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Molecular Mediators Governing Iron-Copper Interactions

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Abstract

Given their similar physiochemical properties, it is a logical postulate that iron and copper metabolism are intertwined. Indeed, iron-copper interactions were first documented over a century ago, but the homeostatic effects of one on the other has not been elucidated at a molecular level to date. Recent experimental work has, however, begun to provide mechanistic insight into how copper influences iron metabolism. During iron deficiency, elevated copper levels are observed in the intestinal mucosa, liver, and blood. Copper accumulation and/or redistribution within enterocytes may influence iron transport, and high hepatic copper may enhance biosynthesis of a circulating ferroxidase, which potentiates iron release from stores. Moreover, emerging evidence has documented direct effects of copper on the expression and activity of the iron-regulatory hormone hepcidin. This review summarizes current experimental work in this field, with a focus on molecular aspects of iron-copper interplay and how these interactions relate to various disease states.

Keywords

intestine; liver; divalent metal-ion transporter 1; ferroportin 1; copper-transporting ATPase1; ceruloplasmin; hephaestin

INTRODUCTION

Iron and copper are unique among the essential trace minerals in that they exist in two oxidation states and are highly redox active; as such, their metabolism must be tightly controlled. This reactivity underlies one of their most important biological roles in electron transfer (i.e., redox) reactions, in which they function as enzyme cofactors. The homeostasis of both minerals is regulated by systemic and cellular mediators. Given their similar physical and chemical properties, it is not surprising that notable physiologic interactions between iron and copper have been described in mammals (18, 25). These points of intersection, although physiologically important, are, however, fewer than the multitude of distinct homeostatic mechanisms by which body iron and copper levels are maintained.

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Iron assimilation from the diet must be tightly controlled because no active, regulated excretory pathways for iron exist in humans. Systemic iron homeostasis is principally controlled by the liver-derived, peptide hormone hepcidin (HEPC), which effectively regulates circulating iron levels by inhibiting intestinal iron absorption and blocking iron release from body stores. Additional cell-based transcriptional and posttranscriptional regulatory mechanisms also exist, which collectively modulate the expression of genes encoding iron homeostasis-related proteins. These mechanisms include transactivation of genes in enterocytes by a hypoxia-inducible factor (HIF2 α) during iron deprivation (with consequent hypoxia) and posttranscriptional control of mRNA levels within many cells via interaction of a stem loop structure within the transcripts (iron-responsive elements) with cytosolic iron-sensing proteins (iron-regulatory proteins). Intracellular modulation of iron levels also occurs by the ferritin complex, which sequesters iron, thus neutralizing its ability to potentiate production of reactive oxygen species.

Copper metabolism is also regulated according to physiologic demand, but mechanistic aspects of this regulation are not understood in detail. Systemic control of copper homeostasis by an unidentified copper-regulatory hormone has been proposed in mice (52). The purported factor was released in response to low cardiac copper levels, and it influenced intestinal copper absorption and copper release from the liver by inducing expression of copper-transporting ATPase 1 (ATP7A). Moreover, copper metabolism is regulated within cells by a host of cytosolic chaperones that mediate intracellular copper trafficking. Copper may also be sequestered within cells by the copper-binding (and zinc-binding) protein metallothionein. Overall body copper levels are regulated by excretion into the bile; biliary copper is not reabsorbed in the gut.

Adequate iron and copper intake is critical for mammals, especially during times of rapid growth, for example in the peri- and neonatal periods. This fact is exemplified by the dire physiologic consequences that arise from deficiency of these essential trace minerals in humans. According to the World Health Organization, iron deficiency is the most common nutrient deficiency worldwide. Infants and children who do not consume adequate iron during critical developmental periods suffer from life-long cognitive impairments (18). Even in developed countries such as the United States, iron deficiency is common, occurring frequently in children and adolescents as well as in women of childbearing age, and during pregnancy and lactation. Dietary intake data indicate that average iron intake is below the Recommended Dietary Allowance of 18 mg/day for adult females. Copper deficiency, in contrast, occurs less frequently and is most often associated with a rare genetic disorder of copper metabolism, Menkes disease. Patients suffering from Menkes disease have inherited mutations in the copper-transporting ATPase 1 (*ATP7A*) gene, resulting in defective intestinal copper transport and consequent severe systemic copper deficiency. The physiologic outcomes of copper deficiency during early life are devastating, particularly with regard to brain development.

Perhaps the first detailed description of iron-copper interactions in humans dates back to the mid-1800s in Italy and France. At that time, young women in particular suffered from a disease called chlorosis (or the green sickness), which was typified by a pale complexion, amenorrhea, lethargy, and decreased work output (25). Although detailed clinical

information regarding this condition is lacking, it is likely that the underlying cause was iron-deficiency anemia (which is still very common today in this demographic group). Interestingly, it was noted that young women working in certain copper factories did not suffer from chlorosis. This suggested that copper exposure positively influenced iron homeostasis. Given our current knowledge of body iron homeostasis, one might then predict that copper enhances intestinal iron absorption during anemia, because this is the physiologic step that ultimately controls body iron levels (since no regulated excretory systems exist). This is a logical postulate because those suffering from iron-deficiency anemia would presumably have depleted iron stores (which could thus not contribute to the correction of the anemic phenotype). It is also conceivable that these chlorotic young women suffered from copper deficiency, which causes an iron-deficiency-like anemia (6). This possibility is perhaps less likely given that copper deficiency has been infrequently reported in humans. Experimental copper deficiency has, however, been extensively studied in animals, and three possible points at which copper could influence iron homeostasis have emerged: (a) intestinal iron absorption; (b) iron availability for, or utilization by, developing erythroid cells; and (c) hemoglobin synthesis. Regardless of whether iron and/or copper deficiency contributed to the development of chlorosis, these observations emphasize the longstanding appreciation of iron-copper interactions in humans.

Whole-body iron and copper homeostasis is summarized and points of iron-copper interaction are highlighted in Figure 1. Dietary iron and copper are absorbed in the upper small bowel, with absorption of both minerals being regulated according to physiologic requirements. Iron absorption is hormonally controlled by circulating HEPC, which modulates iron flux to precisely meet the body's demand for iron, particularly to support erythropoiesis. HEPC functions by inhibiting iron efflux from enterocytes and iron-storing cells in liver, spleen, and bone marrow [termed reticuloendothelial (RE) macrophages]. The mechanism of action involves HEPC binding to the iron exporter ferroportin 1 (FPN1) on the basolateral membrane (BLM) of enterocytes (and RE macrophages in other tissues), leading to internalization and subsequent degradation of FPN1 (75). How intestinal copper transport is regulated is not understood in detail, but it is clear that absorption responds to physiologic demand. After absorption, iron binds to transferrin (TF), and copper binds to albumin or α 2-macroglobulin in the interstitial fluids, followed by delivery to the liver via the portal circulation. Iron may then be stored within hepatocytes or released and bound to TF for distribution throughout the body (Figure 1). Most hepatic copper is incorporated into ceruloplasmin (CP), which is subsequently secreted from hepatocytes into the blood. Both metals are widely distributed to all organs/tissues/cells, as they play critical metabolic roles. Most iron, however, travels to the bone marrow to support hemoglobin production by developing erythrocytes.

Notable areas of interplay between iron and copper include the duodenal epithelium (Figure 1, inset), where both minerals are absorbed. Proteins that mediate potential points of interaction in enterocytes include an iron importer [divalent metal-ion transporter 1 (DMT1)], a metalloredutase [cytochrome B reductase 1 (CYBRD1); also called duodenal cytochrome B], an iron exporter (FPN1), and a ferroxidase (FOX) [hephaestin (HEPH)]. The ATP7A copper transporter is also highlighted in Figure 1; given its strong induction during

iron deficiency (16), it was postulated to influence iron transport. Another site of iron-copper interaction is the liver, where the soluble, multicopper FOX CP is produced and secreted into the blood. Copper contained within CP constitutes the largest serum copper pool. Circulating CP, however, is not necessary for copper delivery to tissues, demonstrating that other copper-binding molecules exist (44). CP and a membrane-anchored form, glycosylphosphatidylinositol (GPI)-CP (encoded by a transcript splice variant) (84), function mainly in iron homeostasis by mediating iron release from sites of iron storage and from the central nervous system. The FOX activity of CP is critical for normal iron homeostasis, as exemplified by the phenotype of patients with aceruloplasminemia, who accumulate iron in the pancreas, retina, liver, and brain. Similar tissue iron loading may also be seen with copper restriction, as CP and GPI-CP require copper for normal FOX activity. This exemplifies one aspect of the reciprocal relationship between iron and copper in the liver, whereby deficiency of one metal precipitates accumulation of the other. How iron deficiency causes hepatic copper loading is unclear, but copper deficiency probably causes iron loading because of decreased CP FOX activity (29). Copper is also required for hemoglobin synthesis in developing erythrocytes within the bone marrow, but the mechanism behind this observation is currently unknown. Furthermore, a novel, multicopper FOX (zyklopen) was recently discovered in the mammalian placenta (8). Additional experimentation is required to elucidate the role of zyklopen in iron homeostasis.

This review is intended to critically analyze current research that pertains to iron-copper interactions. It has been recognized since the nineteenth century that these two minerals have physiologically relevant interactions (25), but in many cases, interplay at the molecular level is not understood. Certainly, the multicopper FOXs, CP and HEPH, mediate some aspect of the interactions, as they are copper-containing proteins with documented roles in iron homeostasis. However, these proteins may not be the only mediators of the homeostatic intersection of these two essential trace minerals. The focus herein is on several cell types where copper has been shown to influence iron homeostasis, including enterocytes of the upper small intestine, hepatocytes, erythroid cells, and RE macrophages. Although important iron-copper interactions also occur in the brain and central nervous system [for a recent review, see (18)], this topic is not considered here.

ABSORPTION OF DIETARY IRON AND COPPER

Dietary iron and copper are absorbed in the proximal portion of the small intestine; molecular aspects of these processes have been recently elucidated. Several transmembrane iron and copper transporters, intracellular storage proteins, and chaperones (mainly for copper) have been discovered, principally by investigations in mutant rodent models and knockout mice. Figure 2 depicts iron and copper homeostasis in a duodenal enterocyte, highlighting points of interaction.

Dietary iron is found in heme and nonheme forms, with the former being derived principally from meat products and the latter from both meat and plant sources. Heme iron transport has been studied recently, but the specific mechanism(s) by which it is absorbed remain unclear (58, 92). Mechanisms of nonheme iron absorption, however, are understood in detail. Nonheme iron exists in foods mainly as ferric iron (Fe^{3+}), which must be reduced to ferrous

iron (Fe^{2+}) to be transported into enterocytes. This process is mediated in part by the brush-border membrane (BBM) ferrireductase CYBRD1 (72). CYBRD1 mRNA expression increases dramatically upon iron deprivation in laboratory rodents (16, 20) along with other genes encoding proteins with proven roles in iron absorption, suggesting that CYBRD1 is an important player in intestinal iron homeostasis. *CYBRD1* knockout mice, however, are not iron deficient and can adequately absorb dietary iron (36). A more recent study provides evidence that CYBRD1-mediated ferrireduction is necessary for maintenance of spleen iron stores and erythropoietic activity during hypoxia (13). Furthermore, in mice, CYBRD1 was shown to be the only iron-responsive ferrireductase, presumably providing additional ferrous iron for transport by DMT1 to support enhanced iron absorption in response to hypoxia (61), iron deprivation, and pregnancy (13). Interestingly, it was also recently reported that CYBRD1 is a potential modifier of the iron-related phenotype in patients with hereditary hemochromatosis (85). CYBRD1 may thus be necessary to maintain iron homeostasis when demand for dietary iron increases, but it is not absolutely required under basal conditions.

After reduction, ferrous iron (Fe^{2+}) is transported across the apical membrane by DMT1 (35), which is a ferrous iron/proton cotransporter. A nonredundant role for DMT1 is best exemplified by the intestine-specific *DMT1* knockout mouse, which suffers from severe iron-deficiency anemia due to impaired duodenal iron absorption (34). Once ferrous iron crosses the BBM of enterocytes, it is presumably delivered to different intracellular locations (e.g., mitochondria, nucleus) by as yet unidentified iron-binding proteins or chaperones. When intracellular iron levels are high, iron can be complexed with ferritin, essentially equating to a mucosal block to iron absorption, as most ferritin-bound iron is subsequently lost via exfoliation of intestinal epithelial cells (IECs). After iron traverses the cytosol, it is transported across the BLM via FPN1 (19). Iron efflux is functionally coupled to an oxidase, HEPH (110), which localizes to the BLM of enterocytes in close proximity to FPN1 (38, 120). HEPH, however, is not absolutely required for intestinal iron absorption, as adult mice expressing a mutant HEPH protein with dramatically reduced activity [sex-linked anemia (*sla*) mice] are only mildly iron deficient (110). Other FOXs, which complement HEPH activity, may thus exist in rodent IECs (94, 95). Another possibility is that CP participates in iron oxidation in the gut. CP was in fact shown to be particularly important for iron absorption in mice during states of hematopoietic stress (11). Once iron is oxidized, it interacts with *apo*-transferrin in the lamina propria of villi for transport in the portal blood to the liver.

Absorption of dietary copper has also been intensively investigated over the past decade. Dietary copper must be reduced from the cupric (Cu^{2+}) state, the form that is present in foods, to the cuprous (Cu^{+}) state for transport across the BBM of IECs. Recent experimental evidence suggests that CYBRD1 can reduce copper (117). Other candidate cupric reductases include the six-transmembrane epithelial antigen of the prostate (STEAP) family proteins (79). After reduction, copper enters enterocytes via copper transporter 1 (CTR1) (76). Interestingly, *CTR1* null mice accumulate copper within enterocytes of the proximal small bowel, but the copper is biologically unavailable because the mice suffer from severe systemic copper deficiency (76). CTR1 may thus mediate endocytosis of copper and subsequent transfer into the cytosol (Figure 2). DMT1 may also transport copper (2, 3), but

the physiologic significance of such remains in question (46). Copper transport by DMT1 certainly seems plausible, particularly during iron deprivation, when DMT1 expression increases dramatically (97, 103, 122).

Given the reactivity of free copper, exquisite regulatory mechanisms have evolved in mammalian IECs whereby absorbed copper is transferred to a series of intracellular chaperones. One such chaperone, COX17 [a chaperone for cytochrome C oxidase (CCO)], delivers copper to the site of CCO biosynthesis in mitochondria (82). Another chaperone, ATOX1 (antioxidant 1 copper chaperone) (60), delivers copper to ATP7A within the *trans*-Golgi network (TGN). ATP7A pumps copper into the TGN to promote the biosynthesis of cuproenzymes. Another chaperone delivers copper in the cytosol to Cu, Zn-superoxide dismutase [copper chaperone for superoxide dismutase 1 (CCS)] (7). Under conditions of copper excess, copper can bind to metallothionein (MT) within the cytosol, which presumably leads to copper loss upon exfoliation of IECs. Copper may then be transported out of IECs by ATP7A, which traffics from the TGN to the BLM when intracellular copper levels are elevated (86). Unlike for iron, an oxidase is probably not required for copper because the oxygen tension in the interstitial fluids is sufficient to cause spontaneous oxidation of cuprous copper to the cupric (Cu^{2+}) state. Copper subsequently binds predominantly to albumin in the interstitial fluids for delivery to the liver via the portal circulation.

IRON-COPPER INTERACTIONS IN INTESTINE

It has been recognized for many decades that copper influences intestinal iron homeostasis (25). Recent studies have begun to provide mechanistic insight into molecular aspects of this interaction. Emerging evidence exemplifies iron-copper interactions in duodenal enterocytes. The BBM ferrireductase CYBRD1 can reduce iron and copper (117), demonstrating a potential point of intersection. Moreover, Matak et al. (71) recently reported that CYBRD1 was induced in the intestinal epithelium of copper-deprived mice. The proposed mechanism related to intestinal hypoxia resulting from copper-deficiency anemia and a concomitant increase in CYBRD1 gene transcription mediated by the hypoxia-inducible transcription factor HIF2 α . Further studies provide evidence that DMT1 is another potential link between iron and copper metabolism. Although the apically expressed DMT1 protein can transport multiple metal ions in heterologous over-expression systems (principally iron, cobalt, and manganese) (46), whether it transports copper remains enigmatic. This is a potentially important issue given the possible influence of copper on intestinal iron transport. When human DMT1 was expressed in *Xenopus* oocytes, copper transport (in the cupric or cuprous state) was not observed (46). Several groups have, however, provided evidence that DMT1 can transport cuprous (Cu^+) copper, for example in Caco-2 cells (3) and in DMT1-overexpressing HEK293 cells (2). Jiang et al. (49) also used the DMT1-overexpression HEK293 cell model as well as a complementary ex vivo duodenal loop model to provide evidence that DMT1 transports copper specifically during iron deprivation. These observations suggest that DMT1 could, at least in part, mediate noted increases in copper content of the intestinal epithelium in rodent models of iron deficiency (22, 97). However, further experimentation is required before a consensus can be reached regarding a physiological role for DMT1 in copper homeostasis.

Recent studies also support the possibility that the iron efflux step is modulated by copper. FPN1 represents the only mammalian iron exporter identified to date. Whether its expression or activity is altered by variations in copper status has been investigated. Copper deficiency in mice was shown to increase FPN1 mRNA expression, possibly by a transcriptional mechanism involving HIF2 α (71). Another recent study, however, provided contradictory results (90). These authors showed that copper restriction of rats and mice had little effect on FPN1 expression or activity. Moreover, it was also recently reported that FPN1 gene transcription was enhanced when intracellular copper homeostasis was perturbed by knockdown of the ATP7A copper transporter in rat IEC-6 cells (33). Increased FPN1 expression was associated with increased iron efflux from ATP7A knockdown cells. Further experimentation is required to clarify how copper influences FPN1 expression and/or activity.

HEPH is a copper-containing protein, and it is thus a logical prediction that its activity could be influenced by copper intake levels. Indeed, FOX activity, attributed to HEPH, decreased in the intestine of copper-deprived mice, likely contributing to the development of systemic iron deficiency (9). It was further noted that iron absorption decreased in copper-deficient rats (98), perhaps due to impaired HEPH activity (101). Moreover, when copper-deprived rats were refed a copper-containing diet, HEPH protein levels increased and iron absorption was restored (99). These rodent studies support an earlier investigation that showed an impairment of iron absorption in copper-deficient swine (62). Copper restriction may thus impair iron absorption via inhibition of HEPH FOX activity, but other mechanisms could also be involved, since none of these studies provided unequivocal proof that HEPH was the sole mediator of the noted effect on iron transport. Furthermore, it was noted that HEPH physically interacted with FPN1 in rat enterocytes and that this interaction was reduced upon iron feeding (120). This finding suggests that copper restriction, via its influence on HEPH biosynthesis, could indirectly affect FPN1 protein levels on the basolateral surface of IECs and thus influence transepithelial iron flux.

Copper homeostasis in duodenal enterocytes of rodents is likely modified by iron deprivation. The initial precipitating event may be the strong induction of DMT1 expression and activity, which enhances copper transport into mucosal cells (49). Altered intracellular copper homeostasis then is exemplified in part by the induction of MTI/MTII mRNA expression (>20-fold) (15, 16, 32). MTs are intracellular metal-ion binding proteins with high affinities for copper (51). Copper can activate MT gene transcription via a metal-responsive transcription factor, MTF-1 (121). MT induction may thus reflect increased intracellular copper concentrations or, alternatively, changes in copper distribution within IECs. Furthermore, the upregulation of ATP7A expression during iron deficiency (16, 32, 97) could also influence intracellular copper homeostasis. Enhanced ATP7A activity would be expected to alter intracellular copper concentrations and/or the biosynthesis of cuproenzymes. The exact influence of ATP7A would, however, ultimately depend upon its relative distribution between the TGN and the plasma membrane. Importantly, these coordinated copper-related molecular events occur in the setting of increased iron transport, suggesting that alterations in copper homeostasis may be functionally linked to iron metabolism.

Increased MT, DMT1, and ATP7A expression in duodenal enterocytes during iron deficiency was associated with not only copper accumulation in the intestinal mucosa but also in the liver (16, 97). Hepatic copper loading during iron deprivation has been frequently observed in several mammalian species (25). Given altered copper homeostasis in duodenal enterocytes and in the liver during iron deficiency, it was logically postulated that the ATP7A copper transporter influenced iron transport. This possibility was directly tested by silencing ATP7A in rat IEC-6 cells using small hairpin RNA (shRNA) technology (33). ATP7A knockdown increased transepithelial iron flux, supporting this hypothesis. Interestingly, this increase was associated with transcriptional induction of FPN1 expression, which was consistent with enhanced basolateral iron transfer in the knockdown cells. Collectively, these observations provide further evidence of the importance of copper in the regulation of intestinal iron absorption.

The intersection of iron and copper homeostasis in the gut is further exemplified by coordinate regulatory mechanisms that mediate the physiologic response of the intestinal epithelium to iron deficiency. Lack of iron decreases hemoglobin production, which impairs oxygen delivery to the intestine (and other tissues), leading to hypoxia. Under these conditions (i.e., low iron and hypoxia), several intestinal genes encoding iron transport-related proteins were upregulated by a transcriptional mechanism involving HIF2 α (70, 103), including *CYBRD1*, *DMT1*, and *FPN1* (107). HIF signaling in the intestine is of particular interest because copper was shown to be required for transactivation of gene expression by the HIF transcriptional complex (which contains a hypoxia-responsive HIF α subunit and a constitutively expressed HIF β subunit) (24, 69). It may then be logically postulated that increased copper levels in enterocytes (22, 97), in the liver, and in the blood (17, 21) potentiate HIF activity, leading to transactivation of genes that participate in intestinal (and whole-body) iron homeostasis. Interestingly, the HIF α subunits are also stabilized by iron deprivation in vivo and in vitro (83). Consistent with this, a recent study in Caco-2 cells demonstrated that iron chelation led to a preferential induction of HIF-responsive genes (45). HIF-related regulation of gene expression is thus likely influenced by iron and copper levels, and importantly, several HIF-target genes are involved in the homeostasis of these metals.

A recent study also provided evidence that the ATP7A copper transporter expressed in rodent IECs is a HIF2 α target (118). A follow-up investigation provided additional experimental support for the coregulation of iron and copper transport-related genes (119). In this study, it was shown that the HIF2 α -dependent upregulation of ATP7A expression during hypoxia required binding of the SP1 transcription factor to the *ATP7A* promoter, a mechanism that was postulated to also relate to *CYBRD1* and *DMT1* promoter activity. Importantly, the gene encoding another intestinal copper transporter, *CTR1*, has also been shown to be basally regulated by HIF2 α (88). The fact that intestinal genes encoding iron and copper homeostasis-related proteins are regulated by a conserved transcriptional mechanism further emphasizes the physiological relevance of iron-copper interactions in the mammalian intestine.

Studies undertaken using the Caco-2 cell enterocyte model have also demonstrated important iron-copper interactions. One investigation showed that depletion of iron or

copper increased uptake of both metal ions (64) and that depletion of iron or copper enhanced iron flux. Copper supplementation of Caco-2 cells enhanced expression of iron transport-related genes in another published study (39). These authors postulated that copper treatment lowered intracellular iron levels, which secondarily potentiated transepithelial iron flux. The molecular mechanisms responsible for these findings are unknown, but nonetheless these observations collectively further exemplify the intimate interactions between iron and copper in the intestinal mucosa and provide rationale for further investigations in this area of scientific pursuit.

IRON AND COPPER HOMEOSTASIS IN THE LIVER

Iron is absorbed predominantly in the proximal small intestine, as detailed above, and is then delivered as diferric- (i.e., *holo*-) transferrin to the liver via the portal circulation. *Holo*-transferrin is endocytosed by hepatocytes via transferrin receptor 1 (TFR1) expressed on the cell surface (Figure 3). Another TFR isoform, type 2 (TFR2), can also mediate uptake of diferric-TF by hepatocytes (50). TFR2 was in fact reported to be the predominant TFR isoform expressed in human liver tissue (12). Subsequent to endocytosis of the diferric-TF/TFR complex, endosomal acidification results in iron being released from TF and then being transferred into the cytosol. Transfer of iron from the lumen of the endosome to the cytosol was suggested to be mediated by DMT1, but *DMT1* null mice do not have defects in iron uptake into the liver (31). This finding was confirmed using hepatocyte-specific *DMT1* knockout mice (112). Another candidate iron transporter, ZIP14, may mediate endosomal iron flux (26, 65). Once in the liver, iron is utilized to synthesize iron-containing proteins or exported via FPN1 followed by oxidation by CP and/or GPI-CP and distribution in the blood as diferric-TF.

After passage through enterocytes, absorbed copper (Cu^{2+}) is bound to albumin or α 2-macroglobulin and transported to the liver via the portal circulation. Transport of copper into hepatocytes (Figure 3) likely requires a reductase (possibly a STEAP protein) (55), as CTR1 transports cuprous (Cu^+) copper (77). The physiologic role of CTR1 in this process was investigated by generating mice lacking CTR1 specifically in the liver (53). These mice had moderate reductions in hepatic copper content (~50%) and similar reductions in the activity of some copper-requiring proteins. An additional, as yet unidentified, copper uptake mechanism thus also exists in the liver. After transport into hepatocytes, copper interacts with intracellular chaperones and is subsequently utilized for the synthesis of copper-dependent proteins. An ATP7A homolog, copper-transporting ATPase 2 (ATP7B), transports copper into the TGN, where it is utilized for the biosynthesis of cuproenzymes, including CP. Under conditions of copper excess, ATP7B traffics to the canalicular membrane and mediates copper efflux for biliary excretion (102). Copper excreted in bile is complexed with bile salts and is thus unavailable for reabsorption in the small intestine. Biliary copper secretion requires interaction of ATP7B with copper metabolism MURR1 domain (COMMD1) (66). Moreover, COMMD1 protein levels are regulated by the ubiquitin ligase X-linked inhibitor of apoptosis (XIAP) (67).

IRON-COPPER INTERACTIONS IN LIVER

A clear link between iron and copper homeostasis in the liver is the multicopper FOX CP. This circulating protein mediates oxidation of ferrous iron released from some tissues (including the liver) to allow binding to TF. The role of CP in iron homeostasis in humans is exemplified by the phenotype of individuals suffering from the rare genetic disease aceruloplasminemia (41). Patients lacking CP develop iron overload in some parenchymal tissues, resulting in oxidative damage to the liver (43, 108), diabetes, retinal degeneration, and progressive neurological symptoms (56). GPI-CP is also expressed in liver (as well as in numerous other rodent tissues) (73). The phenotype of CP-deficient mice mirrors that of patients with aceruloplasminemia (40). Copper-deficient rodents also accumulate iron in hepatocytes, most likely due to reduced CP activity (5). Moreover, CP levels increase during iron deficiency (47, 96), possibly as a result of liver copper loading, which enhances biosynthesis of the *holo* (i.e., active, copper-containing) form of the enzyme. CP activity and body copper levels have also been linked to perturbations in hepatic iron homeostasis in patients with nonalcoholic fatty liver disease (1).

Another potential point of iron-copper intersection in the liver is the STEAP proteins (55). STEAP2, 3, and 4 function as metalloreductases and can reduce iron and copper, enhancing cellular uptake of both metals (28, 79). Recent investigations suggest an important physiologic role for STEAP3 in iron homeostasis, as *STEAP3* knockout mice have defects in uptake of diferric-TF into erythroid cells (78) and develop iron-deficiency anemia. This is likely due to defective iron reduction within endosomes following endocytosis of the TF-TFR complex. Because STEAP3 is abundantly expressed in liver, and hepatocytes take up iron via the transferrin cycle, it is possible that STEAP3 is also important for hepatic iron metabolism. Unexpectedly, though, *STEAP3* knockout mice have higher hepatic iron levels than wild-type littermates (59). These observations do not clarify the role of STEAP3 in hepatic iron/copper homeostasis but rather provide rationale for further investigations in this area of research.

Iron-deprived rats load copper in the liver (17, 81, 97, 106); conversely, copper-deprived rats and mice have increased hepatic iron levels (14, 73, 105, 115). The latter observation may reflect decreases in hepatic ceruloplasmin production (which mediates iron release from the liver) (80). The molecular mechanisms underlying liver copper loading during iron deficiency and the physiological consequences of such are not understood in detail. During iron deprivation, hepatic copper loading could occur as a consequence of increased intestinal copper absorption. This postulate is supported by the findings that under iron-deprived conditions (a) CYBRD1 expression is upregulated (13) and it can reduce copper (117), (b) DMT1 transports copper into IECs (49), and (c) ATP7A expression dramatically increases (15, 16). Studies in iron-deficient rats, however, found no increase in whole-body copper levels (87). It could thus be that the copper accumulating in the intestinal mucosa (22, 97) and liver is not released into the blood circulation but rather has localized effects on iron homeostasis. Alternative explanations for liver copper loading are that biliary copper excretion is impaired, cuproenzyme (e.g., CP) synthesis is perturbed, or mobilization of copper from the liver is otherwise altered during iron deficiency.

Whether the iron-regulatory hormone hepcidin is influenced by copper has also been considered. Recent evidence shows that hepcidin expression is decreased by copper restriction in rats (4, 48) and mice (9). Furthermore, it was shown that hepcidin-25, the active form of the peptide, binds copper with high affinity (109). These authors demonstrated that copper was required for biologic activity of hepcidin. This observation leads to speculation that changes in body copper status could perturb iron homeostasis via dysregulation of hepcidin activity. The copper-binding properties of hepcidin-25 were in fact utilized to develop a novel detection assay for hepcidin in human serum (57), whereby the peptide was labeled with copper, followed by copper quantification by high-performance liquid chromatography and inductively coupled plasma mass spectrometry. Hepcidin also functions as an antimicrobial protein, and interestingly, copper was shown to enhance the bactericidal properties of hepcidin-25 (68).

IRON AND COPPER HOMEOSTASIS IN ERYTHROID CELLS

Cells of the erythron consume 20–25 mg of recycled iron daily to support hemoglobin synthesis. Erythroid cells acquire iron via diferric-TF, in a TFR1-dependent manner, similar to how many other body cells assimilate iron (Figure 4). Once Fe-TF is endocytosed, Fe^{3+} is reduced to Fe^{2+} by STEAP3 in endosomes, and ferrous iron is then transported into the cytosol via DMT1. The role of STEAP3 in this process was revealed by investigation of mice expressing a mutant form of the protein (78) and mice lacking STEAP3 (63). The essential role of DMT1 in iron assimilation by erythroid cells was demonstrated by experimental analysis of mice lacking DMT1 specifically in hematopoietic cells (34) and in a rat model of DMT1 insufficiency (i.e., Belgrade rats) (27).

Iron in developing erythrocytes is transferred preferentially to mitochondria, where Fe-S cluster assembly and heme synthesis occur. Transfer of iron into mitochondria requires an iron transporter on the inner mitochondrial membrane, mitoferrin (104). Once inside this organelle, an iron chaperone, frataxin, likely delivers iron to sites of usage (18). Heme synthesized in developing erythrocytes is predominantly used for hemoglobin production, with hemoglobin in mature red blood cells ultimately accounting for ~70% of total body iron.

Copper is found in human erythrocytes as well. Erythroid cells probably obtain copper via CTR1-mediated transport. Cupric copper may be reduced by a STEAP protein at the cell surface, since at least three STEAP isoforms are expressed in erythroid cells (79). A large proportion of copper (~60%) in mature erythrocytes is associated with SOD1 (23). As in other cell types, copper is transferred to cytosolic SOD1 by the CCS chaperone. Copper is also delivered to mitochondria (possibly by CCS) (111), where it is used for the production of various copper-containing proteins such as CCO. COX17 is likely required to deliver copper to CCO within mitochondria.

IRON-COPPER INTERACTIONS IN ERYTHROID CELLS

The iron-copper connection in erythroid cells has been appreciated since the seminal work of Hart et al. (42), who showed that copper is necessary for hemoglobin synthesis. These authors demonstrated that the anemia in rats consuming an all-milk diet could be corrected

by copper supplementation, whereas administration of iron salts was ineffective. To date, however, the pathological mechanisms underlying copper-deficiency anemia are still not understood in detail. The anemia associated with copper deprivation could result from diminished CP and/or HEPH expression or activity, which would decrease iron release from stores and iron absorption in the intestine, respectively, thereby limiting iron availability to support erythropoiesis. This possibility, however, is not supported by the observation that intravenous iron injection, which obviates the need for absorption of dietary iron or iron release from stores, fails to correct the anemia in copper-deficient animals (89, 100). Moreover, copper-deficiency anemia in mice can occur in the setting of normal plasma iron concentrations (91), demonstrating that lack of iron is not the cause of the anemia. The logical conclusion is then that copper deficiency impairs iron utilization by erythroid cells, which could result from defective copper uptake, impaired intracellular trafficking or transfer into mitochondria, or inhibition of heme/hemoglobin synthesis. Analysis of reticulocytes derived from copper-deficient rats showed that ferritin-iron complexes accumulate in cytosolic vesicular bodies and that copper uptake into mitochondria was impaired, even under conditions of iron excess (30). Furthermore, in reticulocytes isolated from copper-deficient swine, iron assimilation from diferric-TF was decreased 50% and heme synthesis was reduced >65% (116). The underlying molecular defect in iron utilization by developing erythrocytes during copper deprivation in different mammalian species (and in humans) thus remains unclear (89).

IRON AND COPPER HOMEOSTASIS IN RETICULOENDOTHELIAL MACROPHAGES

RE macrophages of the liver, spleen, and bone marrow play key roles in iron storage and recycling, and as such, iron homeostasis in these cells has been intensively studied. Most iron is derived from phagocytosis of senescent red blood cells (RBCs), but iron can also be acquired via the transferrin cycle. Once RBCs are ingested, hemoglobin is degraded in phagolysosomes, and heme is released. Heme oxygenase subsequently cleaves the protoporphyrin ring and liberates iron atoms, which can be utilized for cellular metabolism, stored in ferritin complexes, or exported via FPN1. RE macrophages constitute ~50% of iron stores (54), providing a readily available source of iron when erythropoietic demand increases.

RE macrophages acquire some copper from ingestion of RBCs. Copper may also be acquired by CTR1-mediated uptake. It was recently shown that CTR1 expression and copper uptake increased in a macrophage-like cell line (RAW264.7 cells) during hypoxia (114). This correlated with increased CP expression and secretion into the cell culture medium, suggesting that macrophages utilize the extra copper for CP biosynthesis. Consistent with this observation, CP mRNA expression also increased during hypoxia in mouse liver (69), possibly via HIF-mediated induction of CP gene transcription in hepatocytes (74). Because hypoxia and iron deprivation stimulate erythropoiesis, increased CP production by both cell types (RE macrophages and hepatocytes) may potentiate iron release from stores for mobilization to the bone marrow.

IRON-COPPER INTERACTIONS IN RETICULOENDOTHELIAL MACROPHAGES

Iron and copper homeostasis in RE macrophages is linked by CP (and/or GPI-CP). Iron accumulates in splenic and hepatic macrophages as a result of decreased CP expression/activity, during dietary copper deprivation, and in CP knockout mice (40). This is likely due to reduced (or absent) CP-mediated iron oxidation, which is required so ferric iron can interact with *apo*-TF. In the absence of the functional FOX, iron thus cannot be released from stores.

IRON-COPPER INTERACTIONS: FOCI FOR FUTURE RESEARCH

Although iron-copper interplay has been recognized for more than a century, in many cases the molecular basis for these observations remains obscure. In some instances, however, recent experimental work has begun to provide mechanistic insight into how iron and copper homeostasis are intertwined and the physiological significance of their interactions. This review highlighted some of these areas of recent progress and has pointed out others where little follow-up investigative work has been performed. Further study in these areas will undoubtedly provide novel insight into how copper influences iron homeostasis, which is clinically relevant given the prevalence of iron-deficiency and iron-overload-related pathologies in humans. Moreover, recent studies on copper and copper transporters as they relate to cancer, immunity, and neurodegenerative disease (37, 113) provides the impetus to further investigate how iron influences copper homeostasis. There remain many unanswered questions regarding physiologically relevant interactions of these two metals, several of which are highlighted here.

1. Is DMT1 a physiologically relevant copper transporter? Does it transport copper exclusively during iron deprivation? Does copper influence how DMT1 interacts with and/or transports iron?
2. Is CYBRD1 important for copper reduction and thus absorption into enterocytes? Are there additional metalloreductases of functional significance on the BBM of duodenal enterocytes, and if so, can they reduce both copper and iron?
3. What is the physiologic role of CP in intestinal iron transport? Since CP activity increases during iron deficiency, does it participate in the compensatory response of the intestinal epithelium to increase iron acquisition from the diet? Is the decrease of CP activity during copper deprivation significant with regard to intestinal iron transport?
4. Is decreased HEPH activity solely responsible for the decrement in intestinal iron transport during copper deprivation? Does copper deficiency alter FPN1-mediated iron efflux?
5. What is the biologic significance of increased copper levels in enterocytes during iron deficiency? Is copper redistributed in IECs during iron deprivation? What is the molecular mechanism by which ATP7A influences iron transport?

6. Does copper influence the expression and/or activity of the HIFs in cells important for iron homeostasis (enterocytes, hepatocytes, RE macrophages, and erythrocytes)? Importantly, copper has been shown in other experimental systems to be required for HIF-dependent transactivation of gene expression (24, 93).
7. What physiologic process leads to increases in hepatic copper levels during iron deficiency? Does increased hepatic copper content positively influence whole-body iron homeostasis? Does elevated copper in hepatocytes enhance the biosynthesis of *holo*-CP?
8. Why does copper deficiency cause anemia? What is the specific copper-dependent step in iron utilization by developing erythrocytes? Does it vary among mammalian species?
9. Are rats or mice better models to study iron/copper homeostasis in humans? Survey of the scientific literature shows distinct differences in trace mineral homeostasis between these rodent species. Iron-deficiency anemia in humans, which is relatively common, is associated with increased serum copper levels (likely reflecting higher serum CP) (25). Consistent with this, iron-deficient rats have increased serum copper and increased circulating CP levels, along with increased liver copper content. Copper homeostasis in mice, however, is not altered during iron deprivation or bloodletting (32). So then, does iron-copper metabolism in rats better reflect human physiology?

CONCLUSIONS

Investigations into iron-copper interactions in laboratory rodents have revealed novel aspects of mineral homeostasis that have led to a more detailed understanding of pathological mechanisms of disease, some of which are clearly relevant to human physiology. Perhaps the most biologically important aspects of these studies relate to how copper influences iron homeostasis. Perturbed regulation of iron homeostasis is primarily or secondarily related to the development of numerous clinically relevant pathological states in humans. Strikingly, copper accumulates in tissues important for iron homeostasis during states of iron deprivation, including the intestinal epithelium, the liver, and in blood. Total body copper content likely remains constant under these conditions, thus reflecting a redistribution of copper. Increased copper in the intestine (i.e., enterocytes) may enhance iron transport. In the liver, copper loading may potentiate the production of CP, which is secreted into the blood, where it is necessary for normal iron flux and recycling. Although the multicopper FOXs are certainly important for iron homeostasis, and, logically, their activity could change with copper loading (or deprivation), additional mechanisms are likely involved. Furthermore, studies in models of copper restriction have also revealed novel aspects of the influence of copper and cuproenzymes on iron homeostasis. Given these established relationships, it is important to determine whether copper may also influence iron homeostasis during states of pathologic iron overload.

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Figure 1.

Iron-copper (Fe-Cu) interactions and whole-body homeostasis of both minerals.

Physiological aspects of iron and copper homeostasis are shown, with points of interplay between the two metals indicated by circled numbers. Copper transport is shown with green arrows and lines; iron movement is shown in a dark red color. Both minerals are absorbed in the proximal small intestine. ① Several points where copper may influence iron transport are shown in the inset of a duodenal enterocyte (*asterisks*) (additional details are depicted in Figure 2). After transport to the liver via the portal vein, copper is mainly incorporated into ceruloplasmin (CP) in hepatocytes. ② Copper exits the liver principally as part of CP, which functions predominantly as a ferroxidase to promote iron release from certain tissues. Excess body copper is lost via excretion in bile. ③ Iron is bound to transferrin (TF) in the portal blood and either stored in hepatocytes bound to ferritin or released into the blood, where it is distributed as diferric-TF. ④ Iron-copper interactions in the liver are well established. Iron release from liver is a copper-dependent process as hepatic iron accumulation typifies copper deficiency. Moreover, copper accumulates in the liver during iron deprivation. ⑤ Most Fe-TF is taken up by developing erythrocytes in the bone marrow to support hemoglobin (Hb) synthesis. Iron utilization by these cells is copper dependent, although the precise mechanism is unclear. ⑥ Iron is also assimilated by other tissues, including the

brain and the central nervous system, where iron release requires glycosylphosphatidylinositol (GPI)-CP. ⑦ Another copper-containing ferroxidase (zyklopen) may also be required for iron flux in the placenta. ⑧ Iron contained within senescent red blood cells is recovered and stored by reticuloendothelial (RE) macrophages. Iron release from RE macrophages requires copper, again involving CP or possibly GPI-CP. ⑨ Iron homeostasis is controlled by the liver-derived peptide hormone hepcidin, which alters iron flux by blocking iron absorption in the intestine and iron release from stores. Hepcidin is activated by copper, exemplifying another point of iron-copper interaction. There are no active, regulated excretory mechanisms for iron in humans, but some iron is lost by desquamation of skin cells and exfoliation of enterocytes, and by blood loss. Abbreviations: CTR1, copper transporter 1; CYBRD1, cytochrome b reductase 1; DMT1, divalent metal-ion transporter 1; HEPH, hephaestin; TGN, *trans*-Golgi network.

Figure 2.

Iron-copper homeostasis and interactions in duodenal enterocytes. An intestinal epithelial cell (IEC) is depicted with the iron and copper transport machinery. Points of interaction between the two minerals are indicated by circled numbers. ① Both metals have to be reduced for absorption, and cytochrome B reductase 1 (CYBRD1) (and/or other reductases) may act upon both ions on the apical surface. ② Once reduced, iron enters IECs via divalent metal-ion transporter 1 (DMT1) along with cotransported protons. DMT1 may also transport copper, perhaps only during iron deficiency (FeD). The electrochemical proton gradient across the brush-border membrane (BBM) is maintained via the action of an apical sodium-hydrogen exchanger (NHE) and the basolateral membrane (BLM) Na⁺/K⁺ ATPase (not shown). Absorbed iron may be transported into mitochondria for metabolic use, stored in ferritin or ③ transported across the BLM by ferroportin 1 (FPN1). The expression and/or activity of FPN1 may be influenced by copper. ④ Ferrous iron must then be oxidized by the multicopper ferroxidase hephaestin (HEPH) or other ferroxidases (not depicted) to allow binding to transferrin (TF). Copper, after reduction at the BBM, enters cells via copper transporter 1 (CTR1) and is then distributed by intracellular chaperones. ⑤ Copper enters the *trans*-Golgi network (TGN) via ATP7A for use in cuproenzyme synthesis or exits cells also via ATP7A, which traffics to the basolateral surface when copper is in excess. ATP7A is strongly induced during iron deprivation, suggesting that it may affect iron metabolism in enterocytes. Copper is spontaneously oxidized and then binds to serum carriers (e.g., albumin) for transport to the liver. Abbreviations: ATOX1, antioxidant 1 copper chaperone; CCS, copper chaperone for superoxide dismutase 1 (SOD1); MT, metallothionein.

Figure 3.

Iron-copper homeostasis and interactions in hepatocytes. ① Hepatocytes regulate systemic iron homeostasis by producing and excreting the iron-regulatory peptide hormone hepcidin. Copper may influence hepcidin expression and/or activity. These cells control overall body copper homeostasis as well by regulating copper excretion in bile. Hepatocytes assimilate iron via endocytosis of Fe-transferrin (TF) via transferrin receptor (TFR)1/2. In endosomes, iron is released from TF by the action of a hydrogen ATPase and is then transported into the cytosol by divalent metal-ion transporter 1 (DMT1) or zinc transporter ZIP14. ② The endosomal reductase [perhaps six-transmembrane epithelial antigen of the prostate 3 (STEAP3)] may influence intracellular iron and copper metabolism. Iron is used in cells, stored in ferritin, or ③ exported by ferroportin 1 (FPN1) (which may be influenced by copper levels). ④ After reduction by a potentially multifunctional metalloredutase, cuprous (Cu^+) copper is taken up via copper transporter 1 (CTR1) and distributed within cells by chaperones. Antioxidant 1 copper chaperone (ATOX1) delivers copper to copper-transporting ATPase 2 (ATP7B), which pumps copper into the *trans*-Golgi network (TGN) for ⑤ incorporation into the multicopper ferroxidases (FOXs) glycosylphosphatidylinositol (GPI)-ceruloplasmin (CP) and CP, which oxidize iron released from hepatocytes (and other cells) to permit binding to TF. ATP7B also mediates copper excretion across the canalicular membrane into bile. ATP7B activity is regulated by copper metabolism MURR1 domain (COMMD1) and indirectly by a ubiquitin ligase [X-linked inhibitor of apoptosis protein (XIAP)], which mediates COMMD1 proteasomal degradation. Abbreviation: CCS, copper chaperone for superoxide dismutase 1.

Figure 4.

Iron-copper homeostasis in erythroid cells. Developing erythroid cells acquire iron via the Fe-transferrin (TF)/transferrin receptor 1 (TFR1) cycle. ① An endosomal reductase [perhaps a multifunctional six-transmembrane epithelial antigen of the prostate (STEAP) protein] reduces ferric iron prior to divalent metal-ion transporter 1 (DMT1)-mediated transport into the cytosol. Most iron in these cells enters the mitochondria, where it is transported into the matrix via mitoferrin. The chaperone, frataxin (FXN), may deliver iron to the sites of Fe-S cluster assembly and heme synthesis. Fe-S clusters and heme are incorporated into proteins comprising the electron transport chain complexes I–IV. ② After reduction at the cell surface by a potentially multifunctional reductase, most copper taken up by erythroid cells via copper transporter 1 (CTR1) is delivered to SOD1 by CCS (copper chaperone for superoxide dismutase 1). ③ Copper is also directed to the mitochondrion for incorporation into complex IV [cytochrome C oxidase (CCO)], which also has two iron-containing heme moieties. Mitochondrial copper is delivered to CCO by the cytochrome C oxidase copper chaperone, COX17. Copper deficiency may ④ decrease the assimilation of iron from Fe-TF, ⑤ impair uptake of iron by mitochondria, and/or ⑥ reduce heme synthesis, exemplifying additional points of possible iron-copper interaction.