Expression of copper-related genes in response to copper load[1–4]

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ABSTRACT

Copper is an essential micronutrient for all biological systems. Multiple proteins require one or more atoms of copper for proper structure and function, but excess of copper is toxic. To prevent the consequences of copper deficiency and overload, living organisms have evolved molecular mechanisms that regulate its uptake, intracellular traffic, storage, and efflux. Underlying some of the cellular responses to variations in copper levels are changes in the expression of genes encoding molecular components of copper metabolism. In recent years, genome-scale expression analysis in several eukaryotic models has allowed the identification of copper-responsive genes involved in copper homeostasis. Characterization of the transcriptional changes in response to varying copper levels include both genes directly involved in copper homeostasis and genes involved in different cellular process that, even though they are not directly connected to copper metabolism, change their expression during the cellular adaptation to copper availability. Evaluation of these gene expression patterns could aid in the identification of biologically relevant markers to monitor copper status in humans. *Am J Clin Nutr* 2008;88(suppl):830S–4S.

MOLECULAR MECHANISMS OF COPPER HOMEOSTASIS

The cellular components that participate in copper homeostasis are evolutionary conserved across species. Currently, the most complete description of the regulatory mechanisms in copper metabolism exists for *Saccharomyces cerevisiae* (baker's yeast; 1). This model organism has been useful for understanding the function of the human genes involved in copper metabolism (2), because it shares a high degree of conservation in basic metabolic pathways with humans. The mechanisms of copper uptake and distribution have been reviewed in detail previously (3).

High-affinity copper uptake in yeast is mediated by the transporters Ctr1p and Ctr3p (4, 5). Because extracellular copper is found predominantly as Cu^{2+}, copper ions are reduced by the reductases Fre1p and Fre2p located on the cell surface before transport (6). Once inside the cell, copper binds to the cytosolic copper chaperones Atx1p, Cox17p, and Lys7p, which deliver copper to the secretory pathway, cytochrome oxidase, and Sod1p, respectively (7). Moreover, copper is required for proper targeting and activity of the Fet3p-Ftr1p complex, which is required for high-affinity iron uptake.

In mammals, copper metabolism parallels the pathway previously described for yeast (**Figure 1**). The copper reductases *Steap* (11) and *APP* (12) may be responsible for reduction of Cu(II) before it is transported across the plasma membrane via the high-affinity copper transporter *hCTR1* (13), which is specific for Cu(I). In addition, Cu(II) can be transported directly into the cell by the divalent metal transporter *DMT1* (14). Once in the cytoplasm, copper is delivered to various cell targets with the help of copper chaperones; *HAH1* transfers copper to *ATP7A/B* located in the trans-Golgi network (15), *CCS* to *SOD1* (16), and COX17 through SCO1/2 to cytochrome C oxidase (17). In addition, intracellular copper can be bound to metallothionein (MT) or glutathione (18).

COPPER-INDUCED TRANSCRIPTIONAL CHANGES IN *SACCHAROMYCES CEREVISIAE*

In *S. cerevisiae*, the transcription factors Mac1p and Cup2p (Ace1p) act in a coordinated fashion to maintain copper homeostasis (19). Under copper deficiency conditions, Mac1p induces the transcription of the copper transporters *CTR1* and *CTR3* and the copper-iron reductase *FRE1* (20). In the absence of Mac1p, *FRE1* is down-regulated, whereas several genes involved in copper and iron uptake and transport are up-regulated; the latter are likely mediated by the transcription factors Aft1p and Aft2p, which respond to iron (21) and which are up-regulated under copper deficiency conditions (22). On the other hand, Cup2p induces the transcription of the MT genes *CUP1-1/2* and *CRS5* and *SOD1* in response to elevated copper concentrations (23). Moreover, *FET3* and *FTR1*, which are involved in iron uptake, are also up-regulated under these conditions (24), indicating a disruption in iron homeostasis.

In yeast, copper deficiency and excess result in the opposite response of 3 sets of genes that mostly contain known targets of Aft1p/Aft2p, Mac1p, and Cup2p (22), which suggests that copper homeostasis is primarily coordinated by these transcription factors. In addition, genes associated with respiration are down-regulated in copper deficiency, possibly to preserve iron and copper for other cellular processes, because many mitochondrial proteins utilize copper either as a cofactor or for structure. In the case of copper overload, yeast relies primarily on *CUP2*, and

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[2] Presented at the symposium "Molecular Biomarkers of Copper Homeostasis," held in Viña del Mar, Chile, September 26–29, 2007.

[3] Supported by Fondecyt Chile grant 1071083 and by an unrestricted grant from the Corporación Chilena del Cobre (COCHILCO, Chile) and the Corporación para la Nutrición Infantil (CINUT, Chile).

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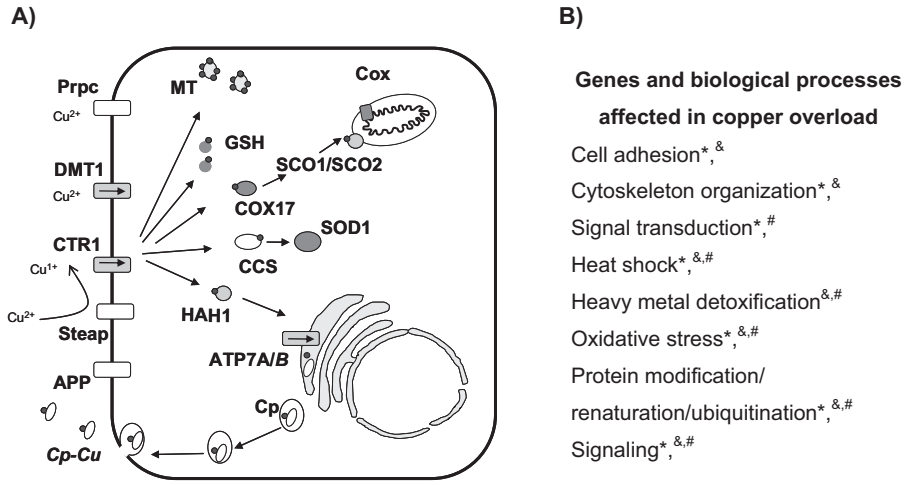

FIGURE 1. (A) Schematic representation of the cellular components involved in copper detoxification in a model eukaryotic cell. *Prpc*, cellular prion protein; *DMT1*, divalent metal transporter; *CTR1*, copper transporter 1; *Steap*, copper reductase; *APP*, amyloid precursor protein; *Cp*, ceruloplasmin; *MT*, metallothionein; *GSH*, glutathione; *COX17*, copper chaperone; *SCO1/SCO2*, copper binding protein; *Cox*, cytochrome oxidase; *CCS*, copper chaperone superoxide dismutase, *SOD1*, Cu/Zn superoxide dismutase; *HAH*, human *ATX1* homologous; *ATP7A/B*, copper ATPase Menkes/Wilson. (B) Genes differentially expressed in response to copper excess are grouped according to biological processes on the basis of data from references 8 (marked with *), 9 (marked with &), and 10 (marked with #).

therefore MT, for protection against high doses of copper and, in its absence, fails to induce a general stress response (25). Differentially expressed genes identified under copper deficiency and excess can be classified according to their cellular role under these conditions and include protection against copper overload, copper import and regulation, and vacuolar function (22).

COPPER-INDUCED TRANSCRIPTIONAL CHANGES IN MAMMALIAN MODELS

In mammals, multiple proteins associated with copper metabolism are regulated by post-translational mechanisms (26). However, regulation also occurs at the transcriptional level. Expression of genes involved in the protection against copper overload involves specific responses, such as the induction of copper-binding MT proteins, and general stress responses, such as the oxidative stress response and proteasomal degradation (10).

MTs are cysteine-rich proteins that bind copper and other metals, thus protecting against free copper ions inside the cell. Supporting the protective role of MTs are their increased expression in copper-treated cells and the elevated copper sensitivity exhibited in MT-null fibroblast cell lines (27). Moreover, MT appears to have an important role in the gene regulatory network associated with cellular adaptation to copper, because fibroblast cells from MT I/II null mice fail to enhance expression of metal metabolism, oxidative stress, heat shock, and ubiquitination genes in the presence of copper, as opposed to wild-type cells (27).

Copper can also bind to both the prion protein, Prnp, and the amyloid precursor protein, App (28). The genes encoding both of these proteins have been found to be up-regulated in 2 different mouse fibroblast cell lines that accumulate copper as the result of inactivation of *ATP7A* (8). Moreover, *Prnp* was also up-regulated in primary hippocampal neurons and PC12 cells after copper treatments (29). These findings could be explained by the presence of a putative metal response element in the promoter region of *Prnp*, which suggests that this gene could be regulated by the presence of heavy metals.

Another promising candidate for copper status detection is copper chaperone for SOD1 (CCS). In animal studies, CCS protein but not mRNA was shown to be consistently elevated in copper deficiency (30). Recently, a reduction of *CCS* mRNA was found after copper supplementation in peripheral mononuclear cells of persons with high serum ceruloplasmin concentrations (31). Clearly, further studies should be undertaken to evaluate the transcriptional and post-translational regulatory mechanism of this protein in response to copper. Other genes that are up-regulated under copper excess conditions include a variety heat shock proteins, with an induction as high as 19-fold (10).

WILSON DISEASE AND ANIMAL MODELS OF COPPER OVERLOAD

Wilson disease is a genetic disorder of copper metabolism in which mutations in *ATP7B* prevent normal biliary excretion of copper. Wilson disease results in the accumulation of copper in tissues and organs, particularly in the liver and brain. Expression of the proapoptotic genes *CD95* and *CD95L* has been detected in livers of Wilson disease patients with hepatic failure (32). The activation of the apoptotic pathway as a consequence of copper overload in Wilson disease is consistent with findings in animal disease models (33).

Several animal models for Wilson disease have been described that carry either loss-of-function mutations or deletions of *ATP7B*. These include the toxic milk mouse (34), the *Atp7b*$^{-}$/$^{-}$ knockout mouse (35), and the Long-Evans Cinnamon (LEC) rat (36). Because these animals accumulate abnormally high amounts of copper in their livers and show diverse pathologies as a consequence of the overload, they provide an additional resource to evaluate the effects of copper excess not only at the transcriptional but also at the organismal level.

Expression profiling in livers of LEC rats has shown up-regulation of genes associated with oxidative stress, DNA damage, apoptosis, and inflammation at different stages of liver injury compared with the wild-type strain. Interestingly, genes

related to oxidative stress and DNA damage were mainly up-regulated in livers from rats with no pathologic signs but with increased copper concentrations, which are consistent with measurements of oxidative stress markers in this rat strain (33). On the other hand, genes associated with cell cycle and chromatin structure, but not oxidative stress, were up-regulated in livers of presymptomatic *Atp7b*$^{-/-}$ mice, whereas genes associated with cholesterol biosynthesis were down-regulated and were correlated with low cholesterol concentrations in the liver (37).

ATP7B interacts with *COMMD1* (38), a protein whose deficiency causes canine copper toxicosis (39). *COMMD1* belongs to the *COMMD* (copper metabolism *MURR1* domain) family of proteins that is widely conserved and that has 10 identified members.

Common mutations in *ATP7B* in patients with Wilson disease increased binding to *COMMD1,* which appears to promote degradation of *ATP7B* (40). In addition to *ATP7B*, *COMMD1* also interacts with nuclear factor-κB (NF-κB) and promotes its ubiquitination (41). However, increased expression of *Commd1* in livers of the toxic milk mouse did not correlate with NF-κB levels (42). In HepG2 cells, the levels of *COMMD1* and of most members of the COMMD family were decreased after prolonged copper exposure, possibly as a response to oxidative stress by allowing the activation of NF-κB (10). Further studies characterizing the interactions of COMMD1 could clarify the role of COMMD1 in the regulation of copper metabolism and in the pathogenesis of Wilson disease.

COPPER-INDUCED TRANSCRIPTIONAL CHANGES IN OTHER MODEL ORGANISMS

In general, expression analysis in copper overload in other organisms has found some correlation with findings in yeast and mammalian models. For example, several genes belonging to the *Cue1* family that encode putative metal-binding proteins were up-regulated in the diatom *Thalassiosira pseudonana* (43). Similarly, oxidative stress-response genes were up-regulated in *Pseudomonas aeruginosa* (44). An interesting finding in the latter is the up-regulation of pyoverdine biosynthetic genes, an iron-specific siderophore, in copper-adapted cultures. This suggests a possible disruption in iron metabolism that has been observed in other species after exposure to copper (45).

Despite some similarities, there are also differences in gene regulation. An interesting case is the transcription factor MTF-1 in *Drosophila melanogaster*, which responds to both copper deficiency and overload by inducing the transcription of the copper transporter gene *Ctr1B* and MT genes, respectively (46). This is in contrast with the case in yeast, where regulation of target genes in these 2 conditions is carried out by 2 transcription factors, Mac1p and Cup2p. Moreover, the yeast and human copper transporters are regulated post-transcriptionally, whereas the *Drosophila Ctr1b* is regulated mainly at the transcriptional level.

ALTERNATIVE APPROACHES USED TO IDENTIFY COPPER HOMEOSTASIS GENES

The phenotypic analysis of yeast deletion mutants provides a different approach in the identification of genes associated with copper metabolism (22, 45). Several genes found to be important in resistance to high copper levels encode members of conserved molecular protein complexes, including the retromer, ESCRTII, and ESCRTIII complexes, which suggests that the mechanisms involved could have relevance in mammalian systems (45). Considering the high structural and functional relation between cupro-proteins of yeast and those of mammals, additional global gene expression analysis may be useful to determine whether adaptation to copper exposure occurs similarly in human cells.

Mutagenesis-based approaches in *Drosophila* have also been used to identify copper homeostasis genes (47). In this case, screening of deletion mutants resulted in the identification of a chromosomal region containing the Ctr1B with resistance and another region containing the *Drosophila* MT genes *MtnB*, *MtnC,* and *MtnD* with copper sensitivity. The MT genes are a target of MTF-1, the deletion of which also induces copper sensitivity (48). Because deletions encompass large fragments of a chromosome, the resolution of this screen is limited and, as the authors point out, it is not possible to establish a direct connection between these genes and the phenotypes observed unless the deletion mutants are screened. However, screening of deletion mutants exhibiting a copper-resistant phenotype identified a chromosomal region containing the *Syntaxin* (*Syx5*) locus, a gene involved in vesicle trafficking. In another approach, de novo mutagenesis, point mutations induced with ethyl methanesulfonate resulted in 10 copper-resistant lines, but in this case, candidate genes were not identified (47).

CONCLUSIONS

The limits of the homeostatic regulation of excess copper in humans are not known, making it difficult to define the mild effects of early copper toxicity. In these regards, the expression analysis of biomarker genes could provide a robust assay to monitor these effects.

Copper is a trace nutrient that is tightly regulated because it is essential for cellular function but toxic if present in excess. As such, its metabolism is highly conserved among different species. Analysis of gene expression in response to varying copper levels has been conducted in different systems using both genomic and candidate gene approaches, and several model organisms have been used to characterize the copper metabolic pathways (**Table 1**).

Genes that change their expression levels in response to copper availability constitute potential biomarkers of copper status in humans. However, caution should be exercised when comparing findings from different test systems, because these have shown a modest degree of correlation. For example, comparison between differentially expressed genes in copper-treated HepG2 cells and livers of diet-induced copper overload mice showed a small degree of concordance (10). Clearly, several factors such as effective dose and length of treatment should be taken into consideration when evaluating available data for the selection of candidate biomarker genes of copper exposure.

Evidence from genomic studies has shown that copper modulates transcriptional changes not only of genes directly associated with copper metabolism but also of genes that participate in general physiologic processes (Figure 1). Therefore, potential copper status biomarkers could be involved in other more general cellular processes and may not be limited to genes directly involved in copper metabolism. In this sense, it is important to consider the time course over which the modifications of copper transport and storage take place and whether these modifications are reversible. Therefore, changes in copper transport rate that

TABLE 1
Published studies that have used genome-wide approaches to identify genes associated with copper (Cu) metabolism

Model organism	Strain/tissue	Condition tested	Number of genes identified	Reference
S. cerevisiae	BY4743/cells	Deregulation in Cu homeostasis by *mac1* knock out	97	(21)
S. cerevisiae	*S*288c/cells	Cu excess and deficiency for several time points	128	(22)
S. cerevisiae	CM66J/cells	Deregulation in Cu homeostasis by constitutive *MAC1* mutant	>50	(24)
S. pombe	972 h-/cells	Cu overload at 2 concentrations	93 and 1259	(25)
H. sapiens	HepG2 cells	Cu overload and deficiency at several time points	157	(10)
M. musculus	129Sv/Ev / liver	Cu supplementation in water	22	—
M. musculus	C57BL/6 fibroblast cell lines	Cu overload induced by mutations in *Atp7a*	181 and 140	(8)
M. musculus	C57BL/liver tissue	Cu overload induced by *Atp7b* knockout	76	(36)
T. pseudonana	CCMP 1335/cells	Cu overload	16	(42)
P. aeruginosa	PAO1/cells	Cu-adapted culture with Cu before incubation	331	(43)
P. aeruginosa	PAO1/cells	Cu-shocked with high Cu concentration	405	(43)
S. cerevisiae	BY4743/cells	Functional profiling of Cu overload using homozygous knockouts	200	(44)
D. melanogaster	Larvae (fourth day of development)	Cu supplementation in food for few hours	82	(45)
D. melanogaster	Larvae (fourth day of development)	Cu deficiency	370	(45)
D. melanogaster	Adult female Celera	Cu in medium for 7 d at 2 concentrations	22 and 94	(46)
D. melanogaster	Embryonic S2 cells	Cu excess	309	—
North Ronaldsay sheep	Liver tissue	Cu supplementation in diet for different time points	8	(49)

are complete in a few minutes would probably depend mostly on post-translational and possibly reversible modifications, whereas changes that take hours would be likely regulated at the transcriptional level. Finally, physiologic and pathophysiologic animal and cellular models could be useful to clarify or confirm the copper response for selected candidate biomarkers, considering factors such as exposure time, age and sex, and others.

The contributions of the authors were as follows—MG and CV: were the primary authors of the article; AR-J, MS, and WJJ: assisted with writing the article and contributed to analysis and discussion of data. None of the authors had any conflicts of interest to disclose.

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