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Review

Trace elements in human physiology and pathology. Copper

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Abstract

Copper is a trace element, important for the function of many cellular enzymes. Copper ions can adopt distinct redox states oxidized Cu(II) or reduced (I), allowing the metal to play a pivotal role in cell physiology as a catalytic cofactor in the redox chemistry of enzymes, mitochondrial respiration, iron absorption, free radical scavenging and elastin cross-linking. If present in excess, free copper ions can cause damage to cellular components and a delicate balance between the uptake and efflux of copper ions determines the amount of cellular copper. In biological systems, copper homeostasis has been characterized at the molecular level. It is coordinated by several proteins such as glutathione, metallothionein, Cu-transporting P-type ATPases, Menkes and Wilson proteins and by cytoplasmic transport proteins called copper chaperones to ensure that it is delivered to specific subcellular compartments and thereby to copper-requiring proteins.

Keywords: Copper; Bioavailability; Deficiency; Enzymes; Ceruloplasmin; Metallothionein

1. Introduction

The effects of trace element are heavily dependent on one another. Thus, high intakes of zinc, cadmium or copper interfere with the utilization and tissue storage of iron. Low concentrations of dietary iron enhance the absorption of not only dietary iron, but also of lead, zinc, cadmium cobalt and manganese. Zinc supplements have been shown to cause anemia secondary to hypocupremia and tetrathiomolybdate inhibits copper absorption. Moreover, little is known about the role of drugs especially diuretics, and intercurrent illness on the development of trace mineral deficiency and the interactions of trace elements with one another, particularly in the situation where the decision is made to replace a single trace element or in older individuals who may be on one trace element supplement.

Copper (Cu) is an essential trace metal found in all living organisms in the oxidized Cu(II) and reduced Cu(I) states. It is required for survival and serves as an important catalytic cofactor in redox chemistry for proteins that carry out fundamental biological functions that are required for growth and development [1]. The average intakes of copper by human adults, vary from 0.6 to 1.6 mg/d [1–5]and the main sources are seeds, grains, nuts, and beans (concentrated in the germ and bran), shellfish and liver. Drinking water does not normally contribute significantly to intake. The concentration of free copper ions has been estimated to be of the order of 10^{-18}–10^{-13} M in yeast cells and in human blood plasma, respectively. In excess of cellular needs, Cu can be cytotoxic. Copper, similar to iron (Fe), can participate in reactions that result in the production of highly reactive oxygen species (ROS), responsible for lipid peroxidation in membranes, direct oxidation of proteins, and cleavage of DNA and RNA molecules (Table 1) [7]. The generation and action of ROS are major contributing factors to the development of different pathologies such as cancer, diseases of the nervous system and aging [8]. In addition to the generation of ROS, Cu may manifest its toxicity by displacing other metal cofactors from their natural ligands. The replacement of Zn(II) byCu(II) in the zinc-finger DNA binding domain of the human estrogen receptor renders this protein defective, altering its role in hormone-dependent signal transduction in vivo [9]. Thus, precise regulatory mechanisms must be in place to prevent the accumulation of Cu ions to toxic levels [6]. The ingested Cu is absorbed and distributed to copper-requiring proteins. Excretion is the main factor controlling homeostasis. Although copper deficiency is rare, it may occur when there is a

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Table 1
Interactions of iron and copper ions with hydrogen peroxide and superoxide

Ferrous iron [Fe^{2+}] + hydroperoxide [H_2O_2]	→ Ferryl [intermediate: iron–oxygen radical complex]
	→ Hydroxyl radical [$OH^{\cdot}$ + OH^-] + ferric iron [Fe^{3+}]
Ferric iron [Fe^{3+}] + superoxide [$O_2^{\cdot -}$]	↔ Perferryl [intermediate: iron–oxygen radical complex]
	↔ Ferrous iron [Fe^{2+}] + oxygen [O_2]

Comparable reactions can be written in which H_2O_2 reacts with cuprous [Cu^+] ion to yield $OH^{\cdot}$, $O_2^{\cdot -}$ reduces cupric (Cu^{2+}) ion to Cu^+. Intermediate copper ion–oxygen complexes are formed

genetic defect in the functioning of a copper transporter (ATP7A), resulting in Menkes disease or the milder Occipital Horn Syndrome. Menkes and Wilson's diseases (WDs), human genetic diseases in Cu transport, have revealed the importance of maintaining appropriate Cu homeostasis [10–14]. Moreover, Cu is essential for efficient iron uptake and mobilization in mammals [15].

2. Dietary copper intake and absorption

2.1. Copper absorption

In humans and animals, dietary copper is absorbed across the mucosal membrane in cells, which line the stomach and the small intestine primarily by those of the small intestine. Cu diffuses through the mucous layer that covers the intestinal wall [1]. In humans with normal intakes (0.6–1.6 mg/d), 55–75% is absorbed and actively recycled between the digestive tract, body fluids and tissues (particularly the liver). Thus, dietary copper contributes only a small proportion of the total reabsorbed from saliva, gastric juices, the bile, pancreatic and duodenal fluids (4–7.5 mg). About 1 mg of Cu is excreted daily by adults, the bile being the main route for copper excretion, with very little excreted by other routes [1,5]. The transport of Cu(II) across the brush border involves both a non-energy-dependent saturable carrier, and diffusion (active at low or high Cu concentrations, respectively) [1]. The rates of copper transport increase with pregnancy and in cancer, and decrease (at least in female rats) upon repeated treatment with estrogen [16]. Within mucosal cells, most newly absorbed copper (about 80%) is retained in the cytosol, bound to glutathione, metallothioneins and/or proteins of similar size. Excess intracellular copper in the intestine, liver, and probably other tissues is immediately bound to glutathione and subsequently to metallothionein [17,18]. Glutathione has been shown to protect cells against copper toxicity [17–19]and inhibition of its synthesis in hepatocytes with buthionine sulfoximine (BSO) reduces the incorporation of ^{67}Cu into metallothionein by more than 50%. Metallothioneins are a group of low-molecular-weight cysteine-rich proteins found in vertebrates, invertebrates, and fungi [20]. Mammalian cells have multiple metallothionein proteins that can buffer the intracellular concentrations of several metal ions, such as copper, zinc, cadmium, and others, which induce and bind to metallothioneins to different extents [21–24]. In mammals, metallothionein induction is mediated by binding of metal-responsive transcription factor 1 (MRF-1) to metal-responsive elements (MRE), in metallothionein promoters. These promoters have multiple copies of MRE elements, which contain a core consensus sequence, 5'-TGCPuCXC-3' conserved in all higher eukaryotes [25]. In yeast, there are two metallothioneins: Cup1 (the most important) and Crs5. Only Cu and Ag induce the CUP1 and CRS5 genes and this induction is mediated through a copper binding transcription factor Ace1 [26–28]. The CUP1 gene promoter contains four Ace1 binding sites, whereas the CRS5 promoter contains only a single site [29,30]. Studies of both yeast and mammalian cells have shown that metallothioneins have no direct role in copper uptake, but are important for the storage of the metal ions [17,31,32] and for protection against copper toxicity. Since the affinity of these proteins for Cu(II) is higher than for most other abundant metal ions (notably Zn(II)), the incoming copper will displace these ions. If metallothionein concentrations in the mucosal cells are high (as when induced with high intakes of zinc), the binding of copper to metallothionein will interfere with its transfer across the serosal surface. Thus, in Wilson's disease, a high dose of zinc is used to inhibit intestinal copper absorption. Several genes and gene products have emerged with regard to the potential transporters and carrier systems for intestinal absorption [33–36]. CTR1, cloned in humans [37] and in mice [38] and a high-affinity Cu transport protein, hCtr1, have been identified in yeast cells defective in Cu transport due to inactivation of both the CTR1 and CTR3 genes. hCtr1 is a 190 amino acid protein with significant homology to yeast Ctr1 and Ctr3, suggesting that mammalian high-affinity Cu transporters may have evolved from both Ctr1 and Ctr3. The amino terminal domain of hCtr1 is rich in methionine and histidine residues. hCtr1 is expressed in many organs and tissues, with liver, heart and pancreas exhibiting the highest levels, with intermediate levels in intestine, while expression in the brain and muscle is low. Transfection experiments have confirmed that hCtr1 promotes copper uptake into mammalian cells [39–41]. A low affinity mammalian Cu transporter, hCtr2, was also identified by sequence homology with hCtr1 [33,37,42]. Similar to hCtr1, hCtr2 mRNA is detected in many organs and tissues; however, the highest levels were observed in the placenta with very low abundance observed in the liver, ovary, intestine and colon [43] Unlike hCtr1, hCtr2 is unable to complement the respiratory defect in yeast strains defective in Cu transport. A second low affinity Cu transporter, is the

Nramp2 protein, that transports divalent metal ions such as Fe(II), Zn(II) and Mn(II) [44]. Nramp2 protein is homologous to the Smf1/Smf2 yeast metal ion transport proteins [45,46]. Ubiquitously expressed in tissues and present in the intestinal brush border, its transport of metal ions is protein coupled and dependent on the membrane potential. A role in iron absorption, as well as homeostatic regulation of its expression by iron within the intestine, has been well established [47–50].

2.2. Uptake and transport of copper

In the portal blood plasma and interstitial fluid, there are proteins, which have specific and high-affinity copper binding. Although albumin is the most abundant plasma protein, only 10–12% of the total plasma copper is bound to albumin (100–150 ng/ml) [51–53]. High-affinity copper binding has been demonstrated for human [54,55] and bovine albumin [54]. Albumin, binds copper with the help of the three amino acids at its N-terminus (including a histidine in the third position). Albumin in some vertebrates, including dogs and pigs, lacks a histidine near the N-terminus. This does not eliminate binding, but lowers copper affinity by 10-fold [56]. In rats and humans, two other proteins: ceruloplasmin, and a macroglobulin, transcuprein were identified [53,57]. Transcuprein and albumin represent the bulk of the exchangeable copper pool in blood plasma [58,59]. Once in the blood plasma, the copper on ceruloplasmin is available for uptake by tissues throughout the rest of the organism [58]. Under normal circumstances, ceruloplasmin is probably the main source of copper for other tissues [60,61] and albumin is not required for copper uptake by the liver and kidney [62]. Ceruloplasmin-copper is not the only form of copper available to most non-hepatic tissues [63–66]. Using various cell lines, it was shown that copper can be taken up from ceruloplasmin, albumin, transcuprein, or Cu-dihistidine [63,67,68] (Table 2). Most non-hepatic tissues, and particularly the heart and placenta, show a preference for ceruloplasmin-copper that must involve interaction with specific receptors in the plasma membranes [69–71].

2.3. Intracellular distribution and metabolism of copper

In cells, due to its highly reactive nature, it would be extremely harmful for Cu(I) to exist as a free ion, where it can participate in reactions whose products ultimately damage cell membranes, proteins and nucleic acids. Thus, Cu is delivered to specific molecules by forming complexes with small cytosolic proteins known as copper chaperone proteins. Crossing the brush border of the enterocyte, most of the copper is shuttled to the trans-golgi network (TGN) and into its channels by ATOX1/HAH1 (corresponding to yeast Atx1) delivering copper to the P-type ATPases located in the TGN [72,73]. In the case of the enterocyte, it would be ATP7A or MNK (the protein defective in Menkes disease) and in the case of the liver, it would be ATP7B or WND (the protein defective in WD). The chaperone protein CCS, delivers copper to the Cu/Zn superoxide dismutase (SOD) in the cytoplasm [74,75], which protects cells against superoxide radicals, whereas, COX17 delivers copper to the mitochondria [76,77], where it is required for cytochrome-*c* oxidase, the terminal enzyme in respiration. Glutathione (GSH) may also play the role of a general chaperone for copper ions [78] by delivering Cu to CTRI in the plasma membranes. In vitro studies have shown that GSH will reduce and bind Cu(I) and deliver it to metallothioneins and to some copper-dependent apoenzymes, like SOD and hemocyanin [78]. Observations that copper binds to chaperones after it has entered suggest that GSH mediation may be involved and direct binding of the chaperone to the membrane transporter might not be required [79]. GSH may not be needed directly for copper distribution within the cell, but might also be needed to restore the abilities of copper binding proteins (with thiol groups) to bind their copper. It might also provide electrons for reduction of copper during transport.

In mammalian serum, the predominant Cu containing protein is ceruloplasmin, a glycosylated multi-Cu ferroxidase synthesized primarily in the liver, which carries 95% of total serum Cu [80]. Ceruloplasmin coordinates seven Cu atoms that are incorporated during its biosynthesis and maturation in the secretory pathway [81]. In patients with aceruloplasminemia, the absence of ceruloplasmin does not alter

Table 2
Copper binding components in human blood plasma

Component	Contribution to total copper content		
	μg/l	μM	%
Ceruloplasmin	650–700	10–11	65–70
Albumin	120–180	2–3	12–18
Transcuprein (macroglobulin)	90	1.4	9
Ferroxidase II	10	0.16	1
Extracellular SOD and histidine-rich glycoprotein	< 10	< 0.16	< 1
Blood clotting factors V and VIII	< 5?	< 0.08	< 0.5?
Extracellular metallothionein and amine-oxidase	< 1?	< 0.02	< 0.1?
15–60 kDa components	40	0.63	4
Small peptides and amino acids	35	0.55	4
“Free” copper ions (source from Ref. [110])	0.0001	0.0000002	0

Cu levels in the peripheral tissues [82,83]. The ability of copper to bind to transcuprein in the presence of abundant albumin (with its high-affinity copper sites) emphasizes its high-affinity for this protein. Rodents express a different spectrum of macroglobulins in their blood plasma than do humans and most other mammals. Rat transcuprein appears to be α_1-inhibitor3, a monomeric macroglobulin with a total molecular weight of about 200,000. The main human macroglobulin (α_2-macroglobulin) and α_1-Inhibitor3 both have a highly homologous, histidine-rich region, suggesting the conservation of specific metal binding domains.

2.4. Release of copper from cells and copper excretion

The regulation of copper excretion appears to be the main mechanism for homeostasis [4,6]. Except for tissues producing secretions for the gastrointestinal tract (salivary glands, the pancreas, and epithelia in the stomach and intestine), most copper must return to the liver for excretion. It is carried by the plasma carriers, transcuprein and albumin, which particularly target the liver and also by ceruloplasmin. Most ceruloplasmin enters hepatocytes after desialylation in endothelial cells [71]. The primary pathway for the excretion of copper from the body is from hepatocytes, via the bile. The importance of maintaining mechanisms for proper Cu homeostasis in the liver is underscored by the existence of the autosomal recessive disorder Wilson s disease (WND). The Wilson and Menkes proteins are highly homologous P-type ATPases. Both the proteins contribute to the cellular export of copper, by direct extrusion of copper from the cell. The biosynthetic loading takes place in the TGN, where the Wilson and Menkes proteins are normally located. The Wilson protein is expressed primarily in the liver, transporting copper to apoceruloplasmin, whereas the Menkes protein is predominant in all other tissues [84,85]. Direct excretion of copper appears to take place when cells are subjected to elevated copper levels. Under these conditions, the Menkes proteins move from TGN to the plasma membrane [86]. On the other hand, an increase in the concentration of copper in HepG2 cells results in the movement of the Wilson protein into a cytoplasmic vesicular compartment [87,88]. The Menkes protein is encoded by the ATP7A gene and mutations in this gene result in the Menkes disease. Menkes' disease is X-linked and characterized by severe neurodegeneration and connective tissue abnormalities, which can be ascribed to the reduced activity of several copper-requiring enzymes. Fibroblasts from patients suffering from Menkes' disease accumulate copper, and this is used diagnostically [89]. WD, which is caused by mutations in the ATP7B gene, is characterized by copper toxicity resulting from the loss of ability to export copper from the liver to the bile and the inability to incorporate copper into ceruloplasmin [90–93]. Patients with WD accumulate Cu in the liver and brain, resulting in liver cirrhosis, neurodegeneration and the formation of apoceruloplasmin [94]. The ATP7B gene, which encodes the 160-kDa WND P-type ATPase, is required for biliary excretion of Cu and incorporation of Cu into ceruloplasmin in the liver [11,14,95]. WND ATPase (7B) has mainly been located in the TGN of liver and brain, where it likely functions to incorporate Cu into ceruloplasmin [96] and perhaps at the plasma membrane of hepatocytes for Cu excretion into the bile [97]. These vesicles are close to the canalicular membrane, where the bile is released [98]. Moreover, a cleaved form of the WND protein of 140 kDa was reported to be localized in the mitochondria of cultured hepatic cells and human tissues, rather than in TGN [99,100] where it is suggested to play a role in mitochondrial Cu ion homeostasis. Although most of the copper is recycled within given cells and tissues, some is released back into the blood. From non-hepatic cells, copper release occurs through MNK. There is evidence that it can occur in two different ways, exocytosis and by trafficking to the plasma membrane particularly when large amounts of copper need to be exported [86,98]. The mechanism involves the cycling of MNK between the TGN and the plasma membrane. This was first demonstrated in CHO cells that developed resistance to copper toxicosis [101,102]. Copper set aside for permanent excretion would be directed to the bile. The route taken by copper to the bile involves HAH1/ATOX1, WND, and exocytosis or trafficking of WND to the brush border of the bile canaliculus [103]. However, copper in bile is less reabsorbable than that in other gastrointestinal fluids. Reabsorbability varies in relation to the amounts of copper in hepatocytes. In addition, it has been proposed that a large fragment of ceruloplasmin, high in copper and resistant to proteolysis, may furnish a means of excreting copper without intestinal reabsorption [104]. Thus, much of copper homeostasis is controlled by the level and form in which it is excreted through the bile. It is clear that no copper is present in cells (or in body fluids) as a free ion [105]. Evidence from human studies with stable copper isotopes indicates that relatively little copper enters and leaves the cells; most is recycled on a daily basis [4,6].

3. The role of copper in mammalian cells

Copper is essential as a cofactor in a number of critical enzymes in metabolism (Table 3).

3.1. Cytochrome-c oxidase

Cytochrome-*c* oxidase [106] sits within the inner mitochondrial membrane. It has four redox active metal sites, two heme sites (hemes a and a3), and two copper sites (CuA and CuB) [107]. The heme is characterized by a hydroxyl farnesylethyl group at position 2 and a formyl group at position 8 of the porphyrin substituted groups. Cytochrome-*c* oxidase is the terminal oxidase in most aerobic organisms and reduces molecular oxygen (O_2) to water [107]. In addition to the O_2 reduction, cytochrome-*c* oxidase pumps protons from the inside to the outside of the membrane. Thus, in addition to the membrane potential produced by the net migration of the positive charges, it produces a proton gradient across the

Table 3
Copper-dependent enzymes in mammals

Enzyme	Function
Cytochrome-*c* oxidase	Electron transport in mitochondria
Cu/Zn-SOD	Free radical detoxification
Metallothionein	Storage of excess Cu and other divalent metal ions [not Fe(II)]. Possible donor of Cu to certain apoproteins
Ceruloplasmin (extracellular)	Ferroxidase, promotes flow of Fe from liver to blood Scavenger of ROS, acute-phase reactant. Cu transport
Protein-lysine-6-oxidase	Cross-linking of collagen and elastin
Tyrosinase (catechol oxidase)	Formation of melanin
Dopamine-β-monooxygenase	Catecholamines production
α-Amidating enzyme	Modifies C-terminal ends of hypothalamic peptide hormones ending in glycine, leaving the COOH of the next to last AA amidated (necessary for hormone maturation)
Diamine oxidase	Inactivation of histamine and polyamines? (cellular and extracellular)
Amine oxidase (extracellular)	Inactivation of histamine, tyramine, dopamine, serotonin?
Peptidylglycine monooxygenase	Bioactivation of peptide hormones
Hephaestin	Ferroxidase, in trans-golgi of enterocytes; aids iron absorption homology to ceruloplasmin
CMGP	Ferroxidase/amine oxidase, homologous to ceruloplasmin (chondrocytes and eye ciliary epithelia)
β-Amyloid precursor protein	Normal function currently unknown
Prion protein (PrPC)	Copper binding properties suggests that it may protect against ROS; has SOD-like activity; may return copper to neurons at synapses (many cells)
S-Adenosylhomocysteine	Sulfur amino acid metabolism hydrolase
Angiogenin	Induction of blood vessel formation
Blood clotting factors V and VIII	Blood clotting

membrane [107,108]. The two heme sites are located within the single polypeptide of subunit I and the two heme iron sites are called hemes α and α3. In the oxidized state, a respiratory inhibitor, cyanide, binds specifically to heme α3 and stabilizes the oxidized state. The cyanide-bound heme α3 cannot be reduced even with an excess amount of dithionite. In contrast, heme α is unreactive to cyanide and is readily reduced by dithionite [109].

3.2. Copper/zinc superoxide dismutase

Copper/zinc superoxide dismutase (Cu/Zn-SOD) converts superoxide anions to peroxide for further disposal (by catalase and glutathione peroxidase). *Drosophila* and microorganisms lacking the enzyme have been shown to be more vulnerable to damage by ROS [110]. In mice where the gene has been knocked out, there is gradual damage to neuromuscular junctions in the hindlimbs [111]. Mutations of Cu/Zn-SOD have also been of interest in connection with amyotropic lateral sclerosis (ALS), where a gain of function may be responsible for the underlying neurological symptomatology [112–114]. Normally, the expression of Cu/Zn-SOD appears to be fairly constitutive. However, when copper becomes less available and hyperoxia induces the expression of SOD (along with metallothionein), it is (at least in certain cells) one of the first enzymes to lose its activity [1,17,115].

3.3. Metallothioneins

Metallothioneins (61 amino acid proteins, with 20 cysteines) come in at least two isoforms encoded by several genes and tightly binding divalent metal ions (except Fe). One characterized function for this group of proteins is to sequester metal ions in an innocuous form when they are present in excess amounts. Thus, cadmium accumulates in metallothionein complexes (particularly in the kidney) throughout life. Excess copper accumulates in MT in Wilson-disease-affected tissues. Zinc and cadmium are particularly good inducers of MT, although copper can also be effective in some tissues. Some other factors, notably the hormones glucagon and cortisol, as well as agents that induce inflammation and the acute-phase response, also enhance MT expression. Although MTs mainly bind Zn, Cu, and Cd ions they can also sequester Hg, Ag, or Ni. However, Cu is bound most tightly and can displace these other ions. Since Zn and Cd ions are not as reactive as Cu with regard to oxygen radical formation, binding of copper to MT is protective for the cell. In addition, Cu–MT, appears to have some SOD activity [116] and in the absence of SOD, oxidative stress induces the expression of MT [115,117].

3.4. Ceruloplasmin

Ceruloplasmin is a single polypeptide chain (about 120 kDa with 12 kDa carbohydrate). Besides its potential role in copper delivery to cells and excretion of copper from the body, ceruloplasmin is a ferroxidase, with the ability to oxidize Fe(II) to Fe(III). This change is helpful to provide iron in the form needed to bind transferrin, (iron plasma carrier) as it emerges from cells for further transport from the bone marrow to red blood cells, where most iron resides. In severe copper deficiency, there is little or no copper-containing ceruloplasmin in the plasma and in tissues and in the absence of active-ferroxidase, iron accumulates in the liver. IV infusion of ceruloplasmin (but not copper–albumin) results in the immediate release of liver iron into the blood [118–121]. However, only 1–2% of the normal plasma level of ferroxidase-active ceruloplasmin can play a role in iron efflux [122]. Since 22 mg (about 0.7%) of iron in the human body fluxes in and out of red blood cells every day, and a

major portion of that enters and leaves liver cells, including hepatocytes, it seems that ceruloplasmin alone cannot be responsible for this process. Recently, a new glycosylphosphatidylinositol anchored form of ceruloplasmin, (GPI-linked ceruloplasmin) has been found in mammalian astrocytes [123] and glia [124,125] in the brain. This form of ceruloplasmin may also be involved in iron transport [126]. However, it appears unlikely that ceruloplasmin plays a role in releasing iron from enterocytes during iron absorption [127]. Ceruloplasmin concentration in plasma increases during inflammation or infection. It appears that the regulation of ceruloplasmin expression is controlled not only just by inflammatory cytokines, but also through hypoxia-inducible factor (HIF1), which is linked to iron metabolism [128].

3.5. Hephaestin

Hephaestin is a ceruloplasmin homolog and shares some of the characteristics of this protein. It is a transmembrane protein of about 134 kDa, primarily located in trans-golgi vesicles. It has ferroxidase activity [129] and is involved in intestinal iron absorption [15]. It may oxidize Fe for binding to apotransferrin, allowing its release into the blood as holotransferrin (through exocytosis).

3.6. Cartilage matrix glycoprotein

Cartilage matrix glycoprotein (CMGP) is another intracellular ceruloplasmin homolog with ferroxidase and oxidase activities [130]. It is located in the vesicular portions of chondrocytes, as well as in the epithelial cells of the eye; composed of four disulfide-bonded subunits (each of 116 kDa), it may play a role in the formation of the extracellular matrix.

3.7. Lysyl oxidase (protein-6-lysine oxidase)

Lysyl oxidase plays a crucial role in the formation, maturation, and stabilization of connective tissue. It is part of the extracellular matrix of organs and tissues in the body (including cartilage and bone). Genes for lysyl oxidase were cloned from rat aorta [131] and human placenta [132]. Recently, several lysyl oxidase-like genes have been identified and cloned. Some of these are particularly expressed by placenta and other reproductive tissues [133,134]. Lysyl oxidase is a multimeric protein composed of 32 kDa subunits, that requires copper for its activity. In Menkes' disease, or in Occipital Horn Syndrome characterized by Cu deficiency, the development of normal connective tissue is altered. The cofactor, lysine tyrosyl quinone (LTQ), is part of the enzyme's active site. It catalyzes the cross-linking of elastin and collagen fibers. Recent evidence suggests that the role of copper may not be catalytic as much as supportive of cofactor formation and structure or enzyme integrity [135,136]. Although copper availability determines enzyme activity, nutritional copper status does not alter the expression of the protein or its mRNA [137].

4. Role of copper in embryogenesis

During embryogenesis, when cell proliferation is very active, respiration and cytochrome oxidase activity are essential. In the last part of gestation, considerable copper is transferred to the fetus from the maternal circulation via the placenta or by ingestion of the amniotic fluid [61]. It is bound to metallothionein and accumulates and is stored in the liver along with similar stores of iron and zinc to be used during the suckling period [138]. The placenta expresses both WND and MNK, but not ceruloplasmin. It was suggested that WND is involved in copper transport at the maternal side of the placenta [139,140], whereas, the MNK protein seems to be active on the fetal side [141]. During embryogenesis, the MNK protein is expressed in all tissues and particularly in the brain, whereas WND expression is initially confined to the central nervous system (CNS), liver, and heart [142]. After birth, much copper is delivered to the newborn via the milk. In human milk, copper is present in various components; ceruloplasmin contributes 20–25% of the copper [143,144]. Most of the ceruloplasmin in milk is produced by the lactating mammary gland [145–149].

5. Role of the pineal night-specific ATPase (PINA) in copper transport

Circadian rhythms are found in virtually all organisms. These rhythms dictate our daily sleep schedule and hormonal fluctuations [150] and even influence our susceptibility to disease such as heart attack [151], strokes [152], and seizures [153]. The pineal gland, an organ situated deep within the brain exhibits dramatic diurnal fluctuations in the secretion of the hormone melatonin, which is known to link environmental light information to the body's physiological responses. Melatonin synthesis is ultimately controlled by the suprachiasmatic nucleus (SCN) of the brain, which uses a biological clock and lighting information to rhythmically control neural pathways. One role of the SCN is to influence neurons of the superior cervical ganglion (SCG), which sends axonal processes directly to the pineal gland. When stimulated, sympathetic SCG neurons release norepinephrine into the pineal, activating β-adrenergic receptors on the plasma membrane of pinealocytes. The receptors initiate a signaling cascade resulting in the production of cAMP, which stimulates the production of melatonin [154]. In animals, the only documented function of the pineal is the synthesis and regulation of melatonin. Melatonin is synthesized from dietary tryptophan by the actions of four enzymes. (1) Tryptophan hydroxylase (TPH), the rate-limiting enzyme in serotonin synthesis, is responsible for the 5' hydroxylation of tryptophan, yielding 5-hydroxytrytophan (5-HTP). (2) A non-specific aromatic amino acid decarboxylase (AAADC) converts 5-HTP to 5-hydroxytryptamine (5-HT, serotonin). (3) A pineal/retina-specific enzyme serotonin *N*-acetyltransferase (NAT) acetylates serotonin to form *N*-acetylserotonin (NAS). (4) And another pineal/retina-specific en-

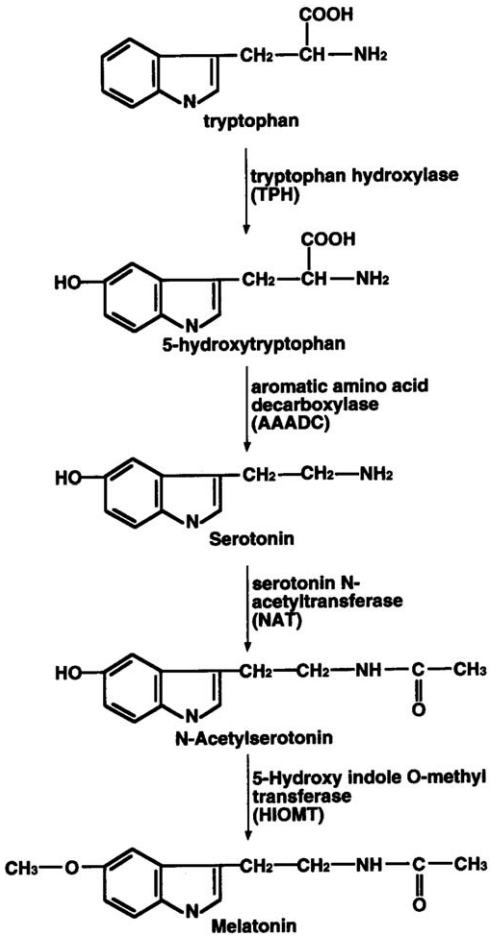

Fig. 1. Melatonin synthetic pathways (see text for details).

zyme, hydroxyindole-*O*-methyltransferase (HIOMT), catalyzes the conversion of NAS to melatonin (Fig. 1).

Sequence analysis of the pineal gland night-specific ATPase (PINA) revealed that it is an alternatively spliced form of the copper-transporting ATPase mutated in WD patients, ATP7B. Sequences encoding the N-terminal half of ATP7B are replaced by a unique untranslated 300-bp leader sequence. The PINA protein, therefore, represents only the C-terminal half of ATP7B. PINA completely lacks the metal binding repeats and the first four putative transmembrane segments of WND and despite these deletions, it is proposed to function as a Cu transporter in rat pinealocytes. It was found to be expressed in the pinealocytes and a subset of photoreceptors in adult rats, and transiently in the retinal pigment epithelium and ciliary body during retinal development [155]. PINA is expressed at 100-fold higher levels at night than in daytime.

6. Copper and human syndromes

6.1. Menkes and Wilson diseases

WD is an autosomal recessive disorder resulting from mutations in the ATP7B gene. Patients with WD suffer from brain disorders and liver disease. The cornea of the eye is also affected in many patients, resulting in the hallmark brown discoloration of the cornea, which is very specific for neurological WD, the "Kayser–Fleischer ring". The etiological significance of copper is supported by the efficacy of treatments, which are principally aimed at chelation of free copper. In the case of copper toxicity (Indian cirrhosis), there is clear liver failure, yet there have been no neurological symptoms described [148,149]. The genes responsible for WD [156,157] and Menkes' disease (and the less severe occipital horn syndrome) were cloned [12,13,158]. The corresponding normal proteins encoded by these genes are both P-type ATPases (ATP7B and 7A, for WND and MNK, respectively). They are usually expressed in different cell types (MNK widely, and WND primarily in hepatocytes and certain areas of the brain). Located in the TGN and vesicular compartments, they can be translocated to the plasma membrane under conditions when copper secretion or efflux needs to be promoted. In contrast to Menkes' disease, WD occurs more gradually, and after birth. It results in the accumulation of excess copper in the liver with other tissues accumulating oxidative damages [110]. Due to the absence of the normal WND ATP7B protein, it is difficult for copper to reach the bile. Accumulation of excess copper in tissues (although mitigated by binding to metallothionein) promotes the formation of ROS, eventually resulting in liver cirrhosis [159–162]. The brain and some endocrine organs are also affected. Recently, a number of small cytosolic copper chaperones have been shown to transport copper to specific copper-dependent target proteins. HAH1, transports copper to the Wilson and the Menkes proteins [163]; CCS, transports copper to the cytoplasmic protein SOD [74] and COX17 transports copper to mitochondria for incorporation into cytochrome-*c* oxidase [164,165]. The presence of these copper chaperones is necessary to ensure that copper can reach its specific target protein. It is not known as to how the chaperones become loaded with copper and whether copper uptake proteins are directly involved in this process.

Menkes' disease is characterized by progressive neurological impairment and death in infancy [166]. Alteration in Cu transport, the entrapment of Cu in intestinal and kidney cells or vascular endothelial cells in the blood-brain barrier leads to Cu deficiency [167]. Menkes' disease gene (ATP7A) has been shown to be expressed in intestinal epithelial cells [168]. The Menkes protein (MNK) is involved in both providing Cu to secreted Cu-metalloproteins, and Cu efflux from intestinal epithelial cells. It contains six successive repeats of the Cu binding motif within the amino terminal region and is regulated by Cu ion concentrations [86]. In the presence of low Cu ion concentrations, the MNK protein is localized in the TGN and at elevated concentrations, Cu stimulates the transport of the MNK protein from the TGN to the plasma membrane to be involved in Cu efflux. It is suggested that in mammalian cells, the reduced form of Cu [Cu(I)], activates this process. The third transmembrane region of MNK functions as a TGN targeting signal [169]and a carboxyl terminal

di-leucine is required for recycling from the plasma membrane back to the TGN [170].

6.2. Aceruloplasminemia

Ceruloplasmin has many functions, including antioxidant defense, copper transport, and iron transport. A genetic absence of the production of active ceruloplasmin has been detected in a few families and has been mimicked by knocking out the gene in mice. The absence of ceruloplasmin does not produce marked changes in copper metabolism. It does, however, produce a gradual accumulation of iron in the liver and other tissues [121,171]. Ceruloplasmin is the only way in which iron destined for transferrin could be oxidized and low levels of ceruloplasmin (1–2% of normal) are sufficient to prevent liver accumulation and promote iron release into the blood [172].

6.3. Alzheimer's disease

Although there is no direct cause–effect relationship for copper in Alzheimer's disease, both copper and zinc are associated with the β-amyloid protein, which forms the damaging "tangles" in the brain pathology of the disease [173,174]. However, zinc precipitates aggregation of the amyloid protein, and copper works against the effect of zinc (except at high concentrations) [175].

6.4. Spongiform encephalopathies (prion diseases)

The prion protein, PrPC, which misfolds in bovine spongiform encephalopathy (mad cow disease) is the causative agent in Creutzfeld-Jacob disease (CJD), kuru, Gerstmann-Straussler-Scheinker (GSS) disease and fatal familial insomnia (FFI), collectively known as prion diseases [176]. PrPC is reported to have copper-dependent SOD-like activity [177–179]. It is a 33-35 kDa protein (varying in glycosylation) with four or five atoms of copper bound to four identical sequences of eight amino acids ("octarepeats") in the N-terminal region of the protein, (probably via the imidazole and glycine or histidine residues [180]). There is evidence that copper lends structural stability not only to the N-terminal region of the protein but also to other parts of the molecule [181,182]. PrPC is expressed in neurons and other cells, including skeletal muscle. Protein concentration is particularly high in neuronal synapses although the two distinct neuronal forms are synaptic or non-synaptic [183].

The prion protein, PrPC is a GPI-anchored to the outside of the plasma membrane found in the brain, spinal cord and peripheral tissues. It has been shown to bind Cu(II) [184–186] and it constantly recycles between the plasma membrane and an early endosomal compartment [187]. Copper stimulates endocytosis of the prion protein [188] and facilitates the renaturation of guanidine-denatured PrPSc molecules to form the protease-resistant infectious prion particle [189]. Disturbance of normal prion protein metabolism, through infection with the protease-resistant form that accumulates in the spongiform encephalopathies (PrPSc), impairs the ability of neurons to respond to oxidative insults [190].

The abnormal form of the prion protein implicated in this disease (PrPSc), which accumulates and is resistant to proteolysis, probably functions normally in the transport of copper at synapses and perhaps also (directly and/or indirectly) in the scavenging of radicals. It has been reported that the enhanced expression of PrpC increases a cell's stability to take up copper [191]. It also appears to increase its resistance to copper toxicity and oxidative stress [192]. Exposure to large non-physiological concentrations of Cu(II) or Zn(II) (but not Mn(II)) induced endocytosis of the protein [193], which would bring its copper (or zinc) into the cell. The addition of octarepeats to the prion protein (as occurs in some forms of spongiform encephalopathy) prevented copper-induced endocytosis, consistent with other evidence that the lack of removal/turnover of the prion protein results in (or contributes to) the brain damage seen in these diseases [194].

6.5. Inflammation, infection, and cancer

Copper metabolism is altered in inflammation, infection, and cancer. In contrast to iron levels that decline in serum in infection and inflammation, copper concentrations and ceruloplasmin rise.

Plasma ceruloplasmin synthesis and secretion by hepatocytes is stimulated by interleukin-1 (IL-1) and IL-6 [5]. Copper itself is important for immune response, including the production of IL-2 by activated lymphocytic cells [195], and supports the activity and effectiveness of cellular and humoral immunity [196,197]. In cancer, plasma ceruloplasmin antigen or oxidase activity are positively correlated with disease stage [198]. Malignant tumors have concentrations of copper that are often higher than those of their tissue of origin. Copper is absorbed by the tumors from ceruloplasmin and from non-ceruloplasmin sources in the blood [60]. Copper may also have a role in angiogenesis [199].

In addition, recent studies provide evidence that limiting the biological availability of copper, by penicillamine [200]or tetrathiomolybdate administration [201], slows tumor growth which is probably due to the inhibition of angiogenesis [199].

The amounts of copper that are normally ingested, and even intakes that are considerably higher, are usually not problematic for humans or rodents. Daily intakes of copper as high as 3 mg/d for children 4–8 years of age and 8–10 mg/d for adults are considered tolerable. (These intakes are 7–11 times higher than recommended daily intakes [202]). For most mammals, copper is a relatively benign trace element. Except in some genetically based special circumstances, copper is not implicated in pathologies. Moreover, copper-containing enzymes and even many copper complexes are capable of protecting the organism against ROS implicated in many chronic diseases. Nevertheless, individuals with inherited propensities to accumulate copper, have been vulnerable to toxicity from ingestion of high copper

doses [203]. Moreover, dogs and sheep appear to have a limited ability to excrete excess copper in the bile [204]. Dogs tend to accumulate copper in their livers throughout their lifespan, and many die of copper toxicosis.

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