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

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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 intracellularly 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.

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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) 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

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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 transmembrane 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

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