Organelles in focus

Mitochondrial copper homeostasis and its derailment in Wilson disease

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ABSTRACT

In mitochondria, copper is a Janus-faced trace element. While it is the essential cofactor of the mitochondrial cytochrome c oxidase, a surplus of copper can be highly detrimental to these organelles. On the one hand, mitochondria are strictly dependent on adequate copper supply for proper respiratory function, and the molecular mechanisms for metalation of the cytochrome c oxidase have been largely characterized. On the other hand, copper overload impairs mitochondria and uncertainties exist concerning the molecular mechanisms for mitochondrial metal uptake, storage and release. The latter issue is of fundamental importance in Wilson disease, a genetic disease characterized by dysfunctional copper excretion from the liver. Prime consequences of the progressive copper accumulation in hepatocytes are increasing mitochondrial biophysical and biochemical deficits. Focusing on this two-sided aspect of mitochondrial copper, we review mitochondrial copper homeostasis but also the impact of excessive mitochondrial copper in Wilson disease.

1. Introduction

Copper is a trace element, essential for neurotransmitter, neuropeptide and collagen biosynthesis, wound healing, angiogenesis, growth and iron utilization (Kaplan and Maryon, 2016; Owen, 1973). Recently, copper has been suggested to regulate the systemic delivery of triglycerides from the GI tract (Pierson et al., 2017; Weiss and Zischka, 2018). Intracellularly, the two most important copper functions are linked to its redox ability as cofactor of either mitochondrial cytochrome c oxidase (CcO) or of the reactive oxygen species (ROS) detoxifying Cu/Zn superoxide dismutase (SOD1) (Blockhuys et al., 2017). These two enzymes manage the biochemical challenge of a safe copper-mediated reduction/disproportionation of oxygen or ROS, respectively. Unbound "free" copper ions and ROS would otherwise inevitably cause the emergence of hydroxyl radicals that are highly detrimental to proteins, nucleic acids and lipids, via Fenton-based chemistry. Indeed, physiologically, copper ions are not "free", i.e., dissolved in water, but strictly bound to carrier molecules and distributed intracellularly by so-called copper chaperones to avoid such cellular toxicity (Rae et al., 1999).

Mitochondria harbor the CcO and around 1–5% of total cellular SOD1 and, therefore, are a major site of intracellular copper utilization (Sturtz et al., 2001). Indeed, especially in yeast, these organelles have been suggested to be the intracellular copper store (Yang et al., 2005; Cobine et al., 2004). This view originates from the rationale that increased cellular energetic needs may be met by enhanced mitochondrial oxidative phosphorylation activities and plausibly by elevated CcO and consequently elevated copper amounts (Cobine et al., 2006; Leary et al., 2009a). Thus, in order to meet the basal but also enhanced energetic cellular demand, there is a constant copper supply to mitochondria, and elevated copper loads can be handled by mitochondria (Cobine et al., 2004; Zischka et al., 2011). However, a steadily increasing and excessive mitochondrial copper load may severely affect these organelles. As it is the case in Wilson disease (WD), hepatic copper overload leads to mitochondrial destruction, hepatocyte death and even liver failure. In this article, we focus on current knowledge but also on controversial

theories about mitochondrial copper homeostasis with a special focus on liver mitochondria. We further outline how a disturbed copper balance induces mitochondrial dysfunction and cellular damage in WD.

2. Mitochondrial copper homeostasis

It has been estimated that a rat liver mitochondrion contains about fifteen to sixteen thousand CcO molecules (Schwerzmann et al., 1986), and that mitochondrially localized SOD1 constitutes around 0.06% of the total mitochondrial protein content (Okado-Matsumoto and Fridovich, 2001). This means that 10^9 mitochondria comprising about 125 μg total protein (Schmitt et al., 2014) would contain around 75 ng SOD1, i.e., about 4.7 pmoles SOD1 (M = 15,943 g/mol) or about 2,800 SOD1 molecules per mitochondrion. Given three copper ions per CcO and one per SOD1, this would amount to around 45,000–50,000 copper atoms per mitochondrion, or around 40 ng/mg mitochondrial protein (assuming $8.1*10^9$ mitochondria per mg of mitochondrial protein (Schmitt et al., 2014)). This value matches reported mitochondrial copper contents of rat liver but also human liver mitochondria ranging from 30 to 50 ng/mg (Zischka et al., 2011; Sokol et al., 1994; Zischka and Lichtmannegger, 2014). As these two mitochondrial copper enzymes are essential for hepatocyte bioenergetics and survival, mitochondria therefore require an adequate copper supply.

The functional mitochondrial copper need is met by copper transporters, so-called copper chaperones (below) and small molecular copper ligands as depicted in Fig. 1. Two prerequisites ensure a safe and robust mitochondrial copper supply. First, in cells, copper is strictly bound to proteins or small molecule ligands to avoid uncontrolled copper redox activity (Rae et al., 1999). Second, the main driving force of copper to be incorporated into CcO and SOD1 is their enormous copper binding affinity (Cu^{1+}-binding dissociation constant K_{Cu} below femtomolar), and an increasing copper affinity of the intermediate copper transporting molecules ensures their directed delivery to CcO and SOD1 (Banci et al., 2010).

As the copper-containing subunits of CcO, COX1 and COX2, are mitochondrially encoded proteins and as metal free apo-SOD1 is imported into the mitochondrial intermembrane space (IMS), copper metalation of these proteins occurs within mitochondria (Field et al., 2003). How is the metal delivered and distributed to and within mitochondria? Most of our current knowledge concerning this issue comes from sophisticated studies in yeast and several, not mutually exclusive, hypotheses have been put forward:

First, copper chaperones, low molecular mass proteins that hand over copper by protein-protein interactions (Banci et al., 2010), have been suggested to transport copper into mitochondria. Indeed, the CcO assembly proteins 19 and 23 (COX19, COX23), as well as COX17, are small soluble proteins containing cysteine residues that bind Cu(I), exhibiting dual localization in cytosol and the IMS (Fig. 1) (Glerum et al., 1996; Nobrega et al., 2002; Barros et al., 2004). However, yeast depleted in these proteins had wild-type mitochondrial copper levels (Cobine et al., 2004; Glerum et al., 1996; Nobrega et al., 2002; Barros et al., 2004). Moreover, CcO deficiency in *cox17Δ*, *cox19Δ* or *cox23Δ* mutant yeast can be restored by external copper supplementation (Glerum et al., 1996; Nobrega et al., 2002; Barros et al., 2004). The same holds true for the dually localized CCS, the SOD1 copper chaperone (Cobine et al., 2004; Field et al., 2003). Thus, while copper chaperones enable mitochondrial CcO and SOD1 activities, alternative mitochondrial copper uptake molecules are likely to be present.

A second potential copper entry or export mechanism to or from the IMS may occur via the tripeptide glutathione (GSH, Fig. 1), as GSH can easily cross the mitochondrial outer membrane (MOM) through porin channels (Mari et al., 2009). However, the idea of such a GSH-copper cotransport into the IMS or mitochondrial matrix has been challenged by experiments in yeast depleted in GSH that had wild-type
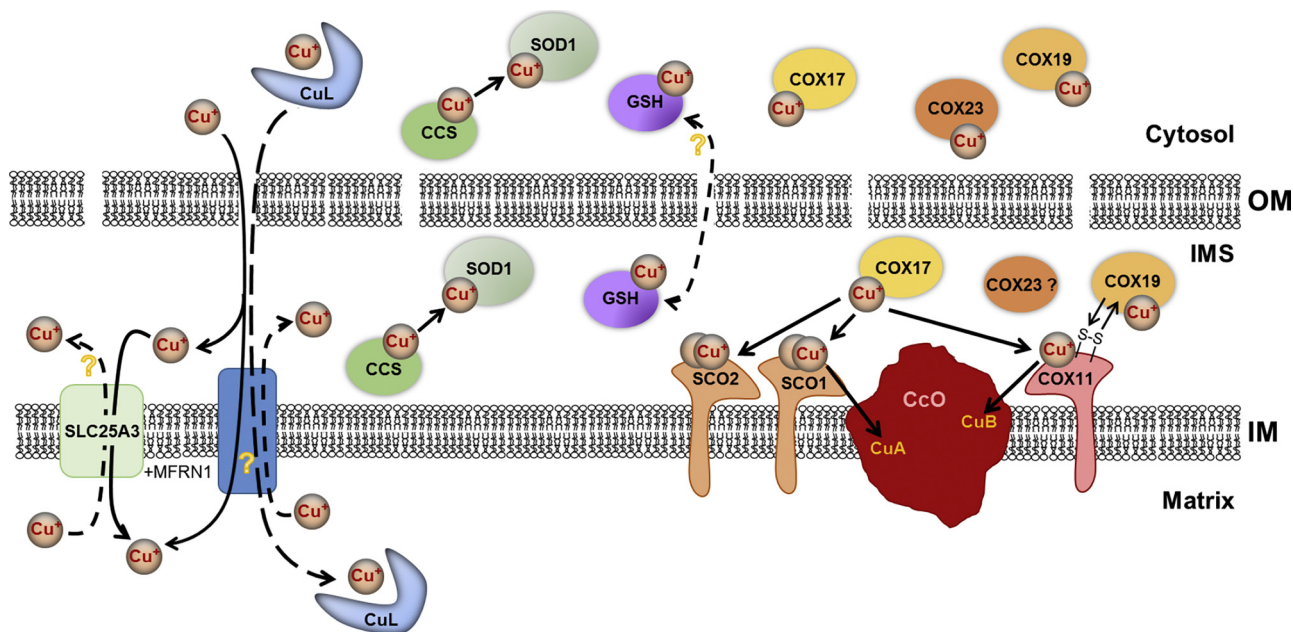

Fig. 1. Copper trafficking within the mitochondrion.

How copper enters mitochondria, either via an anionic non-protein copper ligand (CuL) or via glutathione (GSH), is currently under debate. The inner membrane proteins SLC25A3 or MFRN1 have been suggested to transport copper to the mitochondrial matrix, where it may be stored bound to CuL. In the intermembrane space (IMS), the copper chaperons COX17, SCO1 and SCO2 hand over copper by protein-protein interactions to metalize the CuA and CuB sites of the cytochrome c oxidase (CcO). The copper binding proteins COX23 and COX19 are involved in CcO assembly. COX19 has been proposed to regulate copper transfer to COX11 via modulation of its redox state. In contrast, the specific function of COX23 in copper trafficking is presently unclear. The copper chaperon CCS delivers copper to the Cu/Zn superoxide dismutase (SOD1). *CcO*, cytochrome c oxidase; *CCS*, copper chaperone for superoxide dismutase; *COX17*, cytochrome c oxidase copper chaperone 17; *COX11/19/23*, cytochrome c oxidase assembly protein 11/19/23; *CuL*, copper ligand; *GSH*, glutathione; *IM*, inner mitochondrial membrane; *IMS*, intermembrane space; *MFRN1*, mitoferrin 1; *OM*, outer mitochondrial membrane; *SCO1/2*, synthesis of cytochrome c oxidase protein 1/2; *SLC25A3*, solute carrier family 25 member 3; *SOD1*, Cu/Zn superoxide dismutase.

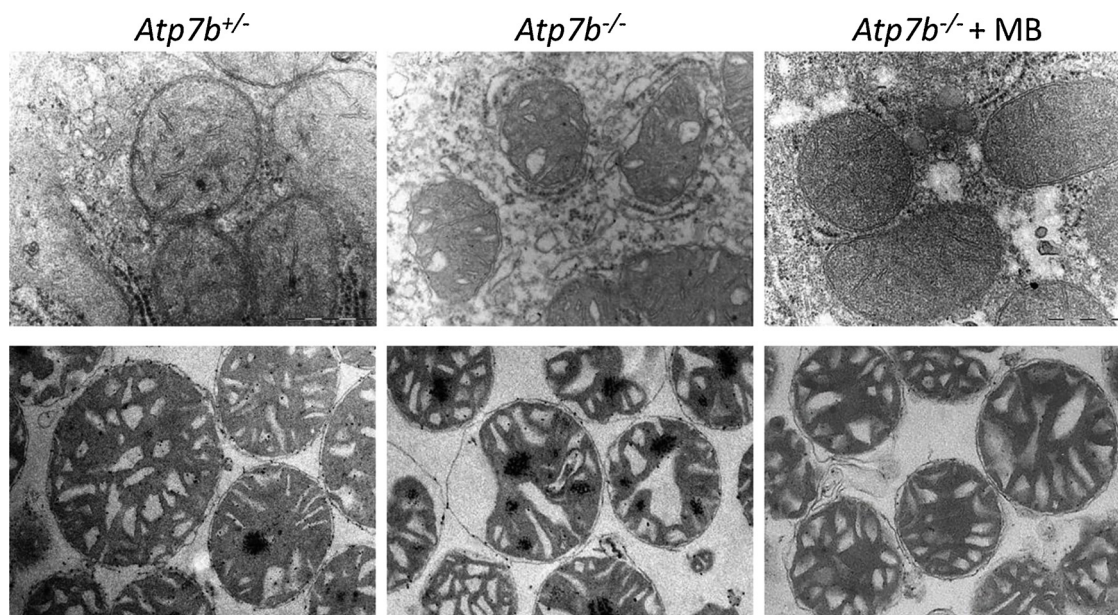## $Atp7b^{+/-}$ $Atp7b^{-/-}$ $Atp7b^{-/-}$ + MB

Fig. 2. Transmission electron micrographs of liver mitochondria from $Atp7b^{-/-}$ LPP rats.
Mitochondria either *in situ* (upper lane) or isolated (lower lane) from $Atp7b^{-/-}$ LPP rats present with dilated cristae and separated inner and outer membranes in contrast to $Atp7b^{+/-}$ controls. Methanobactin (MB) treatment reverses these structural abnormalities.

mitochondrial copper levels (Cobine et al., 2004). Nevertheless, due to its high mitochondrial concentration (around 10 mM, (Garcia-Ruiz et al., 1994)), but comparably low copper affinity (K_{Cu} = 9.1 pM, (Banci et al., 2010)), GSH may indirectly regulate or participate in mitochondrial copper homeostasis, as the redox state of cysteine sulfurs needs to be controlled for proper copper binding of i.e., COX17 or SCO and for its copper transfer to CcO (Leary et al., 2009a; Banci et al., 2008) (Fig. 1).

Third, Winge and coworkers have suggested that mitochondrial copper transport occurs via a non-protein, anionic copper ligand (CuL) of low molecular mass that was consistently found in yeast and mammalian cytosol as well as within the mitochondrial matrix (Cobine et al., 2004, 2006). In thorough studies, CuL was detected via a copper-sensitive fluorescence emission at 360 nm in the copper-rich fraction upon anion exchange chromatography. Gel filtration experiments further indicated a molecular weight of the CuL of about 13 kDa, but neither proteinase K digestion, nor mass spectrometry, SDS-PAGE, and protein detecting Sypro-Ruby stain allowed to establish CuL as a protein (Cobine et al., 2004). Thus, the molecular identity of the CuL is still unclear, and further studies are warranted to support this concept of a CuL-dependent transport into and within mitochondria.

Besides copper entry into the IMS, it was only very recently that the mitochondrial phosphate carrier SLC25A3 (yeast homologue: Pic2) has been demonstrated to import copper into the mitochondrial matrix (Fig. 1) (Boulet et al., 2018; Vest et al., 2013). Copper is also located within the mitochondrial matrix plausibly bound to CuL, and it has been suggested that this matrix copper is redistributed to the IMS for CcO and SOD1 metalation (Cobine et al., 2004). Indeed, *SLC25A3* knockdown and knockout cells (e.g., HEK293) presented with lower CcO activity (Boulet et al., 2018; Vest et al., 2013). Moreover, SLC25A3, reconstituted into liposomes, demonstrated copper transporting activity and restored CcO activity in *pic2Δ* yeast (Boulet et al., 2018). However, lack of SLC25A3 (or Pic2) caused partial copper depletion (30–60%) and lowered (but not absent) CcO activity compared to wild-type mitochondria (Boulet et al., 2018; Vest et al., 2013). This either indicates that copper import to the IMS is still present and copper may metalize CcO via alternative routes, or that further/alternative mitochondrial copper import routes into the matrix may exist, possibly via the mitochondrial iron transporter MFRN1/2 (yeast homologue:

Mrs3/4) that has been reported to transport copper besides iron (Vest et al., 2016; Christenson et al., 2018).

While these molecular players may constitute a large part of the repertoire to supply mitochondrial CcO and SOD1 with copper, a molecularly undefined issue is the removal of copper from mitochondria. Leary et al. have stated that "the [matrix copper] pool can be expanded to a much greater extent than it can be depleted, [which] supports the idea that the organelle's relative priority is to retain sufficient copper" (Leary et al., 2009b). Indeed, mitochondria can accumulate high copper amounts before they ultimately break down (Zischka et al., 2011; Lichtmannegger et al., 2016; Borchard et al., 2018). Thus, at present, it is unclear whether specific mitochondrial copper excretion routes that would counterbalance mitochondrial copper overload exist. This question, however, is of tremendous importance with respect to human pathologies, especially in Wilson disease.

3. Liver mitochondrial impairment in Wilson disease

Wilson disease (WD) is an autosomal recessively inherited disorder, characterized by mutations in the intracellular copper transporting ATPase ATP7B (Tanzi et al., 1993). ATP7B is localized at the membranes of the trans-Golgi network (TGN) or at the apical membrane of hepatocytes to facilitate either metalation of secreted copper enzymes in the TGN or liver copper excretion via the bile (Hung et al., 1997). Consequently, ATP7B mutation results in disrupted hepatic copper excretion, copper overload, hepatocyte death and finally liver failure.

Ultrastructural alterations of mitochondria - besides steatosis - have been amply reported to be early adverse features in hepatocytes of WD patients and WD animal models (Zischka et al., 2011; Lichtmannegger et al., 2016; Sternlieb, 1992; Sternlieb and Feldmann, 1976; Sternlieb, 1968). These include organelle elongations, deformations, inclusions and cristae dilatations (Fig. 2). In their seminal publications, Sternlieb and coworkers reported these alterations in livers of WD patients being especially prominent in (still) asymptomatic patients (Sternlieb, 1992; Sternlieb and Feldmann, 1976; Sternlieb, 1968). In agreement, we have reported similar mitochondrial structure alterations in livers from either LEC or LPP rats that both carry a homozygous ATP7B deletion ($Atp7b^{-/-}$ rats) (Zischka et al., 2011; Lichtmannegger et al., 2016). These alterations were already apparent in $Atp7b^{-/-}$ rat livers at an

animal age of 50 days and steadily increased in severity and incidence with age but also with hepatocellular damage (Zischka et al., 2011; Lichtmannegger et al., 2016). Importantly, the more copper was deposited in these mitochondria the worse their abnormal appearance was. Moreover, an additional fraction containing mitochondrial debris with massive copper load was isolated from diseased but not from still healthy animals (Zischka et al., 2011). These studies are in full agreement with feeding studies with excessive copper in rats (Sokol et al., 1993; Fuentealba and Haywood, 1988; Sokol et al., 1990). Microcystic formations at the mitochondrial cristae were visible after 1 week, and after 3 weeks, mitochondria appeared swollen, indicative of mitochondrial destruction coinciding with a drastic rise in hepatic mitochondrial copper content (Sokol et al., 1993; Fuentealba and Haywood, 1988; Sokol et al., 1990). In contrast, endoplasmic reticulum, plasma and canalicular membranes appeared structurally normal (Sokol et al., 1990). Thus, the mitochondrial structure is a sensitive first responder to an increasing liver copper load. In agreement with these observations in humans and rats, abnormally shaped and sized liver mitochondria already occurred in 6 weeks old $Atp7b^{-/-}$ mice with otherwise unremarkable liver histology (Huster et al., 2006). Of note, the livers of 3 months old toxic milk mice, which carry an $Atp7b$ missense mutation, also showed these changes prior to liver inflammation (first occurring in 6 month old mice) (Roberts et al., 2008).

In further examinations of $Atp7b^{-/-}$ rat liver mitochondria, we found that copper is progressively deposited at the mitochondrial membranes, paralleled by a decreased membrane fluidity and membrane stability (Lichtmannegger et al., 2016). Thus, increased mitochondrial copper deposition causes biophysical and biochemical alterations in mitochondria. Using isolated wild-type rat liver but also brain mitochondria, we further found that mitochondrial protein thiols are important targets of copper exposure (Zischka et al., 2011; Borchard et al., 2018). While there still is a paucity concerning copper toxicity in WD patient brains, these findings indicate that mitochondrial copper toxicity may also be relevant in neurological WD. This suggested mechanism of copper-mediated protein impairment is in agreement with earlier findings about copper toxicity (Nakamura and Yamazaki, 1972) and resembles "classical" protein damage by direct attack of vulnerable target amino acid residues (e.g., cysteine and methionine) (Davies Michael, 2016). Conformational changes and/or loss of protein activity may occur (Mirzaei and Regnier, 2006), which are especially critical for proteins of the mitochondrial oxidative phosphorylation.

In agreement with this suggested toxic mode of action of accumulating mitochondrial copper, functional deficits have been reported in liver mitochondria from WD patients and WD animal models. Patients with acute hepatic failure present with electron transport chain deficits in their mitochondria (Gu et al., 2000). A progressive loss of the mitochondrial ATP production capacity, coinciding with increased copper load and disease severity, was found in $Atp7b^{-/-}$ rats (Lichtmannegger et al., 2016; Borchard et al., 2018). Of note, oxidative damage or elevated mitochondrial ROS emergence, indicative of Fenton-chemistry based copper toxicity were rather late features, only observed in irreversibly damaged mitochondria (Lichtmannegger et al., 2016; Borchard et al., 2018). In agreement with these findings, compared to wild-type controls, 3 to 47 weeks old $Atp7b^{-/-}$ mice appeared with progressively lower respiratory chain function and GSH levels in liver homogenates. However, a significantly elevated GSSG/GSH ratio first occurred at an age of 47 weeks (Sauer et al., 2011).

Maybe the strongest line of evidence for a decisive role of mitochondrial copper overload in the progression from WD comes from treatments that aimed at liver copper removal. Sternlieb and Feldmann demonstrated that the successful treatment of WD patients with the copper chelator D-penicillamine (D-PA) largely resolved the "characteristic mitochondrial abnormalities" and serum parameters indicative of liver damage returned to normal (Sternlieb and Feldmann, 1976). This positive treatment effect is remarkable, as overall liver copper loads were reported to stay high in WD patients, even after years

of D-PA treatment (Scheinberg et al., 1987). Conversely, three WD patients who had responded unfavorably to D-PA treatment were found to have a massive mitochondrial copper load (Sokol et al., 1994). Similarly, four week treatments of $Atp7b^{-/-}$ rats with either D-PA or the copper binding peptide methanobactin (MB) avoided liver damage and significantly reduced the mitochondrial copper burden, but only slightly reduced the overall liver copper load (Zischka et al., 2011). Moreover, intense methanobactin treatments of just a few days primarily caused a significant mitochondrial copper depletion, restored mitochondrial structure and function, and avoided or rescued liver damage (Lichtmannegger et al., 2016). Importantly, upon therapy stop, within weeks, a re-accumulating mitochondrial copper load was paralleled by mitochondrial structural and functional deficits, and by progressive liver damage (Lichtmannegger et al., 2016). This correlation between disease state and copper was not apparent from the overall liver copper load, which was comparable in either still healthy or diseased animals (Lichtmannegger et al., 2016). Mitochondrial copper overload, therefore, is not an innocent bystander or secondary effect but appears to be one, but not necessarily the only key parameter in WD progression (Sternlieb et al., 1973; Abe et al., 1994; Oe et al., 2016). The mitochondrial copper content, structure, and biochemical functionality not only serve as early response markers for disease progression in WD patients or $Atp7b^{-/-}$ rodents, but also as diagnostic biomarkers of treatment efficacy and predictive markers of recurrence of liver damage (Lichtmannegger et al., 2016).

In conclusion, mitochondria are strictly copper-dependent organelles and several molecular players in mitochondrial copper homeostasis have been identified. The "dark side" of mitochondrial copper, however, is that overload is highly detrimental to them, especially in WD and animal models livers. It appears that their progressively impaired biochemical function is a key player in liver demise. Future studies have to reveal how hepatocytes initially try to counterbalance such mitochondrial decay and why they ultimately fail.

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