the subsequent metabolism of α -ketoglutarate to glutamate by glutamate dehydrogenase (McGahan et al. 2005). Glutamate is an important neurotransmitter in the brain and the retina. Our findings are the first to show that glutamate synthesis and secretion are directly regulated by iron. Since both iron and glutamate are

dysregulated in neurological and retinal disease, a physiological link between these two is quite significant.

In a subsequent study aimed at determining the physiological basis for lenticular secretion of glutamate, we found that iron-regulated glutamate production and secretion were functionally linked to increased cystine uptake (by both LEC and RPE) by a cystine/ glutamate antiporter. The increase in transporter activity provided cysteine to these cells. Cysteine is the rate limiting amino acid for glutathione production and the iron-induced increase in antiporter activity caused an increase in glutathione levels (Lall et al. 2008). This may be a protective response to increases in cellular iron, since glutathione is a potent antioxidant providing important protection to the lens and other tissues (Giblin 2000). Since it is now thought that IRP-1 is normally present as c-aconitase in most cells, regulation of this activity by iron is critical, especially when iron metabolism is dysregulated as has been demonstrated in many neurological and retinal diseases.

3. Iron storage

3.1 Ferritin

The ubiquitous iron storage protein, ferritin, is a multimeric protein consisting of 24 subunits of two types designated heavy (H) and light (L). Each ferritin molecule is capable of storing 4,500 iron atoms and provides for the safe storage of iron, preventing it from participating in damaging oxidative reactions. Synthesis of ferritin can limit the size of the labile iron pool (LIP). The LIP is believed to be an intracellular transit pool of iron available for either storage in ferritin or utilization by the cell, but can also contribute to free radical formation.

The ability of cells to store and retrieve iron from ferritin is dependent on the ratio of H:L ferritin chains, with each cell type having a specific H:L ratio. Synthesis of H- and L-chain ferritin is regulated both at the transcriptional and translational level (for Review see (MacKenzie et al. 2008)). As discussed above, ferritin chain translation is controlled by IRP-1 and -2. Regulation of the ratio of H:L chain ferritin is likely dependent on a number of different mechanisms, two of which have been suggested by results from our laboratory and are described below.

3.2. Ferritin in the lens

Ferritin is present throughout the whole lens. The level of ferritin in human lenses is approximately 100–300 ng/mg protein as determined by ELISA (Garner et al. 2000b; Levi et al. 1998). Ferritin content in canine LEC is (60–200 ng/mg protein) (McGahan et al. 1994b) which is similar to that of human lenses. However, the concentration of ferritin in canine lens fiber cells is much lower (0.08 ng/mg protein) (Goralska et al. 2007)). Ferritin was undetectable in guinea pig lenses despite the high level of ferritin L-chain mRNA (Cheng et al. 2000). Lenticular ferritin has a higher content of H- than L-chain (Goralska et al. 2001) and is similar to the H-chain rich ferritins of brain and heart tissues. Cells strictly regulate the tissue-specific ratio of H:L chains but the mechanisms of this regulation are not fully understood. The H-subunit, which has a ferroxidase activity, is responsible for iron oxidation and uptake, but also has other important biological functions. When overexpressed, H-chain reduces the cell proliferation rate (Guo et al. 1998), decreases apoptosis (Cozzi et al. 2003), reduces heme and hemoglobin synthesis (Picard et al. 1996) and can create an iron-deficient phenotype by efficiently chelating intracellular iron (Cozzi et al. 2000).

3.3. Regulation of H:L chain ferritin ratio

We determined that canine LEC, when transiently transfected with a plasmid containing H-chain cDNA, maintain a tissue specific H:L ratio. This is accomplished by increasing the biosynthesis of endogenous L-chain and releasing excess H-chain, present as homopolymeric H-chain ferritin, into the culture medium (Goralska et al. 2003b). The transfection of LEC with a plasmid containing L-chain cDNA resulted in a significant accumulation of L-chain in the cytosol due to limited release of this chain (Goralska et al. 2003b). The other important mechanism by which cells control the level of ferritin subunits is the differential degradation of these chains. The half-life of H-chain in canine LEC is significantly shorter (11 h) than that of L-chain (18 h) due to preferential degradation of H-chain by the lysosomal pathway (Goralska et al. 2005). The half-life of the L-chain is even longer if this chain is assembled into ferritin because L-chain-rich ferritin is more resistant to lysosomal degradation than H-chain-rich ferritin (Goralska et al. 2005; Kohgo et al. 1980). Maintaining the proper level of H-chain, and therefore the tissue specific H:L chain ratio, is important for normal cell function. Strict control of the level of H-chain ferritin could protect cells from the potentially damaging effect of this biologically active protein. Ferritin L-chain, in contrast to H-chain, is not responsible for a wide range of physiological functions and the accumulation of L-chain does not have a negative effect on any tissue other than the lens. In humans with Hereditary Hyperferritinemia Cataract Syndrome, ferritin L-chain is overexpressed due to point mutations within the 3'-untranslated regulatory sequence of its mRNA. These mutations result in a decreased ability of IRPs to bind to mRNA and to inhibit translation of L-chain. Opacities in lenses from these patients are due to the accumulation of intracellular aggregates of L-chain-rich ferritin (Brooks et al. 2002).

The main role of ferritin is to safely store redox-active iron, therefore protecting cells against oxidative damage caused by iron-catalyzed free radicals. The iron storage capacity of ferritin in lens fiber cells and its possible role in prevention of cataractogenesis has not yet been elucidated. We determined that both ferritin H- and L-chains in homogenates of normal human and canine lens fiber cells by Western blot are significantly modified in size and charge (Goralska et al. 2007). The canine lens fiber L-chain ferritin subunit was larger (30 kDa) than the standard canine liver L-chain (19 kDa). The canine lens fiber H-chain ferritin subunit was smaller (12 kDa) than the standard canine heart H-chain (21 kDa). Human lens fiber cells contain normal size L-chain (19 kDa) and truncated H-chain (10kDa; comparable in size to the canine fiber cell H-chain ferritin). The modified chains accumulate with age and their concentration is higher in older than in younger individuals. By in vitro labeling with ⁵⁹Fe we also detected assembled holoferritin and found that the amount of holoferritin declines with age in fiber cells (Goralska et al. 2007). These changes in the structure of ferritin chains are most likely caused by posttranslational modifications. An age-related increase in the accumulation of modified chains and decreased presence of holoferritin likely results in a lowered ability to safely store iron in the fiber cells. This would result in decreased protection against iron-generated free radicals, increased oxidation of lens proteins and contribute to cataract formation (Spector 1995; Truscott 2005b). Interestingly, the ferritin H-chain detected in canine lenses with age-related nuclear cataract was larger (29 kDa) than that found in normal lenses (12 kDa) of the same age and accumulated in deeper fiber layers of the lens (Goralska et al. 2008). There was no difference in the characteristics of L-chain from normal and cataractous canine lenses.

3.4

Mitochondrial ferritin is 80% homologous to H-chain ferritin found in the cytoplasm. A precursor protein of 242 amino acids includes a 60 amino acid recognition signal for the import of this protein to the mitochondrion (Levi et al. 2001). This protein has ferroxidase

activity and stores iron more efficiently than cytoplasmic ferritin (Corsi et al. 2002). There is no information available regarding mitochondrial ferritin in ocular tissues.

3.5 Nuclear ferritin

Little is known about iron metabolism in the cornea. However, there are a series of intriguing studies of an H-chain ferritin that is present solely in the nucleus of chick corneal epithelial cells where it is proposed to protect nuclear DNA from oxidative damage (Cai et al. 1997) (for review see (Linsenmayer et al. 2005)). This ferritin is developmentally regulated and protects against UV damage and iron exacerbation of this damage due to reactive oxygen species (Cai et al. 2008; Cai et al. 1998). This type of protection would be extremely important for a tissue like the cornea, which is constantly exposed to UV light. Deletion constructs were used to demonstrate that there is a tissue specific nuclear translocation mechanism (Cai and Linsenmayer 2001). Indeed, a protein named ferritoid was found that has a functional SV40-type nuclear localization signal and contains numerous sequences with similarity to ferritin. Removal of the nuclear localization signal prevented both ferritin and ferritoid from entering the nucleus. Co-immunoprecipitation experiments determined that ferritin and ferritoid bind to each other and that this complex is found in the nucleus. It was hypothesized that ferritoid serves as a chaperone, guiding ferritin from the cytoplasm to the nucleus (Millholland et al. 2003).

In other cell types, H-chain-rich nuclear ferritin is generally only found in pathological conditions, such as iron overload or in tumor cells (Iancu et al. 1985; Smith et al. 1990). It may serve an important function under these conditions, perhaps protecting against DNA damage.

3.6 Iron uptake into ferritin

It is well-known that ferrous iron, Fe(II), oxygen and the ferroxidase activity of H-chain ferritin are essential to the movement of iron into and formation of the iron core of ferritin. However, a mechanism by which iron moves from its cytoplasmic or nuclear locations to ferritin was not identified until quite recently. It appears that the ubiquitous RNA binding protein poly(C)-binding protein-1 (PCBP1) also functions as a cytosolic iron chaperone, delivering Fe(II) to ferritin. PCBP1 binds 3 iron atoms, binds to ferritin only in the presence of iron and enhances the mineralization of ferritin (Fig. 2). Knock-down of PCBP1 with siRNA greatly decreases the incorporation of iron into ferritin, while having no effect on ferritin levels or iron uptake into the cells (Shi et al. 2008). The mechanism by which PCBP1 levels and activity are regulated is currently unknown. We have found PCBP1 in both LEC and RPE (unpublished data), but its function in these cells is yet to be determined.

4. Iron uptake into cells

4.1 Transferrin

In plasma, iron is bound to the transport protein, transferrin, which has two iron binding sites. Iron is transported from sites of storage (in the liver) and absorption (in the intestine) to the rest of the body. All mammalian cells have transferrin receptors (TfR) on their cell surfaces. Only diferric transferrin can bind to these receptors and the receptor with its cargo is endocytosed. After acidification of the endosome, transferrin releases its iron and is recycled to the membrane where it is released to the extracellular space. At physiological pH and without iron bound, transferrin has low affinity to its receptor. Iron in the endosome is then transported out through ferroportin or by divalent metal transporter-1 (DMT1) (see 4.3 and Fig. 2) and moves to sites of storage and utilization. The regulation of these processes is poorly understood.

Normal levels of transferrin in the IOFs are very high compared to other proteins, indeed, transferrin makes up about 25% of the total protein in the vitreous humor (Dernouchamps et al. 1975). We found that the lens synthesizes and secretes transferrin (McGahan et al. 1995) while others have found that the RPE also secretes transferrin (Beazley et al. 2008; Hunt and Davis 1992). The presence of the blood-ocular barriers prevents the movement of large proteins, such as transferrin, into the eye. It was therefore hypothesized that tissues residing behind such barriers make and secrete transferrin in order to capture iron for maintenance of critical physiological functions.

In addition to its essential role in delivery of iron to tissues, transferrin has another function related to its ability to bind iron. Under normal conditions, transferrin has latent, unoccupied iron binding sites with very high affinity for iron. Transferrin therefore has very potent antioxidant activity because it will bind any “free” iron in its vicinity and iron bound to transferrin is incapable of catalyzing free radical formation. We demonstrated that during intraocular inflammation the iron content and total iron-binding capacity of the IOFs is significantly increased, likely due to influx of iron from plasma through disrupted blood-ocular barriers (McGahan and Fleisher 1986b; McGahan and Fleisher 1988). The antioxidant activity of the IOFs increased in parallel, likely due to the increase in transferrin concentration (McGahan and Fleisher 1986a). Injection of xanthine oxidase into the vitreous humor catalyzes the formation of free radicals and generates an inflammatory response (McGahan and Fleisher 1992; Mittag et al. 1985; Sery and Petrillo 1984). This response was almost completely blocked by co-injection of apotransferrin (iron free), but not by iron-saturated transferrin (McGahan et al. 1994a). These results indicate that the inflammatory response generated by xanthine oxidase is dependent on iron and that transferrin acts as an antioxidant and anti-inflammatory agent by its ability to bind and inactivate iron.

It is likely that transferrin has significant physiological roles in the retina as the levels of its mRNA in human retinas are 6-fold higher than in liver or cerebral cortex (Chowers et al. 2006; Farkas et al. 2004; Yefimova et al. 2000). These roles could include regulation of iron metabolism, antioxidant activity or neurotrophic effects (Bruinink et al. 1996; Hyndman et al. 1991). Recently it was found that retinas from patients with AMD had increased levels of transferrin mRNA and protein compared to retinas from control patients (Chowers et al. 2006).

4.2 Transferrin receptors

Transferrin receptor-1 (TfR-1) is present in all cell types except mature red blood cells and functions as a receptor for transferrin-bound iron uptake (see section 4.1 and Fig. 2). Its levels are acutely regulated at the translational level, with the binding of an IRP protein to a number of stem loops (iron response elements, IRE) in the 5′-untranslated region of the transferrin receptor mRNA. This binding stabilizes the mRNA and promotes increased synthesis of the receptor (Fig. 1). Therefore, with increased iron content in the cell, the IRP is not available to bind to and stabilize transferrin receptor mRNA. Decreased availability of iron increases the binding of IRP and stabilizes the message, thus increasing the ability to transport iron into the cell (Muckenthaler et al. 2008). Importantly, DMT1 colocalizes with TfR-1 in the endosome membrane (Fig. 2) (Gruenheid et al. 1999).

TfR-2 was identified in 1999 and has a high degree of homology with TfR-1. It is expressed predominantly in the liver and is localized on the basolateral membrane of hepatocytes (Merle et al. 2007). However, it has been found in low copy in many other cell types. As discussed in section 5.2 it is now known that TfR-2 plays an important role in regulation of hepcidin and ferroportin expression.

With respect to the eye, transferrin receptors were first documented in the developing chick retina (Cho and Hyndman 1991). Other studies of the location of retinal transferrin receptors followed these initial studies (Yefimova et al. 2000) and expression is increased with age (Chen et al. 2008) and pathology (Hadziahmetovic et al. 2008), but functional studies of iron uptake by these receptors in ocular tissues are yet to be done.

4.3 Divalent Metal Transporter-1 (DMT1)

DMT1 is an iron transport protein with high affinity for Fe(II) and a requirement for co-transport of hydrogen ion (Mackenzie and Hediger 2004). Iron deprivation results in an increase in DMT1 levels. DMT1 is regulated by an IRE in the 3'-UTR of its mRNA and binding of IRP to this IRE confers stability to the message (Fig. 1). DMT1 has 12 transmembrane domains (Cellier et al. 1995) and is responsible for intestinal absorption of Fe(II) as well as iron transport in other tissues. It co-localizes with transferrin in recycling endosomes and acidification of the endosome by a vacuolar (V)-ATPase not only allows for the release of iron bound to transferrin, but also provides the requisite hydrogen ion for co-transport of iron out of the endosome and into the cytoplasm (Fig. 2). This transporter is present in cells in every organ so far tested (Gunshin et al. 1997). However, there is only one study which shows the presence of DMT1 in the eye. Immunostaining of the retina showed the presence of DMT1 in rod bipolar cell bodies, rod bipolar cell termini, horizontal cell bodies and photoreceptor inner segments (He et al. 2007).

5. Control of iron levels in cells by regulation of iron efflux

5.1 Ferroportin

Ferroportin is a protein responsible for the efflux of iron from cells, especially from intestinal cells and from macrophages involved in the recycling of iron from aging red blood cells. Mutations in the ferroportin gene lead to cellular iron accumulation (De Domenico et al. 2005). Expression of ferroportin can cause a decrease in iron stores by increased export of iron and a decrease in ferritin synthesis. Additionally, it was hypothesized that iron entry and exit from ferritin is the result of a cellular iron equilibrium dependent on regulation of iron exit from the cell through ferroportin (De Domenico et al. 2006). Ferroportin not only moves iron out of cells, it is also present in the endosomal membrane and assists movement of iron taken up into the endosomal compartment to the cytoplasm. Ferroportin levels are regulated by hepcidin as described in the next section.

In the retina, ferroportin is found in RPE and Müller cells, colocalizing with ceruloplasmin and hephaestin (Hahn et al. 2004a). Furthermore, ferroportin was found in the photoreceptor layer of the retina in AMD (Dentchev et al. 2005). However, little else is known about ferroportin's presence or function in ocular tissues.

5.2 Hepcidin and HFE

An important peptide hormone, hepcidin plays a critical role in the regulation of iron efflux from numerous cell types, including intestinal cells, macrophages and hepatocytes. Hepcidin binds to and induces the degradation of ferroportin, a protein responsible for iron efflux (Nemeth et al. 2004) (Fig. 2). Therefore, hepcidin is responsible for iron homeostasis, decreasing iron uptake from the intestine and release from the liver in conditions of iron overload. Conversely, hepcidin synthesis is decreased in iron deficiency resulting in increased iron uptake from the intestine and release from liver stores (for review see (Nemeth and Ganz 2006)).

Recent studies point to an important regulatory mechanism for hepcidin synthesis in liver cells. The Hfe gene encodes a protein (HFE) which is an atypical major histocompatibility

class1-like molecule. In Hemochromatosis patients, mutations of the Hfe gene result in iron overload. The concentration of hepcidin is low in these patients as well as in Hfe^{-/-} mice. It was hypothesized that the primary role of HFE was to control hepcidin expression. This was confirmed in a number of studies and is an interesting control mechanism (Bridle et al. 2003; Muckenthaler et al. 2003; Nicolas et al. 2003). HFE binds to TfR-1 where it is sequestered and inactive. Because there is an overlapping binding site for HFE and diferric-Tf, when diferric-Tf is present it competes with HFE for this binding site and displaces HFE which then interacts with TfR-2 to signal for hepcidin production (Schmidt et al. 2008) (Fig. 2). Fe-Tf also induces an increase in TfR-2 stability (Johnson and Enns 2004; Robb and Wessling-Resnick 2004). This model accounts for the finding that hepcidin levels increase in iron overload when there is an abundance of diferric transferrin and that hepcidin levels decrease in iron deficiency. This makes sense for liver and macrophages, which are responsible for regulating of iron metabolism in the whole body. However, the role of such systems in other organs and cell types is unknown.

A recent study found that hepcidin is expressed in Müller cells, photoreceptor cells and RPE in an expression pattern similar to ferroportin's (Gnana-Prakasam et al. 2008). Interestingly, hepcidin expression was regulated by lipopolysaccharide (an experiment designed to mimic a bacterial infection) at the transcriptional level through Toll-like receptor-4. The increase in hepcidin expression correlated with a decrease in ferroportin expression, as well as an increase in oxidative stress and apoptosis, as would be expected from an increase in intracellular iron resulting from decreased iron export. The expression of hepcidin in the retina points to local intraocular regulation of iron metabolism, separate from dependence on the circulating liver-derived hormone. Circulating hepcidin would likely be inaccessible to intraocular tissues due to the presence of blood-ocular barriers.

In another study of the retina, HFE was found almost exclusively in the RPE, specifically in the basolateral membrane and associated with TFR-1 and -2, suggesting that it plays an important role in iron homeostasis in this tissue (Martin et al. 2006).

5.3

Ceruloplasmin (Cp) and Hephaestin have ferroxidase activity and generate Fe (III) from Fe (II). Fe (III) then binds to transferrin and is transported throughout the body to sites of storage and utilization. Although there is only one gene encoding Cp, the final protein exists in two different forms, one which is secreted and one which is linked to glycosyl-phosphatidylinositol (GPI). GPI-Cp is targeted to the cell membrane (Patel and David 1997; Patel et al. 2000) and is closely associated with ferroportin. A recent study (De Domenico et al. 2007) demonstrated that Cp is responsible for removing Fe(II) from ferroportin, thus creating a gradient for the movement of iron out of the cell. In the absence of Cp, iron remains bound to ferroportin. This signals ferroportin internalization degradation, which are independent of hepcidin. The requirement for Cp to stabilize ferroportin appears to be specific to cells that express GPI-Cp. However, in cells that do not express GPI-Cp, the absence of Cp reduces iron export.

The ferroxidase, hephaestin, is a transmembrane homologue of Cp important to the efflux of iron from intestinal cells. Since intestinal cells do not express Cp, hephaestin is believed to substitute for Cp and co-localizes with ferroportin on the basolateral membrane. Absence of either Cp or hephaestin causes a decrease in plasma iron and an increase in iron stores in many cell types (Harris et al. 1998). Importantly, patients with aceruloplasminemia have neurological disease, diabetes and retinal degeneration (Harris 2003).

5.4 Iron export proteins in the eye

While the form of Cp, GPI-linked or not, is not known for any ocular tissue, Cp and hephaestin have been demonstrated to regulate iron metabolism in the eye. For example, double knockout of Cp and hephaestin increased iron concentration in the retina and resulted in a pathological phenotype resembling macular degeneration (Hadziahmetovic et al. 2008; Hahn et al. 2004b). This double knock out indicates that hephaestin's role in iron metabolism is not limited to the intestine.

The retina expresses both the GPI-linked and secreted forms of ceruloplasmin. Photoc injury to the retina increases in the expression and secretion of ceruloplasmin into the vitreous humor (Chen et al. 2003). The ferroxidase activity of ceruloplasmin could reduce oxidative stress due to photic injury, since the conversion of Fe(II) to Fe (III) would increase the binding of iron to labile iron binding sites of transferrin which can only bind Fe (III). Importantly, transferrin is also secreted by the retina and is normally present in the vitreous humor with labile iron binding capacity (McGahan and Fleisher 1986b)

We have found that the lens makes and secretes ceruloplasmin and transferrin. Addition of both proteins increased iron efflux from cultured LEC and their effects were additive (Harned et al. 2006). We propose that ceruloplasmin catalyzes the formation of Fe(III) from Fe(II), which is presented at the cell surface by ferroportin and binds to apotransferrin which is added in excess. The addition of ceruloplasmin and apotransferrin creates a gradient for the movement of iron to the outside of the cells as originally proposed by Osaki (Osaki et al. 1971). Interestingly, in our study extracellularly added ceruloplasmin increased iron incorporation into cytoplasmic ferritin. The mechanism for this effect is unknown, but is likely a protective effect, decreasing the availability of iron for catalysis of free radical reactions.

6. Iron dependent regulation of diverse cellular processes

6.1. RPE65, an iron (II)-dependent isomerohydrolase

The most critical step in the visual cycle is the isomerization of all-trans-retinol to 11-cis-retinal, a reaction which is catalyzed by RPE65. Photon absorption by 11-cis-retinal causes its isomerization to all-trans-retinal and triggers a phototransduction cascade. In order for the visual cycle to continue, 11-cis-retinal must be regenerated by RPE65, an isomerohydrolase found primarily in RPE cells (Bavic et al. 1992; Hamel et al. 1993). RPE65 knock out mice are unable to isomerize all-trans-retinol to 11-cis-retinal, therefore resulting in accumulation of all-trans-retinol in the RPE (Redmond et al. 1998). Briard dogs with mutations in RPE65 are blind, but blindness can be reversed by delivery of the RPE65 gene (Acland et al. 2001). Mutations in RPE65 are associated with retinal diseases such as retinitis pigmentosa and Leber's congenital amaurosis (Marlhens et al. 1997; Morimura et al. 1998; Thompson et al. 2000). This knowledge has formed the basis for gene therapy which has shown success in human patients with Leber's amaurosis (Bainbridge et al. 2008; Cideciyan et al. 2008; Maguire et al. 2008).

Recent studies have shown that RPE65 is an iron-dependent enzyme and that iron chelators reduce its activity and addition of iron restores this deficit (Moiseyev et al. 2006). Therefore dysregulation of iron metabolism could have serious repercussions for the visual cycle.

6.2

Frataxin is a mitochondrial protein encoded in the nucleus which is involved in mitochondrial iron homeostasis and synthesis of heme and iron sulfur clusters, which are integral to the function of numerous proteins including aconitase (for review see,

(MacKenzie et al. 2008)). Hyperexpansion of a GAA repeat in the first intron of the frataxin gene decreases frataxin transcription and causes Friedrich's Ataxia. The biochemical changes associated with a decrease in availability of frataxin are increased accumulation of iron in the mitochondria, decreased heme production, decreased iron sulfur cluster formation, decreased oxidative phosphorylation and increased oxidative stress.

A mouse model of cardiomyopathy caused by knock out of the frataxin gene was employed to study intracellular iron metabolism (Whitnall et al. 2008). An increase in iron accumulation in the mitochondria was observed, which caused a decrease in cytosolic iron concentration resulting in decreased ferritin expression, increased iron uptake and cardiac hypertrophy. Iron chelation decreased cardiac pathology indicating a central role for iron in the pathology of frataxin loss.

There is only one study of frataxin in the eye, which focused on the pathophysiology of optic neuropathy associated with Friedrich's Ataxia (Alldredge et al. 2003). It was suggested that abnormal regulation of intracellular iron in this disease may increase the susceptibility to reactive oxygen species, which results in ganglion cell death.

6.3

Heme Oxygenase 1 and 2 (HO-1 and HO-2) are separate gene products responsible for degradation of heme. HO-1 is an inducible enzyme whose activity increases in response to iron, heme, light, oxidative stress, inflammation and cytokines (for review see (Abraham and Kappas 2008)). HO-2 is a constitutive enzyme present in most tissues. HO degrades heme to iron, carbon monoxide (CO) and biliverdin. The release of iron upregulates the synthesis of ferritin and is part of a cytoprotective mechanism (Eisenstein et al. 1991). CO has important roles in vasodilation (Pannen and Bauer 1998; Wang et al. 2006; Wu and Wang 2005). Biliverdin is subsequently converted to the antioxidant bilirubin by biliverdin reductase (Baranano et al. 2002; Sedlak and Snyder 2004; Stocker et al. 1987). It is these activities that underpin HO's role as a cytoprotective enzyme. However, there can be a negative side to HO's activity since a large release of iron can be deleterious and bilirubin is known to be neurotoxic (Kappas 2004). Therefore, during hemorrhage, excessive generation of iron and bilirubin could have negative consequences.

There is a growing literature which demonstrates the importance of HO-1 in ocular physiology and pathophysiology. Induction of HO-1 with SnCl₂ decreased inflammation and increased wound healing in a mouse model of corneal epithelial injury (Patil et al. 2008). Induction of HO-1, by pretreatment of rats with heme, reduced endotoxin-induced intraocular inflammation (Ohta et al. 2003). Prostaglandin D₂ is a mediator of allergic and inflammatory responses (Kim and Luster 2007). It was recently shown to increase HO-1 promoter activity in RPE cells which could trigger a protective response (Kuesap et al. 2008).

In retina, overexpression of HO-1 in photoreceptor cells provided protection from damage caused by intense light exposure (Sun et al. 2007). It has also been discovered that HO-1 is upregulated in a mouse model of central retinal artery occlusion (Goldenberg-Cohen et al. 2008). In studies of the retinal localization of the constitutive form of heme oxygenase, HO-2 was localized to ganglion cells in the central area of the retina, but not in the inner plexiform layer, inner nuclear layer or outer nuclear layer (Ma et al. 2004). Its presence in the ganglion cells suggests that the byproduct of HO metabolism, CO, could serve as a gaseous signaling modulator of neural activity in the retina.

It has been suggested that HO-1 is involved in regulation of intraocular pressure (IOP) via CO formation (Privitera et al. 2007). Induction of HO-1 by hemin caused a decrease in IOP

in two models of ocular hypertension in rabbits. Inhibition of HO-1 activity abolished the decrease in IOP resulting from hemin treatment. Based on these results the authors suggest that CO is involved in regulation of IOP.

In LEC, UVA irradiation increased HO-1 protein expression. This was followed by an increase in aconitase activity reflecting an increased availability of iron for the formation of the iron sulfur cluster necessary for activation of this enzyme (Rzymkiewicz et al. 2000). Since we have shown that increased aconitase activity increased glutamate secretion (McGahan et al. 2005) and glutathione levels in LEC and RPE (Lall et al. 2008), this is likely an additional protective response due to HO-1 activation. Taken together, these results clearly point to an important role for HO in ocular pathology.

6.4. Hypoxia inducible factor (HIF)

HIF-1 is a transcription factor regulating the expression of more than 60 genes (Semenza 2003). It is a dimer consisting of subunits HIF-1 α and HIF-1 β which are both constitutively expressed. HIF-1 α is rapidly degraded in the cytoplasm under normoxic conditions as long as there is an adequate supply of iron and the cofactor, 2-oxoglutarate, thus preventing dimer (HIF-1) formation (Elkins et al. 2003; Hewitson et al. 2003; Jaakkola et al. 2001; Salceda and Caro 1997). Under hypoxic conditions and in the presence of low iron availability, HIF-1 α is stabilized and is transported to the nucleus where it dimerizes with HIF-1 β forming HIF-1 (Fig. 3). HIF-1 activates numerous genes such as vascular endothelial growth factor (VEGF) and several others relevant to iron metabolism, such as Cp (Mukhopadhyay et al. 2000), transferrin receptor (Lok and Ponka 1999; Tacchini et al. 1999), transferrin (Rofls et al. 1997) and HO-1 (Lee et al. 1997). Iron chelation, which reduces the size of the LIP, induces the activity of this transcription factor (Chong et al. 2002); therefore, the size of the LIP may be central to iron's role in regulating HIF-1 α . Conversely, HIF-1's regulation of many genes relevant to iron metabolism could alter the size of the LIP. Little is known about this complex relationship since the effect of HIF-1 activation on iron metabolism is not clearly understood. In one study using a human renal cancer cell line, increased HIF-1 activation increased iron uptake and decreased ferritin levels, but increased iron incorporation into ferritin and decreased the size of the LIP (Alberghini et al. 2005).

Ischemic preconditioning of the retina prevents severe retinal injury that would normally occur in response to a subsequent episode of prolonged ischemia. Increased ferritin levels appear to provide this protection by safely storing iron and protecting the retina from iron-catalyzed free radical damage (Obolensky et al. 2008). Indeed, this model of ferritin protection against the damaging effect of iron released during ischemia reperfusion has been proposed for other tissues, including the brain (Schaller and Graf 2002) and the heart (Nakano et al. 2000; Sommerschild and Kirkeboen 2002).

The roles of HIF-1 in retinal ischemia, proliferative diabetic retinopathy, retinopathy of prematurity and glaucoma are areas of active investigation (Arjamaa and Nikinmaa 2006; Forooghian et al. 2007; Goldenberg-Cohen et al. 2008; Yanjun et al. 2007; Zhu et al. 2007). A recent study demonstrated that HIF-1 regulates lens cell proliferation and that iron chelation reduced oxygen-induced proliferation (Shui et al. 2008). However, there are no other studies of the regulation of HIF-1 by iron or the effects of HIF-1 on iron metabolism in other ocular tissues. Considering the involvement of iron in ischemia reperfusion injury, this is an important area to explore. In preliminary studies in cultured LEC and RPE we found that iron chelation caused an increase in HIF-1 α movement into the nucleus and increased expression and secretion of VEGF (McGahan et al. 2008).

7. Factors affecting iron metabolism in the eye

We have studied the effects of a number of different treatments on iron metabolism in cultured LEC. These results are summarized in the following sections.

7.1 Ascorbic acid

There is a very high concentration of ascorbic acid in the aqueous and vitreous humor in many species (over 1 mM) which is due to active pumping of this vitamin into the IOFs. Ascorbic acid is an important reducing agent in the lens, helping to keep GSH in the reduced state. Additional protective roles for ascorbic acid include our finding that it caused large increases in ferritin synthesis in cultured LEC (McGahan et al. 1994b) and increased loading of iron into ferritin (Harned et al. 2003). These effects on ferritin synthesis and iron storage in LEC likely protects the lens against oxidative damage.

7.2 UVB irradiation

Epidemiological studies have implicated UVB irradiation in cataractogenesis (Taylor 1989; West et al. 1998). In our studies UVB caused a large increase in the accumulation of newly synthesized H- and L-chain ferritin in LEC (Harned et al. 2003). This increase was additive to that induced by ascorbic acid. While ascorbic acid increased iron storage in ferritin, UVB irradiation had no effect on this process. Ascorbic acid and UVB irradiation increase de novo synthesis of ferritin chains to the same degree. However, while ascorbic acid greatly increased the total amount of ferritin in these cells, UVB had only a very modest effect at one time point. Measurement of newly synthesized holoferritin indicated that UVB had no effect on this parameter, while ascorbic acid caused a very large increase. It was concluded that UVB-induced synthesis of ferritin chains did not result in assembly of these chains into holoferritin, which is consistent with the finding that iron storage was not increased by UVB irradiation. The reasons for such an effect are unclear. However, since H-chain ferritin does have important physiological roles, the UVB effect may be important for regulating these other, non-iron storing, aspects of H-chain ferritin function.

7.3 Tempol

The nitroxide, Tempol, can protect cells from oxidative damage (Krishna et al. 1996; Krishna and Samuni 1994; Rachmilewitz et al. 1994) and has recently been shown to prevent symptoms of neurodegenerative disease in IRP-2 knockout mice (Ghosh et al. 2008). Tempol's activities include the ability to scavenge superoxide (Krishna et al. 1996) and to oxidize Fe(II) to Fe(III) (Mitchell et al. 1990). Our studies in LEC indicate that at the doses used in these experiments, Tempol has a protective effect at the early time point (6h), but loses this protection at later time points; in fact at 20h it is toxic (Goralska et al. 2000). We also found a decrease in ferritin synthesis and a decrease in iron incorporation into ferritin. This finding is similar to that of Tempol's effects in the IRP-2 knockout mice. However, Ghosh, et al. (Ghosh et al. 2008) did not find any toxic effects of Tempol fed to the mice over a period of 8 months. This difference from our data could be dose and/or species dependent.

7.4 α -Lipoic acid

The antioxidant properties of α -lipoic acid have been well studied (Packer et al. 1995; Petersen Shay et al. 2008) and its potential clinical use in numerous pathological situations is substantial (Novotny et al. 2008; Salinthon et al. 2008). The central role of iron-catalyzed reactions in oxidative stress, and the fact that lipoic acid can act as an oxidant in the presence of iron (Scott et al. 1994) led us to investigate the effects of lipoic acid on ferritin synthesis, iron incorporation into ferritin and the resistance of cultured LEC to hydrogen

peroxide toxicity (Goralska et al. 2003a). We found that lipoic acid increased ferritin levels and iron incorporation into ferritin. Additionally, lipoic acid decreased iron uptake from transferrin. The combination of an increased incorporation of iron into ferritin and reduced iron uptake would result in a decrease in the LIP available for catalyzing free radical reactions. Indeed, lipoic acid significantly decreased hydrogen peroxide-induced cytotoxicity.

7. Summary

There are numerous proteins that were originally thought to be made almost exclusively by the liver (ceruloplasmin, transferrin, hepcidin) or intestinal cells (hephaestin). However tissues existing behind blood-ocular and blood-brain barriers apparently are capable of synthesizing these proteins and therefore provide local regulation of iron metabolism. A complete understanding of iron metabolism in the eye and brain is essential in light of the preponderance of evidence that dysregulation of iron metabolism occurs in neurological and retinal degenerations as well as diseases such as glaucoma and inflammation. On the other hand, proper regulation of iron proteins can have profound protective effects against oxidative damage. Unfortunately, as described in this review, very little is known about iron metabolism in ocular tissues, leaving a field ripe for investigation.

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Abbreviations

IRP	iron regulatory protein
IRE	iron response element
LEC	lens epithelial cells
RPE	retinal pigmented epithelial cells
GSH	glutathione
HO	heme oxygenase
IOFs	intraocular fluids
PCBP1	poly-c binding protein-1
TfR	transferrin receptor
DMT1	divalent metal transporter-1
GPI	glycosyl-phosphatidylinositol
AMD	age-related macular degeneration
Cp	ceruloplasmin

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Figure 1. Functions and regulation of iron regulatory protein-1 and -2 (IRP-1 and IRP-2)
 IRP-1 exists in two conformations depending upon the availability of iron. When iron is present IRP-1 attains aconitase activity and does not bind the iron response element (IRE) in either the 3' or 5'-untranslated regions (UTRs) of the mRNAs of numerous proteins including those listed here (ferritin H- and L-chains, ferroportin (Fpn), transferrin receptor (TfR) or divalent metal transporter-1 (DMT1)). The presence of iron also causes the degradation of IRP-2, thus also decreasing IRP binding to IREs. When the iron content of the cells is low, both IRP-1 and IRP-2 can bind to the IREs. Current knowledge indicates that IRP-1 is generally present in the cytosolic aconitase (c-aconitase) conformation and that IRP-2 is mostly responsible for IRE binding and regulation of the translation of many proteins.

Figure 2. Regulation of intracellular iron content

Transferrin has two iron binding sites which have high affinity for Fe (III). Diferric transferrin binds to transferrin receptor-1 (Tfr-1) which is then endocytosed. The endocytic vesicle is acidified by the actions of a vesicular ATPase (V-ATPase) which is a proton pump, actively moving H⁺ into the vesicle. At low pH, transferrin releases its cargo of iron which can leave the endocytic vesicle either through ferroportin or by co-transport with H⁺ through the divalent metal transporter-1 (DMT1). Iron may then transit through what is called the labile iron pool (LIP) to sites of storage or utilization, or may leave the cell through ferroportin present in the plasma membrane. Iron can also enter the LIP from heme which is broken down by heme oxygenase. Iron brought into the cells can regulate the activities of the iron regulatory proteins-1 and -2 (Fig. 1), which in turn regulate proteins involved in iron metabolism. It can also be chaperoned by PCBP1 to safe storage in ferritin. The protein hepcidin regulates ferroportin levels by triggering its degradation. In turn, hepcidin is regulated by HFE which triggers an increase in hepcidin synthesis. HFE is normally bound to transferrin receptor-1 (Tfr-1), but the binding site is also recognized by diferric transferrin which competes with HFE for binding. The HFE is released and binds to transferrin receptor-2 (Tfr-2) which triggers a signaling pathway up-regulating hepcidin. The movement of iron out of the cell by ferroportin is enhanced by the ferroxidase activities of ceruloplasmin (Cp) and/or hephaestin. In this figure, dotted lines indicate movement and solid lines indicate descriptors. The labile iron pool (LIP) is surrounded by a dotted line indicating that the LIP is not a discrete entity located within an organelle, but likely exists throughout the cytoplasm.

Fig. 3. Iron regulation of hypoxia inducible factor-1α and glutamate production

In the presence of normoxia, iron and 2-oxoglutarate, HIF-1α is targeted for degradation by the proteasome. However, in hypoxic conditions and when iron is limited in availability, HIF-1α moves into the nucleus where it dimerizes with HIF-1β to form the transcription factor HIF-1 which regulates the synthesis of VEGF, genes regulating iron metabolism and many other genes (see Section 6.4). Iron also regulates c-aconitase activity resulting in control of glutamate synthesis, cystine uptake and glutathione levels in RPE and LEC (see Section 2.1.2).