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Increased copper levels in in vitro and in vivo models of Niemann-Pick C disease

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Abstract Niemann-Pick type C disease (NPC) is a hereditary neurovisceral atypical lipid storage disorder produced by mutations in the *NPC1* and *NPC2* genes. The disease is characterized by unesterified cholesterol accumulation in late endosomal/lysosomal compartments and oxidative stress. The most affected tissues are the cerebellum and the liver. The lysotropic drug U18666A (U18) has been widely used as a pharmacological model to induce the NPC phenotype in several cell culture lines. It has already been reported that there is an increase in copper content in hepatoma Hu7 cells treated with U18. We confirmed this result with another human hepatoma cell line, HepG2, treated with U18 and supplemented with copper in the media. However, in mouse hippocampal primary cultures treated under similar conditions, we did not find alterations in copper content. We previously reported increased copper content in the liver of $Npc1^{-/-}$ mice compared to control animals. Here, we extended the analysis to the copper content in the cerebella, the plasma and the bile of NPC1 deficient mice. We did not observe a significant change in copper content in the cerebella, whereas we found increased copper content in the plasma and decreased copper levels in the bile of $Npc1^{-/-}$ mice. Finally, we also evaluated the plasma content of ceruloplasmin, and we found an increase in this primary copper-binding protein in $Npc1^{-/-}$ mice. These results indicate cell-type dependence of copper accumulation in NPC disease and suggest that copper transport imbalance may be relevant to the liver pathology observed in NPC disease.

Keywords Copper · Niemann-Pick type C · Liver · Ceruloplasmin

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Introduction

Niemann-Pick type C disease (NPC) is a neurovisceral atypical lipid storage disorder involving endocytosed cholesterol (Patterson et al. 2001). NPC is a fatal autosomal recessive disease caused by mutations in the *Npc1* or *Npc2* genes. The *Npc1* gene encodes a lysosomal transmembrane protein, and the *Npc2* gene encodes a soluble lysosomal protein that binds cholesterol. Both proteins are involved in cholesterol trafficking from lysosomes (Kwon et al. 2009). Mutations in the *Npc1* gene account for approximately 95 % of NPC cases (Wraith et al. 2009). NPC is characterized by an inability to process cellular cholesterol from the endocytic pathway, leading to late endosomal/lysosomal accumulation of cholesterol and glycosphingolipids as well as abnormal tubulovesicular trafficking, progressive neuropathology and neurodegeneration.

Evidence of oxidative stress has been shown in the thalamus of $Npc1^{-/-}$ mice, where a positive mark for Nitrotyrosine was detected (Smith et al. 2009) in different NPC cellular models such as human SH-SY5Y neuroblastoma cells and human fibroblasts from NPC patients and healthy controls (Zampieri et al. 2008). Furthermore, previous data from our laboratory showed an increase in the levels of oxidative stress markers in vitro in NPC cell models and in vivo in the cerebellum of $Npc1^{-/-}$ mice (Klein et al. 2011). We also found that in a neuronal model of the disease, treatment with an antioxidant compound prevents cellular death and apoptosis (Klein et al. 2011).

Copper is an important micronutrient that plays an essential role in human physiology (Uauy et al. 1998). It serves as a cofactor for redox-catalyzing enzymes such as cytochrome C oxidase (cell respiration), superoxide dismutase (reactive oxygen species (ROS) catabolism), dopamine β-hydroxylase (biosynthesis of catecholamines), lysyl oxidase (formation of connective tissue) and ceruloplasmin (the main plasma copper-binding protein and copper-dependent ferroxidase) (Vulpe and Packman 1995), and it is required for embryonic development, neuronal myelination, radical detoxification and numerous other physiological processes (Linder 1991). An increase in copper, iron or zinc levels has been described as a risk factor for oxidative stress damage in neurodegenerative pathologies such as amiotrophic lateral sclerosis, Alzheimer's Disease and Parkinson's Disease (Barnham and Bush 2008). Furthermore, mutations in copper-binding proteins have been linked to those devastating disorders (Gaggelli et al. 2006) as well as Wilson disease and Menkes disease (Kaler 1994; Langner and Denk 2004). Therefore, appropriate levels of copper are essential to avoid cellular damage by oxidative stress due to the rapid oxidation of copper, which causes damage to the biomolecules listed above and generates ROS, leading to cell death (Linder 1991). In this delicate maintenance of copper homeostasis, the liver plays a major role because most of the newly absorbed copper enters the liver after absorption from dietary sources in the small intestine. Also, the liver regulates the distribution of copper through release into the plasma when bound to ceruloplasmin or by excretion via bile (Mercer and Llanos 2003).

In vitro studies have demonstrated the impaired recycling of the mannose-6-phosphate receptor (M6PR) from the late endosomal/lysosomal compartment to the TGN in NPC fibroblasts (Ganley and Pfeffer 2006; Kobayashi et al. 1999; Walter et al. 2003). In this pathway, copper can also be transported in recycling vesicles, which are positive for M6PR (Griffiths et al. 1988; Harada 2002). ATP7B, a cation-transporting P-type ATPase, identified as a Wilson disease gene product, functions in copper secretion into plasma coupled with ceruloplasmin synthesis and biliary copper excretion (Terada et al. 1999; Terada et al. 1998). Yanagimoto et al. described the late endosomal localization of ATP7B in hepatocytes during normal conditions; however, in a pharmacological NPC model of a hepatoma cell line, ATB7B localized in late endosome/lysosome hybrid organelles. Furthermore, in this model, copper incorporation in apo-ceruloplasmin and holo-ceruloplasmin secretion is decreased (Yanagimoto et al. 2009, 2011). These data suggest that ATP7B function may be impaired in NPC hepatic cells due to the disruption of the late endosome to TGN transport, supporting the hypothesis of altered copper trafficking in NPC disease.

Here, we show a correlation between the NPC phenotype and copper accumulation in in vitro models as well as an increase in copper levels in the plasma and the cerebella of $Npc1^{-/-}$ mice, whereas in the bile, a decrease in copper levels occurs. These results, together with recently reported data from our lab (Vazquez et al. 2011), show a significant increase in

copper content in the livers of *Npc1*$^{-/-}$ mice, strongly suggesting that copper homeostasis is altered in NPC disease. Finally, we also evaluated the levels of ceruloplasmin, the primary plasma copper-binding protein and copper-dependent ferroxidase, which is also increased in NPC disease model mice.

Materials and methods

Cell culture

For neuronal primary cultures, hippocampi from Sprague–Dawley rats at embryonic day 18 were dissected, and primary hippocampal cultures were prepared as previously described (Alvarez et al. 2004). Hippocampal cells were seeded in polylysine-coated wells at 1×10^5 cells/cm^2 and maintained in Neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA, USA) plus antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) for 5 days before the cell treatments. Glial proliferation was inhibited by adding 2 μM cytosine arabinoside on the third day (Alvarez et al. 2004).

HepG2 cells were cultured in DMEM supplemented with 10 % FBS and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) in a 5 % CO_2 atmosphere at 37 °C. For the experiments, cells were seeded in 6-well multiplates at an initial density of 4×10^4 cells/cm^2, 24 h before treatments.

U18666A treatment and Filipin staining

U18666A (U18) is a sterol derivative that triggers intracellular cholesterol accumulation as observed in NPC cells. This drug has been widely used to induce the NPC phenotype in different cell types (Liscum and Faust 1989; Sparrow et al. 1999; Koh et al. 2006). Here, we treated hippocampal neurons as well as HepG2 cells for a total time of 24 h with or without U18 and in the presence or absence of Cu:Histidine (Cu:His) complexes (1:10 ratio) at a final concentration of 5 μM copper. HepG2 cells were treated with several doses of U18 (0–2 μg/mL) for 24 h, and hippocampal primary cultures were treated with 1 μg/mL for the last 2 h of treatment. Filipin staining, for free-cholesterol accumulation detection, was performed as described previously (Yévenes et al. 2011). Briefly, cells were fixed in 4 % paraformaldehyde/4 % sucrose in PBS for 30 min. Next, cells were washed with PBS and treated with 2.5 mg/mL glycine for 20 min. Finally, cells were treated with 25 μg/mL Filipin (Sigma Chemicals Co, St. Louis, MO) for 30 min, washed with PBS and covered with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Images were captured with an Olympus BX51 microscope (Olympus, Tokyo, Japan) and analyzed with the Image-Pro Express program (Media Cybernetics, Bethesda, MD, USA).

Animals and diets

BALB/c mice carrying a heterozygous mutation in the *Npc1* gene (Loftus et al. 1997) were used to generate wild-type control (*Npc1*$^{+/+}$) and homozygous-mutant (*Npc1*$^{-/-}$) animals. Genotypes were identified as previously described (Amigo et al. 2002). All mice had free access to water and a chow diet (<0.02 % cholesterol; Prolab RMH 3000, PMI Feeds Inc., St. Louis, MO) until they were used for the studies. For experiments, 7-week-old *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ male mice were fasted for 2 h before liver, bile and plasma sampling. Protocols were performed according to the Public Health Service Policy on Humane Care and Use of Laboratory Animals in the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals and approved by the review board for animal studies at our institution.

Sample collection

Mice were anesthetized by intraperitoneal injection of ketamine (80–100 mg/kg) and xylazine (5–10 mg/kg). The cyst duct was ligated, and a common bile duct fistula was performed using a polyethylene catheter. This cannulation process was performed continuously for 30 min in order to collect hepatic bile into an eppendorf tube. The liver was sectioned, and 20 mg of wet weight was used for copper measurement and was stored at −80 °C. The remaining tissue was also stored at−80 °C for Western blot analysis. Plasma was obtained from hepatic vein blood.

Quantification of copper

Metal content was quantified as previously described (Vazquez et al. 2011). Briefly, 20 mg of liver tissue was disrupted in 65 % concentrated suprapure nitric acid (Merck, Chemical Co., Darmstadt, Germany) for 24 h at

60 °C and then diluted to a final concentration of 5 %. Plasma and bile samples were not treated with nitric acid prior to copper measurement. For cell culture samples, cells were collected with TBS-EDTA and resuspended in TBS-EDTA, 0.1 % NP-40 plus protease inhibitors (Leupeptin, PMSF) and finally digested in 1:1 volume ratio with suprapure HNO_3 at 60 °C overnight. Copper determination was made using a graphite furnace atomic absorption spectrophotometer (Perkin Elmer, SIMMA 6100). Calibration was against a Cu standard curve (J.T. Baker), and the sample values were normalized to the values of fresh weight (FW). Copper concentration in cell suspension solution (less than 10 % of the sample) was also measured and subtracted from the samples measurements.

Western blot analysis

For ceruloplasmin (Cp) detection, plasma samples from *Npc1*$^{+/+}$ to *Npc1*$^{-/-}$ mice were diluted 1:4 in 0.9 % NaCl, and 20 μL of the diluted sample plus 5 μL of non-reducing loading buffer was resolved in 12.5 % SDS-PAGE and transferred to nitrocellulose membranes. Ponceau Red staining was used for normalization.

Immunoblots were conducted using sheep anti-mouse ceruloplasmin (1:1,000) (Abcam, cat # AB8813), and secondary antibody conjugated with horseradish peroxidase goat anti-mouse IgG (1:2,000) (cat # 12-349, Upstate).

Statistical analysis

Mean and standard error of the mean (SEM) values with the corresponding number of experiments are indicated in the Results section. Probability values were calculated using Student's *t* test via the GraphPad Prism version 5.03 program (GraphPad Software Inc., La Jolla, CA, USA).

Results

Copper content and cholesterol accumulation in in vitro models of NPC disease

U18did not induce alterations in copper content in HepG2 cells after 24 h of treatment in the absence of Cu:His complexes in the media (Fig. 1a, left panel). However, in the presence of copper in the form of Cu:His complexes, we observed a significant increase in intracellular copper content in the media (Fig. 1a, right panel). Treatment with 0.5 μg/mL of U18 induced an increase that was not statistically significant in intracellular copper content, whereas 1 and 2 μg/mL induced a robust increase in copper content compared to U18-untreated cells ($p < 0.001$, Two-way ANOVA, Fig. 1a, right panel). As a control, the NPC phenotype was demonstrated by Filipin staining for intracellular free cholesterol (figs. 1b, c). Copper accumulation was detected only when cells were co-incubated with copper in the form of Cu:His complexes; however, copper did not alter U18 induced cholesterol accumulation (Fig. 1b).

In NPC disease, the liver and the cerebella are the primary affected tissues. Therefore, we also evaluated copper accumulation in a neuronal NPC model. To this end, neuronal hippocampal primary cultures were incubated in the absence or presence of Cu:His complexes for 24 h with or without U18 (1 μg/mL) for the last 2 h. In these primary cultures, we performed the assay for a shorter period of time because it has been reported that at this concentration U18 toxic effects were produced (Cheung et al. 2004). We detected a decrease in intracellular copper content that was not statistically significant in U18-treated neurons compared to controls (Fig. 2a). Cholesterol accumulation after U18 treatment was detected by Filipin staining (Fig. 2b).

Copper content in plasma, bile and cerebella in in vivo models of NPC disease

Following the analysis of copper levels in an in vivo model of NPC disease, we measured copper content in the plasma and the bile of *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice (Fig. 3a). These results indicated a significant increase in copper levels in plasma; however, in bile, a statistically significant decrease was observed ($^{*}p < 0.05$). When we measured copper content in cerebella (Fig. 3b), we did not observe a significant change in copper content in *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$mice. Taken together, these results suggest that copper release to plasma is enhanced, and copper excretion to bile is impaired in NPC disease.

Ceruloplasmin levels in plasma of Npc1$^{-/-}$ mice

Plasmatic copper is primarily bound to ceruloplasmin, a copper-dependent ferroxidase. Therefore, in our in vivo

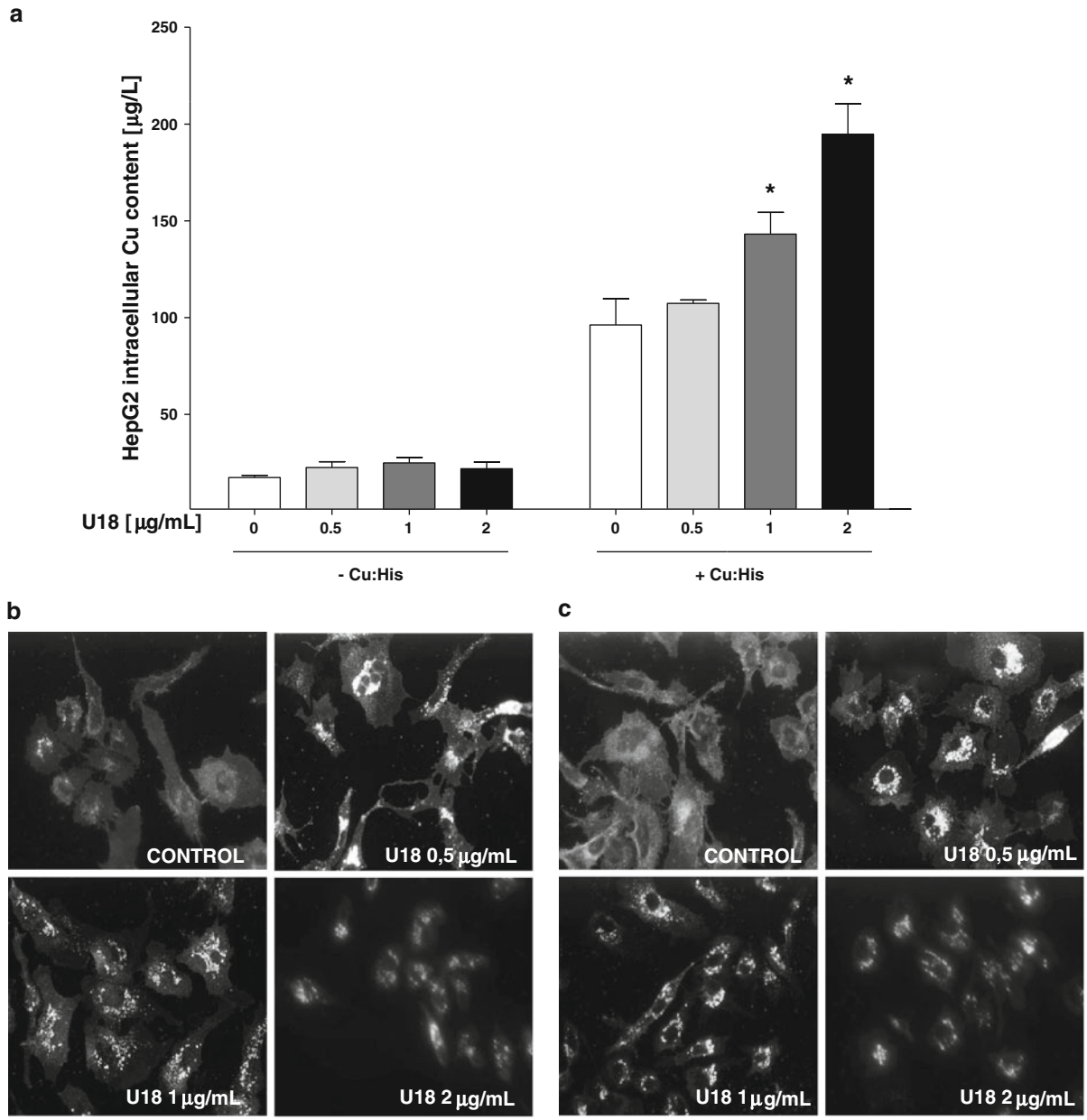

Fig. 1 Copper content is correlated with cholesterol accumulation in a human hepatoma NPC model cell line. HepG2 human hepatoma cell line treated with U18666A (U18) in the absence or presence of Cu:His complexes (1:10) at 5 µM copper for 24 h. **a** Copper content was measured by AAS. Mean values ± SEM are presented. U18-treated cells (1 and 2 µg/L) show a significant increase in copper content compared to control (0 µg/L) in the presence of Cu:His complexes. Statistical analysis: Two-way ANOVA, $*p < 0.001$; $n = 6$. **b**, **c** Filipin staining for cholesterol detection in the same populations; without copper (**b**) and with 5 µM copper (**c**) in the media for 24 h

NPC model, we examined the levels of plasmatic ceruloplasmin by Western blot analysis (Fig. 4a). We found an increase in ceruloplasmin levels in the plasma of $Npc1^{-/-}$ mice compared to $Npc1^{+/+}$ mice (Fig. 4b), and this increase was statistically significant ($*p < 0.05$).

Discussion

The data presented here show alterations in some parameters of copper homeostasis in in vitro as well as in vivo models of NPC disease. In a hepatic NPC

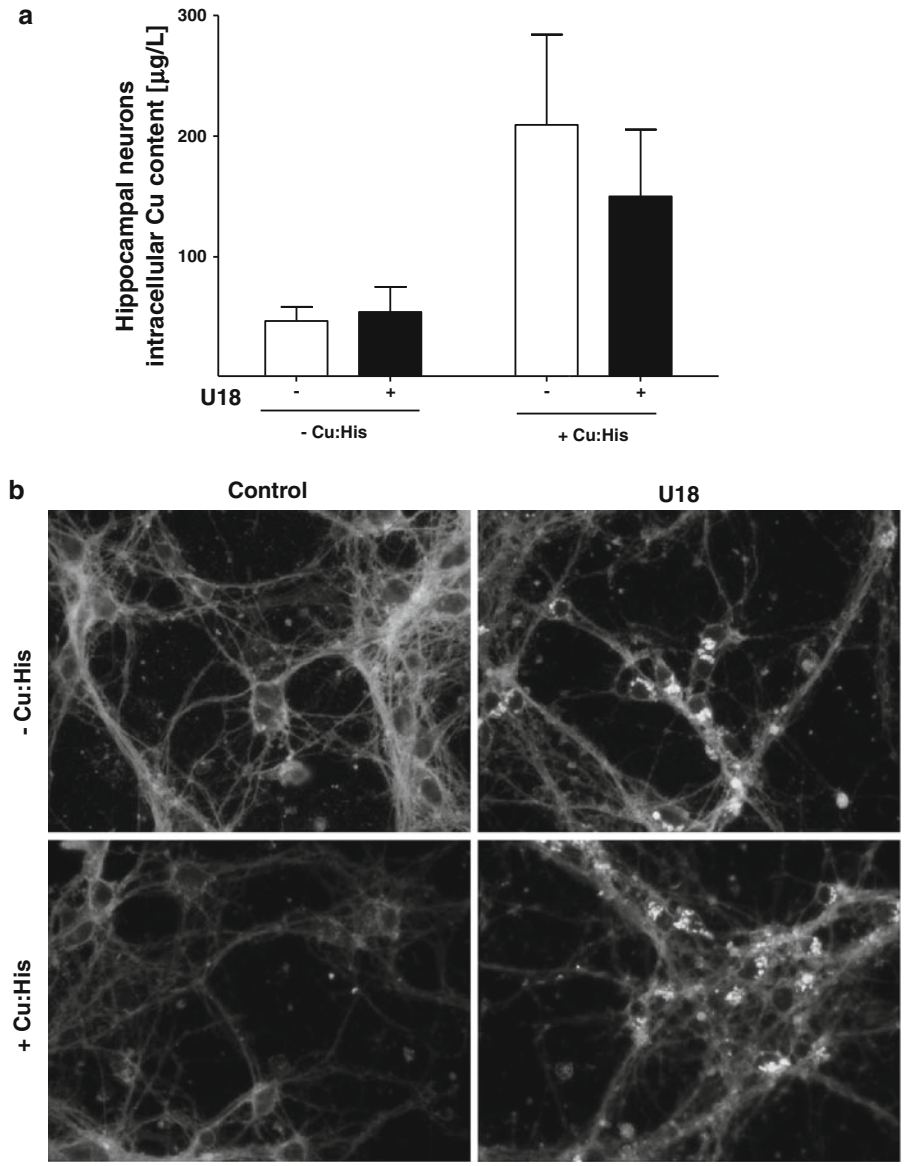

Fig. 2 Copper content in U18666A-treated primary hippocampal neurons. Rat hippocampal neuron primary cultures treated with U18666A (U18) (1 µg/mL) for the last 2 h, in the presence or absence of Cu:His complexes (1:10) 5 µM copper for 24 h total. **a** Copper content was measured by AAS. Mean values ± SEM are presented. **b** Filipin staining for cholesterol detection in the same populations

cellular model, we found an accumulation of copper along with cholesterol accumulation, the main characteristic of NPC disease. This result is in agreement with our previous result showing an increase in copper content in the liver of *Npc1*$^{-/-}$ mice (Vazquez et al. 2011), although we did not observe any significant change in copper content in cerebella, the other major affected organ in this disease**.** However, we also found a significant increase in plasmatic levels of copper as well as of ceruloplasmin, the primary copper transport protein. In addition, we detected a decrease in copper levels in the bile of *Npc1*$^{-/-}$ mice. Overall, these results suggest that copper release to plasma is enhanced, and copper excretion to bile is impaired in NPC disease.

The detected increases in copper content in the liver (Vazquez et al. 2011) and the plasma along with a decrease in copper content in the bile of *Npc1*$^{-/-}$ mice

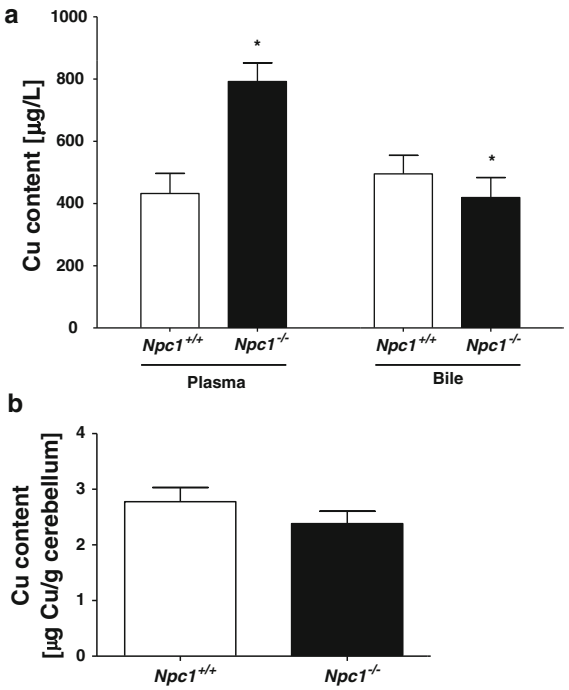

Fig. 3 Copper content in $Npc1^{+/+}$ and $Npc1^{-/-}$ mice. Copper content measured by AAS in a murine model of NPC disease **a** Copper content in the plasma and the bile of $Npc1^{+/+}$ and $Npc1^{-/-}$ mice. Mean values ± SEM are presented. Plasma: $Npc1^{+/+} = 431.9 \pm 64.8$ µg/L v/s $Npc1^{-/-} = 792 \pm 59.8$ µg/L; $p = 0.0006$, unpaired t test ($n = 12$ $Npc1^{+/+}$ and 10 $Npc1^{-/-}$), and the bile: $Npc1^{+/+} = 524.3 \pm 53.8$ µg/L v/s $Npc1^{-/-} = 389.8 \pm 52.9$ µg/L; $p = 0.0484$, unpaired t test($n = 11$ $Npc1^{+/+}$ and 6 $Npc1^{-/-}$). **b** Copper content in cerebella of $Npc1^{+/+}$ and $Npc1^{-/-}$ mice. Mean values ± SEM are presented. $Npc1^{+/+} = 2.78 \pm 0.25$ µg Cu/g tissue v/s $Npc1^{-/-} = 2.38 \pm 0.22$ µg Cu/g tissue; $p = 0.3011$, unpaired t test ($n = 15$ $Npc1^{+/+}$ and 9 $Npc1^{-/-}$)

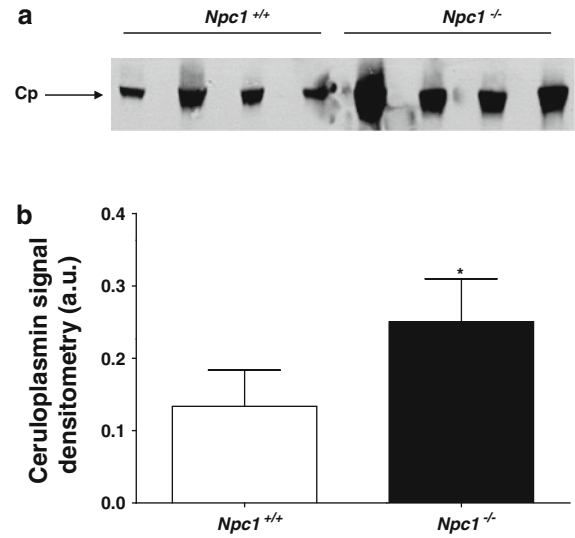

Fig. 4 Ceruloplasmin levels in the plasma of $Npc1^{-/-}$ mice. **a** Ceruloplasmin levels in the plasma of $Npc1^{+/+}$ and $Npc1^{-/-}$ mice. Representative immunoblot analysis of ceruloplasmin (Cp; 130 kDa). **b** Densitometric analysis; Cp signal was normalized by Ponceau staining. Mean values ± SEM are presented. $Npc1^{+/+} = 0.14 \pm 0.03$ v/s $Npc1^{-/-} = 0.26 \pm 0.04$; $p = 0.023$, unpaired t test ($n = 9$, both groups)

compared to $Npc1^{+/+}$ mice suggest that in NPC disease there is an alteration in intracellular copper transport, which leads to an accumulation of copper in tissues and plasma along with an impairment in copper excretion to bile. The increase in plasmatic copper content was correlated with the increase in ceruloplasmin plasma levels. The diminished excretion into bile may be due to a mislocalization of ATP7B at the bile canaliculus (Schaefer et al. 1999; Guo et al. 2005; Cater et al. 2006). Further analysis of ATP7B expression levels as well as its localization in the liver of $Npc1^{-/-}$ mice compared to $Npc1^{+/+}$ mice must be performed to clarify this issue.

Copper is an essential micronutrient that plays a fundamental role as a co-factor and/or prosthetic group for numerous enzymes involved in several metabolic pathways. Alternatively, in pathological conditions where copper homeostasis mechanisms may be compromised, copper also participates in reactive oxygen species generation, which in turn can irreversibly damage proteins, lipids as well as nucleic acids in cells (Videla et al. 2003; Cervantes-Cervantes et al. 2005). Therefore, a delicate balance of copper levels is necessary for essential metabolic functions in order to avoid oxidative stress damage (Uauy et al. 1998; Linder 1991). In the context of the data presented here, copper accumulation in the late endosome/lysosomal compartment can enhance oxysterol production, which may be another pro-oxidative agent. The in vitro models developed in this work represent adequate alternatives for further studies to elucidate the mechanisms involved in cellular death observed in NPC disease and its relationship with copper level alterations.

Oxidative stress damage in NPC disease has already been reported by several groups. Data from our lab demonstrate an increase in the expression of several oxidative stress damage-related genes in the cerebella and the liver of $Npc1^{-/-}$ mice (Klein et al. 2011; Vazquez et al. 2011). We also published an increase in fibrosis and inflammation as well as an

increase in carbonyl adduct formation together with a decrease in antioxidant capacity in the livers of *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$ mice (Vazquez et al. 2011). In this scenario, copper accumulation can be related to the increase in oxidative stress damage observed in the disease.

Yanagimoto et al. (2009, 2011) showed an accumulation of copper intracellular content in hepatic cells treated with U18, and they also showed that NPC1 protein and ATP7B co-localize in the lysosomes of these cells. The authors propose that the proper function of ATP7B depends on NPC1 function. They also indicated a role for NPC1 in copper incorporation into ceruloplasmin (Yanagimoto et al. 2011), although they detected an increase in secreted holo-ceruloplasmin only when NPC1 protein function was restored by overexpression. The difference between their results and ours could be explained because we analyzed plasma levels of ceruloplasmin in an in vivo model of NPC disease instead of ceruloplasmin secretion into the media from hepatic cell line cultures.

No alterations in copper content were found in U18-treated neurons. In relation to the pharmacological model used in this study as well as in the work by Yanagimoto et al. (2009), it is important to consider that U18 has been widely used as an inducer of lipid accumulation in late endosomes/lysosomes; however, in hippocampal primary cultures, this agent is toxic at high concentrations and prolonged incubation times (Cheung et al. 2004). Nevertheless, under the conditions used in this study for primary hippocampal neurons (0.1 μg/mL for 2 h) and for the HepG2 cell line (0.5–2 μg/mL for 24 h), we did not observe significant differences in cell viability, and no major morphological changes were observed (data not shown). Due to the intrinsic limitations of this type of pharmacological model, it is necessary to corroborate these results with a genetic model or an endogenously-disrupted NPC1 model system.

The study of copper traffic alterations in NPC disease models contributes to the better understanding of copper metabolism and physiology as well as previous studies regarding Wilson and Menkes diseases. NPC1 may be a new player in copper intracellular transport, and cholesterol- and lipid-related proteins should be further studied in copper homeostasis in mammals. Indeed, connections between copper and cholesterol metabolism have been previously observed, and it is well known that copper deficiency results in elevated plasma levels of cholesterol (Klevay 1973, 2000a, b; Hooper et al. 1980; Klevay et al. 1984). Therefore, cholesterol and copper metabolic pathways appear to be connected at some level and would be interesting to explore further.

In summary, we observed copper homeostasis alterations in a hepatic cellular model as well as in a murine model of NPC disease. Further studies are required in order to establish the relevance of these findings in the pathophysiology of NPC disease, particularly in human patients.

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