Liver as a key organ in the supply, storage, and excretion of copper\textsuperscript{1–4}

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**ABSTRACT**

The liver plays an important role in the disposition of copper. Most dietary copper passes through the liver where it can be used for protein and energy production or excreted through the biliary route. Because copper is a prooxidant, its intracellular handling is tightly managed. In Wilson disease, in which synthesis of ceruloplasmin and biliary excretion of copper are defective, copper accumulates in the liver and leads to progressive liver damage. The features of hepatic Wilson disease are highly variable. The spectrum of liver disease includes mild inflammation, fatty liver, an autoimmune disorder, and cirrhosis. Wilson disease thus resembles drug hepatotoxicity, and indeed it can be regarded as a prototypic example of endogenous hepatotoxicity. Biomarkers developed for detecting drug hepatotoxicity may be relevant to Wilson disease. Biomarkers developed through metalloproteomics, which for copper seeks to define a set of proteins that have copper-binding capacity, or through genomic studies may also be relevant to Wilson disease and other disorders of copper handling, whether copper is deficient or overloaded. *Am J Clin Nutr* 2008;88(suppl):851S–4S.

**INTRODUCTION**

Copper is essential for the action of diverse enzymes, performing a broad range of physiologic functions. An ordinary diet provides sufficient copper because typical daily intake of copper ranges from 1 to 10 mg, usually ≈2–5 mg/d, depending on the amount of meat, legumes, shellfish, and chocolate consumed. The recommended daily intake is 0.9 mg/d. Efficiency of intestinal absorption is high (55–75%) and intake is not regulated; normal copper balance is maintained by regulation of excretion for which the predominant route is hepatobiliary. Most (85%) of dietary copper is excreted. The renal pathway accounts for <5% of copper excretion, unless renal tubular reabsorption capacity is exceeded.

Dietary copper, as well as copper found in saliva and gastric and pancreatic juices, is absorbed in the proximal small intestine. Absorption is probably by hCTR1 expressed on enterocytes, although the divalent cation transporter (DMT1), which is mainly involved in iron uptake, may play a limited role. Once absorbed, copper is in an exchangeable pool, bound reversibly to serum albumin and to various amino acids, histidine being the most important. Copper-albumin and copper-histidine distribute to various amino acids, histidine being the most important. Copper-albumin and copper-histidine distribute to various amino acids, histidine being the most important. Copper-albumin and copper-histidine distribute to various amino acids, histidine being the most important. Copper-albumin and copper-histidine distribute to various amino acids, histidine being the most important. Copper-albumin and copper-histidine distribute to various amino acids, histidine being the most important. 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The majority (95%) of copper in blood is incorporated in ceruloplasmin, which is itself an acute-phase reactant and therefore subject to variation independent of total body copper. It is not routine to measure serum copper-albumin or copper-histidine directly, and estimates of non–ceruloplasmin-bound copper are faulty unless ceruloplasmin is measured as ferroxidase activity. Basal 24-h urinary copper excretion reflects non–ceruloplasmin-bound copper and provides valuable diagnostic information in Wilson disease (WD). Very low plasma concentrations of ceruloplasmin, if measured enzymatically, may characterize copper insufficiency in patients with WD, who have been too aggressively treated (2).

**COPPER IN THE LIVER**

Dietary copper loosely bound to albumin or histidine reaches the liver by the portal vein and is taken up into hepatocytes across the sinusoidal plasma membrane (Figure 1). Because copper associated with these transporters is Cu(II), it must be reduced to Cu(I) before hepatocellular uptake. After the available copper is reduced either by a reductase on the outer aspect of the hepatocyte membrane or possibly by dietary reductants, it is transported into the hepatocyte by hCTR1, a member of the solute ligand carrier superfamily, encoded by the gene SLC31A1 (3). hCTR1 appears to exist as a trimer, forming a channel in the hepatocellular plasma membrane. Analogous to the Wilson adenosine 5′-triphosphatase (ATPase), hCTR1 has copper-binding domains near the amino terminus, but these domains consist of a methionine cluster motif (MXXM), as opposed to a cysteine cluster (CXXC). Copper uptake may be linked to potassium transport. Although hCTR1 is not the regulatory control point for copper homeostasis, it is degraded when copper concentrations are high. A second copper transporter, known as hCTR2, may

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Copper does not exist in ionic form within cells. In hepatocytes and other cells it is always bound to low-molecular-weight proteins, called metallochaperones, which each deliver copper to different specific target molecules within the cell (4). In all likelihood, the entire fleet of copper-metallochaperones has not yet been identified. CCS1 guides copper to SOD1, a principally cytoplasmic defense against oxidant stress. ATOX1 directs copper to the Wilson ATPase. It has 1 CXXC copper-binding domain, similar to the 6 founding the Wilson ATPase. It interacts with the Wilson ATPase (5) and with copper (6). Metallothioneins and glutathione are also found in the cytoplasm; they interact with the Wilson ATPase and with copper (6). Metallothionein is then secreted into the blood. When intracellular copper concentrations are elevated, the Wilson ATPase expedites biliary excretion of copper by a process that may also involve COMMD1. Biliary copper excretion can also take place by the transporter MRP2. The complete network of proteins involved in hepatocellular copper disposition has not yet been identified. BC, bile canaliculus.

Mechanistic details of the intracellular action of the Wilson ATPase remain unclear. Positioned in the trans-Golgi network, it participates in the synthesis of holoceruloplasmin, apparently by making copper available for incorporation into the nascent holoprotein. Remarkably, when intracellular copper concentrations are elevated, copper excretion can also take place by the transporter MRP2. The complete network of proteins involved in hepatocellular copper disposition has not yet been identified. BC, bile canaliculus.

In hepatocytes the Wilson ATPase plays a pivotal role in copper disposition. It is an intracellular copper-transporting P-type ATPase with molecular weight \( \approx 165 \text{ kDa} \) (1411 amino acids). Like other P-type ATPases, it has a cation channel and phosphorylation domain with a highly conserved DKTGT motif in which the aspartate residue is transiently phosphorylated during the transport cycle. The Wilson ATPase has 6 copper-binding domains (GMXCXXC); 8 transmembrane segments forming a pore, including the distinctive CPC motif in segment 6; and an ATPase region. Its structure has been determined by homology mapping with the use of the sarcoplasmic Ca\(^{2+}\) P-type ATPase Serca1 as model (8), and structures of portions of the protein have also been solved (5). Conformational changes associated with copper binding may influence Wilson ATPase function; accordingly, the N-terminal region may play a regulatory role. Recent functional studies were performed with a truncated human Wilson ATPase lacking the copper-binding domains 1–5 (9).

The role of copper in normal mitochondrial metabolism principally involves cytochrome-\(c\) oxidase (complex IV in the OX-PHOS chain) and associated proteins such as Cox17, Cox19, Sco1, and Sco2 (7). Cox17 is the main copper chaperone for targeting delivery of copper to mitochondria; Cox19 is required for complex IV expression and may have copper-transport functions. Sco1 and Sco2 are assembly factors for complex IV. Thioredoxin-2 (Trx-2) and some metallothioneins are also found in the mitochondrial matrix.

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WD is a disorder of hepatic copper disposition, first described in 1912. It is due to mutations in the gene ATP7B. Because the Wilson ATPase has 2 main intracellular actions (promoting incorporation of copper into apoceruloplasmin and expediting excretion of excess copper into bile), patients characteristically have hepatic copper overload, with low serum concentrations of enzymatically active ceruloplasmin and thus proportionally low serum concentrations of copper, except when there are high serum concentrations of non–ceruloplasmin-bound copper. More than 300 mutations in ATP7B have been identified. Most of them are low-abundance mutations, and most patients are compound heterozygotes. WD occurs worldwide; the average incidence is ≈30 affected persons per million population. WD can lead to liver disease, progressive neurologic disorder, or psychiatric illness. The hepatic presentation usually occurs at younger ages. As yet, clear-cut genotype-phenotype patterns remain elusive. Different mutations have differing effects on the structure or function of the Wilson ATPase; eg, some mutants fail to relocate intracellularly when copper concentration is elevated. WD is fatal if not treated, but with effective treatment, especially if commenced early, the outlook for a normal healthy life is excellent. Treatment is lifelong with either an oral chelating agent or zinc salts; liver transplantation is reserved for patients who do not respond to medical treatment or present initially with Wilsonian fulminant hepatic failure (15).

Liver damage in WD appears to involve oxidant stress. Copper is a prooxidant because it can exist in 2 different valence states; indeed, this is the basis of its metabolic utility. It participates in the Haber-Weiss reaction; its ability to generate reactive oxygen species has been shown in HepG2 cells (16). Apoptosis is prominent in Wilsonian liver damage. Recent studies suggest that one mechanism leading to apoptosis is activation of acid sphingomyelinase and consequent production of ceramide, which can initiate apoptosis (17); increased plasma acid sphingomyelinase activity was proposed as a biomarker of WD.

One of the striking features about WD is its clinical diversity as liver disease. The pattern of the liver disease is highly variable: an acute illness resembling viral or autoimmune hepatitis, fatty liver, cirrhosis, or simply asymptomatic elevation of serum aminotransferases. WD may present as fulminant hepatic failure with acute intravascular hemolysis and rapidly progress to renal failure; this is apparently due to widespread hepatocellular apoptosis in an already damaged liver. Hepatocellular carcinoma is uncommon. This clinical diversity is highly reminiscent of drug-induced hepatotoxicity. Drug-induced liver disease can resemble almost any type of liver disease described, and notably a single drug can lead to more than one pattern of liver injury. Accordingly, we propose that WD can be regarded as a prototype of drug-induced hepatotoxicity because of abnormal metabolic pathways. In fact WD may be an excellent paradigm for studying reactive oxygen species–associated hepatotoxicity. The important operational implication of this concept of WD is that genomic and proteomic techniques being used to develop biomarkers for drug hepatotoxicity may be relevant to WD (18).

METALLOPROTEOMICS

Proteomics encompasses protein expression, including structure-function relationships, physiologically and in disease states. An organism’s versatility and variability—reflecting its developmental program as well as its cumulative responses to external stimuli or stressors—is indicated in protein expression, reflecting its genome but subject to additional posttranslational modifications. To analyze protein expression, we may limit it by restricting our examination to proteins of a single organ or cell type or subcellular organelle, by examining only a physiologic state or a disease, or by specifying a time point or developmental stage. We have modified standard proteomics strategies to examine proteins interacting with a metal; we call this "metalloproteomics" (19). In defining a metalloproteome, we seek to determine the set of proteins that have metal-binding capacity, by virtue either of being metalloproteins or of having metal-binding sites. We are interested in the metalloproteome for copper, but equally a metalloproteome could be defined for any metal.

We examined the copper metalloproteome in HepG2 cell lysates to determine more completely the proteins involved in hepatocellular handling of copper. Our initial strategy was to use a copper-loaded immobilized metal affinity column to capture copper-binding proteins; thus, we identified 38 high-abundance proteins, in addition to some nonspecific proteins. Some of the proteins of interest were known to have copper-binding capability, but others were not previously known to be able to bind copper. We also examined copper depletion and found a novel variation in posttranslational modification of elongation factor 1α, depending on whether HepG2 cells were copper depleted (20). A more focused examination of HepG2 cytosol and microsomes showed a broader spectrum of copper-binding proteins (21). We have shown that protein disulfide isomerase, a highly abundant classic chaperone protein of the endoplasmic reticulum, is a copper-binding protein with the classic CXXC binding motif. Its copper-binding properties were confirmed by extensive biophysical analysis (22). Others have shown that it is upregulated in the liver of copper-challenged North Ronaldsay
Copper toxicosis in sheep (23). We have also examined expression of some of the proteins we found in the copper metalloproteome in the toxic milk mouse, a model for WD. Noting that peroxiredoxin-1 was identified in the copper-metalloproteome, we found that the mitochondrial protein peroxiredoxin-3 was indeed up-regulated in hepatic mitochondria as the liver disease progresses during the first 6 mo of life. Similarly, the mitochondrial protein Trx-2 was also up-regulated (24). Although Trx-2 has the canonical CXXC primary sequence, we expect that preoxiredoxin-3 forms a “virtual” CXXC copper-binding domain through dimerization as peroxiredoxin-1 does. Thus, a proteomics strategy can identify functional copper-binding proteins, in addition to those predicted by their primary sequence. Quantitative assessment of the metalloproteome can be attained by adding an isotope-coded affinity tag component to the method, followed by mass spectrometric analysis.

Other groups have taken some different method approaches. Protein-chip plus the surface-enhanced laser desorption ionization method was used to investigate copper-binding proteins in HepG2 cells that were incubated with either physiologic or elevated (100 μM) concentrations of copper. This method has advantages relating to reproducibility and high throughput; however, lack of immediate identification of the proteins detected is a drawback. Among the proteins found, various metallothioneins and hCTR2 were identified (25). In a non-Wilsonian hereditary copper toxicosis in sheep, a proteomic study showed important changes in certain hepatic proteins with copper challenge in the copper-sensitive North Ronaldsay breed. Up-regulated proteins included NADP+-dependent isocitrate dehydrogenase, retinol-binding protein, and protein disulfide isomerase (23). Combination of results relating to copper disposition in a variety of experimental models or diseases or both may help to focus on candidate biomarkers. Gene expression profiling of copper-loaded HepG2 cells has shown similar changes to those identified by proteomics, also with genes relating to drug metabolism and immune response (26). Examination of the transcriptome in the Atp7b knockout mouse has shown changes in lipid metabolism, metallothioneins, and various proteins controlling the cell cycle (27).

In conclusion, our knowledge of how copper is handled in hepatocytes has progressed greatly because the identification of the gene abnormal in WD. Metalloproteomics offers a strategy for identifying other proteins involved in copper disposition and can provide candidate biomarkers. Some of these biomarkers may be valuable for diagnosing WD, which is often difficult to diagnose by currently available clinical and genetic methods.

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The author’s responsibilities were as follows—EAR: organized and wrote the first draft, provided key concepts and the figures, and managed all revisions of the manuscript; BS: reviewed the entire manuscript in its original and revised forms and clarified all technical points relating to the bioinorganic chemistry of copper. None of the authors had a personal or financial conflict of interest.

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