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Copper: Toxicological relevance and mechanisms

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Abstract

Copper (Cu) is a vital mineral essential for many biological processes. The vast majority of all Cu in healthy humans is associated with enzyme prosthetic groups or bound to proteins. Cu homeostasis is tightly regulated through a complex system of Cu transporters and chaperone proteins. Excess or toxicity of Cu, which is associated with the pathogenesis of hepatic disorder, neurodegenerative changes and other disease conditions, can occur when Cu homeostasis is disrupted. The capacity to initiate oxidative damage is most commonly attributed to Cu-induced cellular toxicity. Recently, altered cellular events, including lipid metabolism, gene expression, alpha-synuclein aggregation, activation of acidic sphingomyelinase and release of ceramide, and temporal and spatial distribution of Cu in hepatocytes, as well as Cu-protein interaction in the nerve system, have been suggested to play a role in Cu toxicity. However, whether these changes are independent of, or secondary to, an altered cellular redox state of Cu remain to be elucidated.

I. Introduction

Copper (Cu) is found in a variety of cells and tissues with the highest concentrations in the liver and brain (Turnlund, 1998). Cu is largely present in biological systems as cupric form (Cu^{++}), although several distinct types of the bound cation can be found in Cu containing enzymes, often in combination within a single protein (Su et al 1982; Divertie et al. 1982). The Cu enzyme, lysyl oxidase, for example, is essential for cross-linking collagen and elastin, both are required for the formation of connective tissue. The Cu protein, ceruloplasmin or ferroxidase I, facilitates transport from the interstitial lumen and storage sites to sites of erythropoiesis. Cu is required for the formation and maintenance of myelin, a protective layer covering neurons, and is involved in the formation of melanin pigment in skin, hair, and eyes. Also, Cu is a component of cytochrome c oxidase, which catalyzes the reduction of oxygen to water, the essential step in cellular respiration, and is a part of copper, zinc-superoxide dismutase (Cu, Zn-SOD) which scavenges the free radical superoxide. Non-specific Cu^{++}-binding to thiol enzymes may modify the catalytic activities of cytochrome P450 monooxygenase, and Cu^{++} could both oxidize and bind to some amino acid residues of the P450 monooxygenase but not to its heme group (Letelier et al. , 2009). Additionally, Cu is a constituent of dopamine-beta-hydroxylase, a critical enzyme in the catecholamine biosynthetic pathway (Turnlund, 1999; Uauy et al., 1998). Therefore, it is not surprising that Cu-enzyme/protein-related malfunctions contribute to the development of hepatic, neurological, and other disorders.

Essentially all of the body's Cu in normal healthy humans is linked to enzyme prosthetic groups or tightly bound to Cu transport or chaperone proteins (Rosenzweg, 2001, Prohaska, 2008, Boal and Rosenzweig, 2009). Cu chaperones help minimizing the probability of unbound (free) Cu from participating in redox reactions (Burkitt, 2001; Evans and Halliwell, 1994) and ensure deliver of Cu ions to specific target proteins (Boal and Rosenzweig, 2009, Fields et al., 2001, Prohaska, 2008). Cu absorbed in excess of metabolic requirements is normally excreted through bile. The amount of Cu ingested in food and water is relatively low, and the body is able to control excess amounts of Cu in the body by either decreased absorption or increased excretion under normal conditions. As tight control of Cu homeostasis prevents excess accumulation of Cu in the body, acute and chronic Cu toxicity are relatively rare. However, Cu toxicity may result from exposure to excess Cu caused by accident, occupational hazard, environmental contamination, as well as adrenal gland insufficiency, inborn errors of Cu metabolism and other factors. Recent investigations examining how copper imbalance develops and alters metabolic functions have provided a better insight into the pathophysiology, and into therapies and prevention strategies for health problems associated with Cu toxicity.

II. Cu source exposure

Cu is a transition metal with atomic mass of 63.54 daltons (Da). Its malleability, low corrosion, alloying ability, high thermal conductivity, and high electrical conductivity make Cu one of the most important metals for industrial application. Cu is used as a metal or alloy in machinery, construction, transportation, and military weapons (Barceloux, 1999; Winge and Mehra, 1990) and is an important component of white gold and other alloys used for imitation jewelry, dental products, and in many cosmetics (Okereke et al., 1972; Vilaplana et al., 1991; Lucas and Lemons, 1992). Cu metal has low reactivity due to its high nuclear charge, small size, and consequent high ionization potential (Georgopoulos et al., 2006.) Cu, however, can exist in nature in an elemental form and in a wide range of compounds, and Cu ions exist in both oxidized, cupric, or reduced, cuprous state (Linder et al., 1996; Linder et al., 1998). The cuprous ion (Cu^{+}) can disproportionate rapidly in aqueous solution to form Cu (II) and Cu (0). The cupric ion (Cu^{++}) is the most important oxidation state of Cu generally encountered in water and is coordinated with six water molecules in solution.

The natural concentration of Cu in soil is approximately 50 ppm Cu (Barceloux, 1999). Cu in the air is released from such natural sources as windblown dust, volcanoes, and forest fires, and man-made sources such as Cu smelters, iron and steel production, and municipal incinerators. Cu content of the atmosphere ranges from 5-20 ng Cu/m^3 and natural water has a mean concentration of 4-10 μg Cu/L with most of the Cu bound to organic matter (Barceloux, 1999). Sources of Cu in the environment include Cu water pipes, Cu cookware, drinking water, birth control pills and Cu intrauterine devices, vitamin and minerals supplements, fungicides with added Cu for swimming pools, and foods. Plumbers, welders, machinists and others who work with Cu are at risk for Cu toxicity. Exposure to Cu through environmental or occupational exposures often includes exposure to other metals, such as arsenic, iron, or mercury, and chemicals such as ethanol, polychlorinated biphenyls, and pesticides (Pohl et al., 2011).

Cu intake varies greatly for individuals depending on food choices and dietary customs, as well as environmental factors. Most diets contain enough Cu (1-5 mg) to prevent a deficiency and not enough to cause toxicity. In the United States, the dietary or nutrient reference (recommended) intakes for Cu are based on the Food and Nutrition Board, Institute of Medicine recommendations of 0.9 mg/d for adults of both genders, 19-70 years (Food and Nutrition Board, 2006, Trumbo et al., 2001). The tolerable upper intake level in the same age group is 10 mg/d. Drinking water contributes about 6-13% of the average daily intake of Cu. The United States Environmental Protection Agency (2013) has set the maximum contaminant level goals for Cu at 1.3 mg/L or 1.3 ppm. This amount is based on possible health risks and exposure over a lifetime with an adequate margin of safety to prevent potential health problems. Determining the upper intake level of Cu consumption is more difficult because of health consequences from both Cu deficiency and Cu excess (Stern, 2010). The regulatory framework for risk assessment of essential trace elements introduced by the International Program on Chemical Safety has proposed a homeostatic model to determine the Adequate Range of Oral Intake of essential trace elements. Evidence available suggests that the upper level intake of Cu needs to be reevaluated, and that developing the scientific basis for a tolerable upper intake level of Cu and Cu deficiency are critically important (de Romaña et al., 2011).

III. Cu Status and markers

In addition to dietary intake and environmental exposure, Cu status can be influence by other factors including supplemental vitamins and minerals, as well as activity of the adrenal gland. Adrenal hormones, for example, promote hepatic production of ceruloplasmin, the main Cu binding protein in the body. Thus, malfunction of liver and adrenal gland insufficiency can cause Cu to accumulate in the tissues. When the liver is unable to release ceruloplasmin, Cu becomes bio-unavailable. Also, dietary zinc (Zn) and a number of nutrients may alter Cu status. As an antagonist of Cu, Zn deficiency is associated with Cu accumulation in various storage organs.

Increasing evidence suggests that Cu deficiency may be more prevalent than previously thought and Cu toxicity is uncommon under customary daily life conditions (de Romaña et al., 2011). The occurrence of mild Cu deficiency or excess Cu exposure is not easily recognized. Due to lack of sensitive and specific indicators, blood, urine and hair analysis are used to detect Cu toxicity. Serum Cu concentration and ceruloplasmin are the most frequently used indicators. However, both indicators only detect more pronounced changes of Cu status. The synthesis of ceruloplasmin has been proposed as a possible biomarker of Cu status, because it is regulated by the amount of available Cu in the liver (Goodman et al., 2004). Also, effects of milder degrees of Cu deficiency and excess Cu exposure are not well described.

The activities of several cuproenzymes are decreased in mild Cu deficiency. However, due to the lack of standardized assays and a large inter-individual variability, their use is limited. Also, no laboratory tests have been identified as potential early markers of Cu excess. As Cu is stored mainly in the brain, liver and other organs, and not in the blood or urine, specific, sensitive and noninvasive biomarkers are needed to detect an increase in body Cu before the

appearance of functional or clinical effects. Of the many proteins assessed as potential markers of Cu status, the chaperone of Cu,Zn-SOD seems to yield promising results. Dietary restriction of Cu has been shown to impair catalytic functioning of Cu,Zn- SOD in tissues, and Cu supplementation restored the enzyme activity in animals deprived of Cu (Harris, 1992). Also, protein expression of the Cu chaperone for SOD is increased in erythrocytes of rodents with mild Cu deficiency, and Cu chaperone for SOD mRNA abundance in mononuclear blood cells significantly decreased after Cu supplementation (Olivares et al., 2008). These studies suggest altered activity and/or expression of Cu,Zn-SOD may serve as a sensitive marker of Cu status, although further studies under different conditions are needed to confirm its use as an indicator of early Cu deficiency.

As adverse health consequences can result from both Cu deficiency and Cu excess, defining Cu requirements and upper safe limits of consumption is a complex process. At this range of intake, physiological mechanisms allow for normal Cu homeostasis, and there are no detectable adverse effects. Toxic levels are, by definition, intakes above the upper level. However, due to the lack of noninvasive, sensitive biomarkers of storage or early damage from excess Cu, toxic effects from excess Cu are normally based on the infrequent occurrence of clinical disease, such as unexplained liver cirrhosis (Uauy et al., 2008). Biomarkers that are capable of predicting the risk of elevated hepatic Cu stores and thus the possibility of disease are still lacking. Yang et al. (2010) have shown that high dose of Cu induced increases in alanine aminotransferase, aspartate aminotransferase, triglyceride, total bilirubin, total bile acid levels, and scattered, dotted hepatocytic necrosis in male Wistar rats. They also show that genes related to oxidoreductase activity, metabolism, and signal transduction were involved in the development of the observed phenotypes, and that altered gene expression patterns were induced by exposure to a low, sub-toxic dose of Cu. These findings suggest that changes in gene expression may serve as more sensitive indicators of potential adverse effects of Cu than traditional measurements of toxicity.

IV. Cu homeostasis

Organisms have evolved into a number of systems for the efficient uptake, intracellular transport, protein loading and storage for Cu and other metal ions to ensure that the needs of the cells can be met while minimizing the adversary effects associated with deficiency or excess. Cu homeostasis is generally well maintained by intestinal absorption, biliary excretion and intrahepatic storage, and no significant changes in body Cu occur with mild-to-moderate Cu exposure (Turnlund et al., 1997, 1998). In mammalians, the liver is the major captor, distributor and excreter of Cu.

The rate of Cu absorption depends on Cu intake, chemical form and presence of other factors in the diet that may either promote or inhibit its absorption, and the Cu status of the individual. Cu is not stored in a significant amount in the body. About 30 to 50% of ingested Cu, mostly in Cu^{++} form, is absorbed in the small intestine, and very small amounts are absorbed in the stomach (Turnlund et al., 1997). Cu absorbed from the small intestine is transported in the blood bound predominantly to albumin, but also to transcuprein (Turnlund et al., 1998). Cu taken up by the liver may be stored within hepatocytes, secreted into plasma, or excreted in bile. The Cu in hepatocytes is mostly bound to metallothionein, or

synthesized into cuproenzymes. Cu released from the liver is primarily bound to ceruloplasmin for transport to the tissues, but may also bind to albumin, transcuprein, and histidine. Ceruloplasmin, the main Cu binding protein and an acute phase protein, contains up to eight Cu atoms in both the cupric and cuprous states. Approximately 60 to 90% of the circulating Cu in the blood is in the form of ceruloplasmin (Harris, 1993).

Transfer of Cu to newly synthesized cuproenzymes and Cu disposal is carried out by the individual or concerted actions of Cu-chaperones and Cu-transporting ATPases (Cu-ATPases) expressed in tissues (Prohaska, 2008, Boal and Rosenzweig, 2009). Cu trafficking pathways participate in Cu homeostatic regulation by providing Cu for essential enzymes and proteins, and at the same time, preventing Cu from reaching toxic concentrations (Prohaska, 2008, Boal and Rosenzweig, 2009). Intracellular Cu trafficking has been defined with the clarification of Cu handling proteins or Cu chaperones. There are three major Cu trafficking pathways that entail Cu chaperones (Boal and Rosenzweig, 2009, Fields et al., 2001). The Atx1 Cu chaperone delivers Cu to transport ATPases in the secretory pathway, the chaperone encoded by the Cu chaperone for superoxide dismutase (CCS) gene delivers Cu to Cu,Zn- SOD, and Cu supplied to the mitochondria for activation of cytochrome *c* oxidase.

Cu transport protein Atox1 is a Cu-dependent suppressor of oxidative damage in yeast lacking superoxide dismutase. Kelner et al. (2000) have shown that neuronal cell lines transfected with the Atox1 gene to increase the endogenous level of Atox1 expression are protected against serum starvation and oxidative stress, and suggested that Atox1 may also play a role in preventing neuronal cells against oxidative damage induced by Cu. By examining the role of Cu delivery to the secretory pathway in Cu utilization and homeostatic maintenance, Hatori et al. (2012) found that the glutathione/glutathione disulfide (GSH/GSSG) pair controls the Cu transport pathway by regulating the redox state of a Cu chaperone Atox1. GSSG oxidizes Cu-coordinating cysteines of Atox1 with the formation of an intra-molecular disulfide. GSH alone is sufficient to reduce the disulfide, restoring the ability of Atox1 to bind copper. In cells, high GSH reduces Atox1 and is required for cell viability in the absence of Atox1. Atox1 has a redox potential similar to that of glutaredoxin, which becomes essential for cell survival when GSH levels decrease. The findings suggest that GSH balance and Cu homeostasis are functionally linked, and jointly maintain conditions for Cu secretion and cell proliferation.

The biosynthetic incorporation of Cu into secreted and plasma membrane-bound proteins requires activity of Cu-ATPases ATP7A and ATP7B (Prohaska, 2008, Boal and Rosenzweig, 2009). ATP7A and ATP7B exert their functions in Cu transport through a variety of interdependent mechanisms and regulatory events, including catalytic ATPase activity, Cu-induced trafficking, post-translational modifications and protein-protein interactions. ATP7A and ATP7B are evolutionarily conserved polytopic membrane proteins with essential roles in human physiology. The Cu-ATPases are expressed in most tissues, and their transport activity is crucial for central nervous system development, liver function, connective tissue formation, and many other physiological processes (Lutsenko et al., 2007a, 2007b). The influx of reduced copper ions is controlled by two functionally homologous transmembrane solute carrier transporters CTR1and CTR2. The molecular characteristics of

Cu transporters CTR1 (encoded by SLC31A1), CTR2 (encoded by SLC31A2), ATP7A and ATP7B, their roles in mammalian Cu homeostasis and the physiological consequences of their inactivation have been examined (Gupta and Lutsenko, 2009; Wee et al., 2013). These Cu transporters vary in their expression profiles and intracellular localization patterns. CTR1 plays roles in the developing embryo as well as regulating homeostasis in the adult mammal (Wee et al., 2013). In contrast, the regulation, expression and function of CTR2 are not well-defined. Both are capable of transporting other divalent metal ions and are the primary transporters for platinum-based chemotherapeutic drugs such as cisplatin. The SLC31 (CTR) family of Cu transporters is a major gateway of Cu acquisition in eukaryotes, ranging from yeast to humans. Characterization of the function, modes of action, and regulation of CTR and other molecular factors that functionally cooperate with CTR for Cu transport, compartmentalization, incorporation into cuproproteins, and detoxification has revealed that organisms have evolved fascinating mechanisms for tight control of Cu metabolism (Kim et al., 2013).

The Golgi complex harbors copper-transporting ATPases, ATP7A and ATP7B that transfer copper from the cytosol into Golgi lumen for incorporation into Cu-dependent enzymes. Incorporation of Cu into the secreted and plasma membrane-targeted cuproenzymes takes place in Golgi, a compartment central for normal Cu homeostasis (Polishchuk and Lutsenko, 2013). The Golgi complex also sends these ATPases to appropriate post-Golgi destinations to ensure correct Cu fluxes in the body and to avoid potentially toxic Cu accumulation. Mutations in ATP7A or ATP7B or in the proteins that regulate their trafficking affect their exit from Golgi or subsequent retrieval to this organelle. This, in turn, may disrupt the homeostatic Cu balance, resulting in Cu deficiency (Menkes disease) or Cu overload (Wilson disease). Wilson disease (WD) is caused by mutations in ATP7B, a transporter that loads Cu(I) onto newly synthesized cupro-enzymes in the *trans*-Golgi network (TGN) and exports excess copper by trafficking from the TGN to the plasma membrane (Braiterman et al., 2014). While these studies have yielded a better insight into the enzymatic properties and cell biology of the Cu ATPases, the mechanism by which the Golgi regulates trafficking of ATP7A/7B and, thereby, maintains Cu homeostasis remains unclear.

V. Cu toxicity

Most organisms possess a combination of regulated import, sequestration, and enhanced export mechanisms to protect against metal-induced toxicity. These mechanisms regulate metal status through metal-binding proteins at transcriptional, translational, and enzymatic levels. As stated above, the presence of a complex system of metal ion transporters and chaperones to regulate Cu homeostasis ensures Cu is provided to essential proteins without causing cellular damage. Disruptions in the homeostasis of Cu is associated with tissue damage and a number of diseases (Bleackley and Macgillivray, 2011; de Romana et al., 2011). In addition to the direct interact with essential macromolecules and minerals, several mechanisms, notably free radical-induced oxidative damage, have been proposed to explain Cu-induced cellular toxicity.

a. Free radical-induced oxidative damage

Transition metal ions, such as Fe and Cu, are capable of undergoing redox cycling reactions and promote the formation of reactive oxygen species (ROS). A number of studies have attributed Cu toxicity to the propensity of Cu ions to participate in the formation of ROS that modify the structure and/or function of essential biomolecules (Halliwell & Gutteridge, 1984; Lippard, 1999). In the presence of superoxide or reducing agents such as ascorbic acid or GSH, Cu^{++} can be reduced to Cu^{+}, which is capable of catalyzing the formation of hydroxyl radicals from hydrogen peroxide via the Haber-Weiss reaction (Bremner, 1998; Kadiiska et al., 1993). As only one electron is needed to reduce Cu^{++}, the other electron may be involved in the production of hydroxyl radicals (Multhaup et al., 1999). The hydroxyl radical is the most powerful oxidizing radical likely to arise in biological systems. It is capable of initiating oxidative damage by abstracting the hydrogen from an amino-bearing carbon to form a carbon-centered protein radical and from an unsaturated fatty acid to form a lipid radical (Chow, 1991; Letelier et al., 2010; Powell, 2000; Burkitt, 2001), and inducing DNA strand breaks and oxidation of bases (Kawanishi et al., 1989; Hayashi et al., 2000; Buettner, 1993; Liang and Dedon, 2001). Also, peroxynitrite, the reaction product of nitric oxide and superoxide, may promote the release of Cu ions from such protein complex as ceruloplasmin (Harris, 1992). The underlying mechanism of action for transition metal ions may involve the formation of superoxide, hydroxyl radical and other ROS, and subsequently producing malondialdehyde, 4-hydroxynonenal and other exocyclic DNA adducts (Rossi et al., 2006, Gaetke and Chow, 2003; Jomova and Valko, 2011).

Results obtained from a number of experimental studies support the view that ROS-induced oxidative damage plays an important role in Cu toxicity. For examples, Cu has been shown to involve oxidative modification of low-density lipoprotein (LDL) and promote atherogenesis by enhancing the transformation of macrophages into foam cells and by developing vasoconstrictor and prothrombotic properties (Haidari et al., 2001). Excess Cu may lead to peroxidative damage to membrane lipids via the reaction of lipid radicals and oxygen to form peroxy radicals (Powell, 2000), and causes peroxidation in the membranes of hepatocyte lysosomes (Bremner, 1998; Sokol et al., 1990). Cu-overloaded rats exhibit oxidative injury including decreased levels of hepatic GSH and α-tocopherol, increased levels of mitochondrial lipid peroxidation products, decreases in state 3 respiration and the respiratory control ratio in hepatic mitochondria, and decreased complex IV (cytochrome C oxidase) activity and increased hepatic Cu (Sokol et al., 1990; Ohhira et al., 1995; Sansinanea et al., 1998; Zhang et al., 2000). Cu overload also reduces the activity of cytochrome c oxidase and impairs liver mitochondrial respiration (Myers et al., 1993), and increase in rat liver chemiluminescence, while the activities of catalase and GSH peroxidase are significantly decreased (Ossola et al., 1997). By overwhelming body antioxidant systems and inducing DNA damage, lipid peroxidation, protein modification and other effects, ROS may lead to the development of degenerative diseases, including cancer, cardiovascular disease, diabetes, atherosclerosis, neurological disorders and chronic inflammation (Chow, 1979; Chow and Chow-Johnson, 2013; Jomova and Valko, 2011).

A role of oxidative stress in Cu toxicity is supported by the protective effect of metal chelators, such as ammonium tetrathiomolybdate and ethylenediaminetetraacetic acid, which

prevent neuronal death of rats caused by intra-hippocampal injections of cupric sulphate (Armstrong et al., 2001). While the hyperoxia-mediated induces the metal-binding proteins ceruloplasmin and metallothionein in the lungs of mice expressing varying amounts of Cu,Zn-SOD, it has no effect on tissue levels of Cu, Fe, or Zn (Levy et al., 2001). As mice with increased expression of Cu,Zn-SOD have significant reduction in circulating ceruloplasmin and Cu concentrations, ceruloplasmin may function as a store of Cu for Cu,Zn-SOD synthesis. Also, as chronic reduction in Cu,Zn-SOD impairs vascular tone probably mediated by a direct inactivation of nitric oxide production and an increase in lipid peroxidation (Lynch et al.,1997), dietary Cu may affect the endothelium-dependent arterial relaxation.

In humans, the disruption of normal Cu absorption and excretion is associated with two severe disorders, Menkes disease and Wilson's disease. The consequences of insufficient Cu supply that is characteristic of Menkes disease have been linked to the inactivation of key metabolic enzymes, although other non-enzymatic processes may be involved (Song et al., 2011). In contrast, the consequences of Cu accumulation in Wilson disease are generally ascribed to Cu-induced oxygen radical-mediated damage (White et al., 2009; Sayre et al., 2000). Wilson's disease is phenotypically variable and can present with predominantly hepatic or neurologic manifestations. Patients with Wilson's disease have evidence of lipid peroxidation in liver mitochondria and reduced liver and blood concentrations of the antioxidant vitamin E (Myers et al., 1993).

Chronic Cu toxicity in the form of liver cirrhosis and damage to other organs is seen in genetic abnormality of Cu metabolism (Wilson's disease) and in the presumed environmental disorder Indian Childhood Cirrhosis (ICC). ICC, non-ICC, and possibly idiopathic Cu toxicity appear to be caused by ingesting milk that has been boiled or stored in corroded Cu or brass vessels, although a genetic predisposition has also been linked to ICC-like illness (Pankit and Bhave, 1996; Pankit and Bhave, 2002, Wijmenga et al., 1998). Cu has also been implicated in the pathogenesis of such common neurodegenerative diseases as Alzheimer's, Parkinson' and Huntington's diseases as well as amyotrophic lateral sclerosis. Alzheimer's disease is characterized by neuronal degeneration, increased concentrations of Cu, Fe, and Zn, and increased deposits of amyloid-β protein in the brain (Strausak et al., 2001, Zatta et al., 2008; Squitti et al., 2009). In Alzheimer's disease, the amyloid precursor protein gene molecule, which has been directly linked to early-onset forms of the disease, contains a Cu-binding site (Multhaup, 1997; Multhaup et al., 1998; Multhaup et al., 1999), and the binding of amyloid-β protein to Cu and Zn could promote ROS generation in the brain (Strausak et al., 2001).

b. Other possible mechanisms

In addition to the free radical-induced oxidative damage, information available suggests that the cellular response to Cu overload, particularly at the early stages of Cu accumulation, involves more specific mechanisms and pathways. This includes regulation of lipid metabolism, antimicrobial defense, neuronal activity, resistance of tumor cells to chemotherapeutic drugs, kinase-mediated signal transduction, and other essential cellular processes (Hasan and Lutsenko 2012). While the mechanism of these actions remains to be

established, many regulatory and signaling events are associated with changes in the intracellular localization and abundance of Cu transporters, as well as distinct compartmentalization of Cu itself. Several other possible mechanisms of Cu toxicity are listed below:

Altered lipid metabolism—Using an animal model of Wilson disease to characterize the pre-symptomatic effects of Cu accumulated in the liver, Huster and Lutsenko (2007) found links between Cu metabolism, cell-cycle machinery, and cholesterol synthesis, and identified several candidate proteins that may mediate the cross-talk between Cu status and lipid metabolism. Significant down-regulation of lipid metabolism is observed at all stages of Wilson's disease irrespective of Cu distribution. These findings suggest that altered lipid metabolism may be involved in Cu toxicity. Also, using animal models of Wilson's disease to study gene and protein profiling, Burkhead et al. (2011) have revealed the link between molecular players and pathways, including cell cycle and cholesterol metabolism, mRNA splicing and nuclear receptor signaling, and Cu imbalance, and uncovered cellular processes that are primarily affected by Cu accumulation in the liver.

Altered hepatic gene expression—Using a toxicogenomic approach in a fish model to investigate the signaling pathways mediating the effects of exposure to Cu, Santos et al. (2010) found that Cu exposures resulted in DNA strand breaks in blood cells at all exposure concentrations, alterations in hepatic gene expression occurred in a concentration-dependent manner, and that genes associated with the cholesterol biosynthesis pathway were significantly over-represented and consistently down-regulated, similar to that occurring in a mouse model for Wilson's disease. Additionally, Cu exposure induces metallothionein and catalase, and increases the concentrations of NAD (+) and lactate, which are consistent with a shift toward anaerobic metabolism, and changes in gene expression. The expression of hepatocyte GP73, a Golgi membrane protein, in hepatocytes in response to acute and chronic liver disease, is more frequently observed in Wilson's disease patients with hepatic versus neurologic presentation, and is significantly higher in patients with hepatic phenotype (Wright et al., 2009). In ATP7B(−/−) mice, GP73 mRNA is significantly elevated at 20-46 weeks of age, coincident with extensive hepatic inflammation and fibrosis, but not at 6 weeks, when hepatic histology is normal despite significant Cu overload. GP73 mRNA levels normalized concomitantly with the resolution of hepatic injury at 60-weeks. However, in tumor-like nodules GP73 is strikingly elevated. The findings suggest that increased hepatocyte GP73 expression is more commonly a feature of hepatic than neurologic Wilson's disease, and is triggered in response to inflammation, fibrosis, and dysplasia, rather than Cu overload.

Altered alpha-synuclein aggregation—Alpha-synuclein, a natively unfolded protein that aggregates and forms inclusions that are associated with a range of diseases that include Parkinson's disease and dementia with Lewy Bodies has been shown to bind metals including Cu and Fe. Using a cell culture model of alpha-synuclein aggregation to examine the relationship between metals and formation of aggregates, Wang et al. (2010) have shown that Cu is important for both aggregation and cellular localization of alpha-synuclein, and that reduction in cellular Cu results in a dramatic decrease in aggregate formation both in

terms of large aggregates visible in cells and oligomers. They also show that reduction in Cu results in a change in localization of the protein, which became more intensely localized to the plasma membrane with low Cu, and these changes are reversed when Cu is restored to the cells. Additionally, mutants of the Cu binding domains alter the response to Cu, and increased expression of alpha-synuclein increase cell sensitivity to the toxicity of Cu. These results suggest that the potential pathological role of alpha-synuclein aggregates is dependent upon the Cu binding capacity of the protein.

Altered activation of acidic sphingomyelinase (Asm) and release of ceramide—Apoptosis is a highly regulated and crucial process found in all multicellular organisms. It has been implicated in regulatory mechanisms of cells, and attributed to a number of diseases, including inflammation, malignancy, autoimmunity and neurodegeneration. Transition metals, including Cu, cadmium, chromium and nickel, may promote apoptosis along with DNA base modifications, strand breaks and rearrangements. Cu may induce apoptosis by p53 dependent and independent pathways, and suggests that disorders of apoptosis may play a critical role in Cu-induced hepatotoxicity and neurotoxicity (Rana, 2008). Lang et al. (2011) studied the role of Asm and ceramide in liver cell death and anemia in patients with Wilson's disease, and found that Cu^{++} triggers hepatocyte apoptosis through activation of Asm and release of ceramide. The production of ceramide, an apoptotic signal, in hepatocytes may lead to hepatocyte apoptosis. Genetic deficiency or pharmacological inhibition of Asm prevented Cu^{++}-induced hepatocyte apoptosis and protected rats, genetically prone to develop Wilson disease, from acute hepatocyte death, and liver failure. Cu^{++} induced the secretion of activated Asm from leukocytes, leading to ceramide release and phosphatidylserine exposure on erythrocytes, events also prevented by inhibition of Asm. Phosphatidylserine exposure resulted in immediate clearance of affected erythrocytes from the blood in mice. Asm may downregulate the liver-specific methionine adenosyltransferase 1A, and contribute to tumor necrosis factor-induced lethal hepatitis. Also, individuals with Wilson disease showed elevated plasma levels of Asm, and displayed a constitutive increase of ceramide- and phosphatidylserine-positive erythrocytes. Asm may downregulate the liver-specific methionine adenosyltransferase 1A, and contribute to tumor necrosis factor-induced lethal hepatitis (Mari et al., 2004). These findings suggest a central role of Asm activation in liver cirrhosis and anemia in Wilson's disease, and that Cu^{++} may trigger hepatocyte apoptosis through activation of Asm and release of ceramide (Brewer et al., 2007).

Altered hepatic distribution of Cu—Cu accumulation in Wilson's disease is different from accumulation due to excess dietary Cu builds up in lysosomes and causes liver injury when it is released into the cytoplasm (Fuentealba et al., 2000, Medici et al. 2006). The impaired transport also interferes with incorporation of Cu into ceruloplasmin, thus decreasing serum concentrations of ceruloplasmin. Consequently, hepatic fibrosis develops, ultimately producing cirrhosis, and Cu diffuses out of the liver into the blood, then into other tissues. The excess Cu is deposited in other extra-hepatic tissues including the brain and cornea of the eye. The accumulation of Cu in the liver and other organs is associated with the development of hepatic or neurologic symptoms. The most common symptoms are hepatitis (acute, chronic active and fulminant), central nerve system development (motor

deficits) and cognitive or psychiatric abnormalities. Temporal and spatial distribution of Cu in hepatocytes may play an important role in Wilson's disease pathology. Using high resolution synchrotron-based x-ray fluorescence imaging in situ, Ralle et al. (2010) have shown that Cu does not continuously accumulate in ATP7B(−/−) hepatocytes. The lack of further accumulation is associated with the loss of Cu transporter CTR1 from the plasma membrane and the appearance of Cu-loaded lymphocytes and extracellular Cu deposits. Also, the progression of Wilson's disease is characterized by changes in subcellular Cu localization and transcription remodeling in the initial response to Cu overload and the metabolic pathways show compartmentalization that parallels changes in subcellular Cu concentration.

Altered protein-metal interaction—The interaction between metals and proteins in the nervous system seems to be a crucial factor for the development or absence of neurodegeneration, and metal accumulation within the nervous system observed in those diseases could be the result of compensatory mechanisms to improve metal availability for physiological processes (Rivera-Mancía et al., 2010). Using a computational approach and employing quantum mechanics/molecular mechanics methods to examine the molecular mechanism of protein-mediated Cu^{+} transfer from the human Cu chaperone Atox1 to the fourth metal-binding domains of Wilson's disease protein, Rodriguez-Granillo et al. (2010) found that both Atox1 and Wilson's disease protein have solvent-exposed metal-binding motifs with two Cys residues that coordinate Cu^{+}. Those data suggest that the Cu-transfer reaction from Atox1 to Wilson's disease protein appears to be kinetically accessible, and that altered protein-metal interaction in the nerve system may play a role on Cu toxicity.

As a number of biological response modifiers are redox-sensitive, intracellular production/level of ROS may alter the expression and activation of vital biological modifiers, which in turn may alter cell proliferation, differentiation, apoptosis, and other cellular events (D'Autréaux and Toledano, 2007; Chow and Chow-Johnson, 2013; Long et al., 2014; Caliceti et al., 2014). Therefore, Cu-induced changes in the redox properties may alter neurotransmitter biosynthesis, angiogenesis and other physiological processes. However, it remains possible one or more of the proposed mechanisms of Cu toxicity may be secondary to the formation of ROS or consequence to oxidative damage.

IV. Summary and Conclusion

Cu is an essential trace mineral for many important enzymes and proteins in living organisms. Cu homeostasis is generally well-maintained with effective regulatory mechanisms, and Cu toxicity resulting from disturbed homeostasis is an important contributor to numerous different symptoms and disease conditions. These disease states are most often linked to the role of Cu as a redox-active transition metal that may initiate oxidative damage. Recently, altered lipid metabolism, gene expression, alpha-synuclein aggregation, activation of Acidic sphingomyelinase and release of ceramide, and temporal and spatial distribution of Cu in hepatocytes, and Cu-protein interaction in the nerve system, have been suggested to play a role in Cu toxicity.

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