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Human copper homeostasis: a network of interconnected pathways

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Abstract

Copper plays an essential role in normal human physiology. Copper misbalance affects heart development, CNS and liver function, influences lipid metabolism, inflammation, and resistance to chemotherapeutic drugs. Recent studies yielded new information on the structure, function, and regulation of human copper transporters, uncovered unanticipated functions for copper chaperones, and established connections between copper homeostasis and other metabolic pathways. It has become apparent that the copper trafficking machinery is regulated at several levels and that the cross-talk between cell compartments contributes to the intracellular copper balance. The human copper regulon is emerging.

Introduction

Copper is required for respiration, connective tissue formation, iron metabolism, and many others processes. In human cells, copper is utilized in several cells compartments, and the intracellular distribution of copper is regulated in response to metabolic demands and changes in cell environment. Significant progress has been made in characterizing the copper trafficking pathways in mammalian cells. This review will discuss recent advances and emerging concepts in the following areas:

1. Copper entry: machinery and regulation
2. Copper chaperones: an expanding repertoire of functions and mechanisms
3. Cross-talk between copper trafficking pathways
4. Regulation of copper delivery to the secretory pathway and copper efflux
5. Copper homeostasis and cells' resistance to cisplatin await integration

Copper entry: machinery and regulation

The transporter CTR1 plays the key role in high affinity copper uptake. In cells, CTR1 is found in two locations: at the plasma membrane and in intracellular vesicles (Figure 1). The distribution of CTR1 between these two compartments and the extent to which copper influences this distribution is cell-specific. In the liver, kidney, placenta, and mammary gland, the predominant location of CTR1 is at the basolateral plasma membrane [1,2*,3,4],

where it transports copper from circulation by, most probably, retrieving copper from specific carrier(s) [5]. In intestine, CTR1 has an additional role in making dietary copper available for further utilization, presumably, by facilitating its release from intracellular vesicles [6*]. Consistent with this role, a significant fraction of CTR1 in enterocytes is intracellular and is located in the vicinity of the apical membrane.

The intracellular distribution of CTR1 is dynamic. Elevation of extracellular copper induces endocytosis of CTR1 to vesicles (thus decreasing copper uptake across the membrane), whereas a decrease in extracellular copper restores the CTR1 levels at the plasma membrane [7]. In addition to acute modulation of plasma membrane levels of CTR1, the total amount of CTR1 is regulated at the mRNA level in response to prolonged changes in copper status [8*]. The homeostatic control of the steady-state levels of CTR1 mRNA is illustrated by the observation that the expression of exogenous hCTR1 is accompanied by the decrease in the amount of mRNA for the endogenous hCTR1 [8*].

Fluctuations of copper levels in a serum are likely to be low keeping the steady-state CTR1 levels (both total and at the plasma membrane) fairly constant. However, physiologic conditions that increase metabolic demands for copper can provide a notable CTR1 upregulation. Larger amounts of CTR1 protein (and an increase in copper uptake) are observed in macrophages in response to hypoxia [9**] or treatment with gamma-interferon [10*], both of which increase the flow of copper towards the secretory pathway. The physiologic signals such as 17- β estradiol may also modulate the response of CTR1 to changes in copper levels [11]. The complex kinetics and tissue specificity of CTR1 regulation is well illustrated by recent studies in fish [12]. The molecular machinery that controls CTR1 abundance is largely unknown; however, recent study suggests a direct role for nuclear protein Sp1 in *CTR1* transcription [8*].

Copper enters the cell even if CTR1 is markedly down-regulated, although in this case the amount of entering copper is insufficient to fully support the activity of copper-dependent enzymes [1]. Which protein/process mediates copper uptake in the absence of CTR1 is uncertain. A low-affinity copper transporter CTR2 is largely intracellular [13,14] and may function to release copper from the lysosome or lysosome-like compartments for re-utilization (by analogy with the yeast CTR2 [15]). However, a small fraction of CTR2 is detected at the plasma membrane, and the overexpression of CTR2 is associated with increased copper uptake [14]. Perhaps, under certain conditions, CTR2 can be recruited to the plasma membrane to facilitate copper import. Whether more CTR2 is present at the plasma membrane when CTR1 is downregulated remains to be explored.

Yeast CTR2 works together with a reductase [16], which converts Cu(II) to Cu(I), a form required for translocation. In mammalian cells, the lumen of the lysosome-like compartments is likely to be oxidizing compared with the cytosol and specific reductases might be needed to maintain Cu in the reduced form. STEAP family of reductases are candidates for such a role [17]. STEAP3 and STEAP4 are located in intracellular vesicles and reduce copper *in vitro* [17]. STEAPs have little effect on copper transport across the plasma membrane, thus it would be interesting to examine if they facilitate the intracellular transport activity of CTR2.

Copper chaperones: an expanding repertoire of functions and mechanisms

The current model for copper homeostasis suggests that upon entry into the cell copper binds to cytosolic copper chaperones, CCS and Atox1, which then transfer copper to specific cellular destinations. CCS activates cytosolic Cu, Zn-dependent superoxide dismutase (SOD1) by inserting copper and a disulfide bond; while Atox1 transfers copper to the copper-transporting ATPases in the secretory pathway (Figure 1). A low molecular weight carrier and/or a yet-to-be characterized protein initiates a series of transfer reactions that result in copper delivery to cytochrome *c* oxidase (CCO) in mitochondria [18^{*}]. This attractive and widely cited model of ‘one carrier per cell compartment’ needs updating, because recent data paint a more complex picture of chaperone-mediated copper distribution (Figure 1).

Specifically, SOD1 is also found in the inter-membrane space of mitochondria (IMS), where it provides protection from superoxide that cannot be neutralized by the matrix-based MnSOD. The entry, folding, and metallation of SOD1 in the IMS require CCS. In turn, the presence of CCS in mitochondria is influenced by oxygen [19^{**}]. High oxygen concentration promotes oxidative folding of CCS in the cytosol and precludes its import into IMS thus retaining more SOD1 in the cytosol. Lower oxygen concentrations favor CCS mitochondrial localization and SOD1 translocation into IMS. Thus, in addition to its important role as a copper carrier, CCS acts as an oxygen sensor [19^{**}] and a factor regulating SOD1 compartmentalization.

Another novel function for the CCS/SOD1 pair was recently reported in yeast [20]. The CCS-dependent activity of SOD1 and SOD1 entry into the nucleus facilitates the binding of transcription factor Mac1 to its target genes and upregulation of copper uptake [20]. Inactivation of either CCS or SOD1 impairs yeast ability to upregulate the Mac1 target genes and combat copper deficiency [20]. A role in transcription was also proposed for Atox1 [21]. In mouse fibroblasts, the copper-dependent increase in cell proliferation and cyclin D expression require Atox1 [21]. The translocation of Atox1 into the nucleus and direct binding to the cyclin D promoter region suggest that this effect is direct [21]. At the same time, genetic deletion of Atox1 does not prevent copper entry into the nucleus [22] indicating the presence of more than one pathway for copper delivery/action in mammalian nuclei.

The proteins important for copper insertion into cytochrome *c* oxidase (CCO) in mitochondria (Cox17, Cox11, Sco1 and Sco2) also have multiple roles regulating the stability, redox state, and/or metallation of their partners. In the IMS, copper chaperone Cox17 transfers copper to Sco1 and Cox11, which are implicated in the formation of the Cu(A) and Cu(B) sites of CCO, respectively. A recent study uncovered a fascinating ability of Cox17 to couple the transfer of copper and electrons [23^{**}]. *In vitro*, copper-bound Cox17 with two intact disulfide bonds simultaneously transfers Cu(I) and two electrons to oxidized Sco1. As a result, Sco1 is reduced and binds copper, while the metal-binding cysteines of Cox17 are oxidized into a third disulfide bond [23^{**}]. Further transfer of copper to CCO requires the formation of a complex with Sco1 and Sco2. In this complex, Sco2 appears to

oxidize the copper-coordinating cysteines in Sco1 as a prerequisite step for copper transfer [24*]. The subsequent steps in CCO maturation are still being explored.

A new potential regulator of copper metabolism has recently been described. CutC is a highly conserved cytosolic copper binding protein [25]. The function of this protein in human cells remains unclear; however downregulation of CutC in *C. elegans* increases sensitivity to high copper [26].

The cross-talk between copper trafficking pathways

It remains unresolved how human cells allocate copper to different intracellular routes. Copper carriers may bind free ion that emerges from a CTR1/CTR2 pore or retrieve copper from a Cu–glutathione complex, which would easily form given high concentrations of glutathione in the cytosol. In this mechanism, the preferential route of copper distribution will be determined by the copper-binding affinity and/or abundance of copper carriers. Alternatively, chaperones may play an ‘active’ role in copper uptake through direct interactions with the transporter. In this case, copper allocation towards a certain route will be established on the basis of preferential interactions between the transporter and a chaperone. *In vitro*, the exchange of copper between the C-terminus of CTR1 and Atox1 was reported, favoring the above scenario. An ‘active’ role of metallochaperones in copper uptake is also suggested by observation that the human CTR1 expressed in *Drosophila* has a low transport activity unless it is co-expressed with CCS [27].

The extramembraneous regions of CTR1 form structural elements that are well positioned to regulate copper access to and from the pore [28*], and it was suggested that metallochaperones can dock to CTR1 (Unger, meeting report). From this perspective, significant structural similarity between Domain I of CCS and Atox1 [29*] is intriguing. Structural alignment illustrates that the identity between these proteins is not limited to the copper-binding CxxC motif and residues involved in protein folding, but also involves several charged residues at the protein surface (Figure 2), which can be involved in protein-protein interactions. Given this similarity, one would expect the Domain 1 of CCS and Atox1 to compete for copper. This prediction finds supports in recent studies on copper distribution in macrophages in hypoxia [9**]. Oxygen deprivation redirects copper flow from mitochondria and the cytosol towards the secretory pathway (demonstrated by the decreased activity of CCO and SOD1 and increased biosynthesis of ceruloplasmin in the secretory pathway [9**]). The mechanism of limited copper delivery to mitochondria is unclear, but in the cytosol hypoxia decreases the CCS levels. Thus, CCS downregulation appears to be necessary to shift copper distribution from the cytosolic targets towards the Atox1-mediated route. A similar effect (re-directing copper for efflux) is caused by mutations in Sco proteins that impair copper incorporation into CCO in mitochondria [18*]. By contrast, simultaneous downregulation of Atox1 and metallothionein leads to the sequestration of copper in vesicles and functional copper deficiency in the cytosol despite overall elevation of copper, and simultaneous upregulation of CTR1 and CCS [30]. Elucidating the mechanisms that coordinate copper distribution pathways in a cell is the next important frontier in studies of copper biology. Better understanding of such a regulon would help to decipher the role of copper homeostasis in resistance of cancer cells to cisplatin (see below).

Copper delivery to the secretory pathway and copper efflux

Copper-transporting ATPases (Cu-ATPases) maintain intracellular copper levels. Their activity, post-translational modification, and intracellular localization are modulated by intracellular copper [31]. The Cu-ATPases accept copper from Atox1 and use the energy of ATP hydrolysis to transfer copper into the secretory pathway, where copper is incorporated into copper-dependent enzymes (Figure 1). In response to copper elevation, Cu-ATPases undergo kinase-mediated phosphorylation and relocalization to vesicles in proximity to either basolateral (ATP7A) or apical (ATP7B) membrane. Copper is subsequently exported via vesicle-mediated fusion. Metabolic factors that upregulate copper uptake also trigger trafficking of Cu-ATPases [32,9^{**},10^{*}]. Recent reports suggest the presence of Cu-ATPase in new cellular locations (ATP7B in tight junctions [32] and late endosomes [33], and ATP7A in the nucleus [34]); further studies are needed to understand the mechanistic significance of these observations.

Similarly to CTR1, regulation of endogenous Cu-ATPases is cell-specific. ATP7B traffics in response to copper in hepatocytes, intestinal, and placental cells, but does not traffic in renal or ovary cells [35,36^{*}]. The lack of trafficking implies that in these cells the role of ATP7B is either to maintain a continuous supply of copper to enzymes in the secretory pathway (when ATP7A traffics away to mediate copper export) or to sequester copper for storage. In many cells, two Cu-ATPases are co-expressed, and dissecting their specific roles is essential for better understanding of human copper homeostasis. Recent studies of hormone-dependent regulation in placental cells revealed a coordinated but different response of two Cu-ATPases to insulin and estrogen. Expression and trafficking of ATP7A to the basolateral membrane is stimulated, while the expression of ATP7B is reduced with a net increase in copper transport to the fetus [37^{**}]. Significant regulation of Cu-ATPases also occurs during development [38,39] and tumorigenesis [40].

Information on the structure and regulation of Cu-ATPases has been steadily accumulating [31,41]. The structure of the ATP-binding domain has been solved [42,43], further illustrating the uniqueness of ATP coordination in Cu-ATPases compared with other P-type pumps. Studies of the archaeal CopA revealed the presence of two copper-binding sites in the transmembrane portion of Cu-ATPases [44^{*}], and demonstrated conformational changes in response to ligand binding [45]. Characterization of CopA has yielded immensely valuable information, however mechanistic differences exist between archaeal and eukaryotic Cu-ATPases and should be considered. In CopA, the chaperone CopZ delivers copper directly to the transmembrane sites [46^{*}]. By contrast, in yeast the transfer of copper by Atx1 to the transmembrane portion of Cu-ATPase Ccc2 requires the N-terminus of the pump [47^{*}]. Also, recent study demonstrates that proteins that accept copper from human Cu-ATPases may influence both the transport activity and trafficking of Cu-ATPases [48^{*}]. Such regulation by acceptor protein(s) can be common for mammalian Cu-ATPases but be absent in archaea and prokaryotes.

Important progress has been made in understanding the directionality of Cu-ATPase trafficking [49^{*}]. The highly conserved sequence, F³⁷AFDNVGYE⁴⁵ of ATP7B was shown to comprise an apical targeting determinant for trafficking in elevated copper as well as TGN

retention under low copper conditions [49*]. This targeting motif may work together with other copper-dependent signals, such as kinase-mediated phosphorylation. Multiple phosphorylation sites have been identified in ATP7A and ATP7B [50,51,52*] perhaps reflecting the variety of metabolic signals to which Cu-ATPases respond. Mutagenesis of candidate Ser residues in ATP7A results in mislocalization in the presence of added copper [51], consistent with the role of these residues in vectorial trafficking. Interestingly, *in vitro* the membrane-bound ATP7B becomes phosphorylated in the absence of soluble cell extract [50,52*], and an increase in a kinase-mediated phosphorylation parallels an increase in the ATP7B catalytic activity [52*]. These observations raise an intriguing possibility that ATP7B is either tightly associated with a membrane-bound kinase or may have a kinase activity of its own.

Consequences of Cu-ATPases inactivation further illustrate existence of a fine-tuned copper regulon, which involves proteins beyond 'classic' copper homeostatic genes. In the liver, ATP7B inactivation leads to copper accumulation in the cytosol and nuclei [53], downregulation of lipid metabolism and increased cell proliferation [53,54*]. Similarly, copper elevation due to ATP7A inhibition in macrophages decreases the promoter activity and protein amounts of cPLA₂ α , a cytosolic phospholipase [55]. That elevated copper does not simply inhibit transcription is evident from studies showing the upregulation of amyloid precursor protein and prion protein in human fibroblasts lacking ATP7A [56,57]. It is interesting that the transcription factor SP1 appears to contribute to this copper-dependent regulation [56,57]. Thus, SP1 may play a dual role in modulating copper uptake [8*] and regulating the response to copper elevation.

Copper homeostasis and cells' resistance to cisplatin await integration

Recent years saw an explosion of interest in the role of copper metabolism in resistance of cancer cells to cisplatin-like drugs [58,59*]. The format of this review does not permit detailed discussion of this important and complex subject. It is clear that the components of copper trafficking machinery can directly bind cisplatin [60,36,61]. The structure of the Atox1-cisplatin complex has been solved [59*] illustrating the potential mode of interaction between the drug and copper-binding proteins. The *in vivo* downregulation of copper efflux pump ATP7B in tumors [36*] was shown to increase tumor sensitivity to cisplatin [36], illustrating clinical potential of modulating copper metabolism. It has also become apparent that cisplatin does not simply mimic copper [61] and that detailed characterization of copper regulon may help to understand how changes in copper balance influence cells resistance to chemotherapeutic drugs.

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•• of outstanding interest

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Figure 1.

Copper distribution pathways in a generalized mammalian cell. CTR1 accepts copper from the extracellular copper carriers and transfers copper into cytosol; changes in copper levels induce reversible trafficking between the plasma membrane and intracellular vesicles. CTR2 is predominantly intracellular but can be found at the plasma membrane. Entering copper is either binds directly or is retrieved from CTR1/CTR2 by copper chaperones that have multiple functions; CCS distributes copper to SOD1 in cytosol and mitochondria, while Atox1 transfers copper to the secretory pathway and nucleus. An ensemble of proteins regulates copper delivery to cytochrome *c* oxidase (CCO) in mitochondria. Cu-ATPases transport copper to the secretory pathways for incorporation into cuproenzymes and mediate copper excretion by sequestering excess copper in vesicles. The trafficking of Cu-ATPases between these two locations is associated with phosphorylation by a kinase (indicated by stars), which increases in response to copper elevation.

Figure 2. Structural comparison of human Atox1 (blue, 1TL5) and Domain I of CCS (green, 2CRL). Sequence alignment illustrates significant similarity of mammalian Atox1 and CCS (identical residues are in red, conserved residues in orange). The conserved residues around the metal-binding site are highlighted by grey (top); their location in the superimposed structures (left) and surface exposure (right) are shown (bottom).