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Cellular Lipid Metabolism Is Influenced by the Coordination Environment of Copper

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Transition metals such as cobalt, copper, iron, nickel, and zinc play important roles in biology and can even influence gene expression.[1] Proteins can act as sensors of metal ions such as Cu leading to cellular responses that avoid metal imbalance.[2] The improper trafficking of Cu ions within the human body is a contributing factor in many diseases including Wilson's,[3−5] Menkes',[6,7] and Alzheimer's diseases.[6,8,9] In Wilson's disease, Cu ion homeostasis is disrupted. Mutations to the ATP7B protein required for the proper export of Cu from liver cells to bile and ceruloplasmin cause Cu levels to increase in the cell to toxic levels.[10−13] Studies of mice in which the ATP7B gene is knocked out have identified a link between Cu and lipid metabolism,[13] showing that upon inducing a state of Cu excess, the expression of genes for cholesterol and lipid biosynthesis are affected.[12−14] In these mice Cu concentrations in the liver rise over time to 30−40 times the normal concentration. However, the bioavailable form of Cu that is absorbed in the liver that gives rise to increases in lipid metabolism is unknown. The bioavailable form of Cu in the blood is histidine bound,[15] and histidine complexes are used to treat Cu deficiency such as that which arises in Menkes' disease.[16] Therefore, the bioavailable form of Cu that reaches the liver likely involves at least one histidine residue. Herein we have studied the effects of $Cu(his)_2$, Cu(EDTA), and $CuCl_2$ on lipid metabolism in liver cells.

To probe the effect that these three Cu species have on lipid metabolism, we used coherent anti-Stokes Raman scattering (CARS) microscopy to observe the size and distribution of lipid bodies in liver cells. This technique allows for the noninvasive label-free observation of lipids based on the strong CARS signal from the CH_2 stretch at 2850 cm^{-1} and allows us to directly observe the changes in lipid phenotypes that result from treatment with Cu without disturbing the cellular environment.[17−19] Previously, CARS microscopy has been shown to be an excellent tool for examining the lipid droplets in live fibroblast cells,[17] the effects of transcriptional regulators of lipid metabolism,[20] and the effects of hepatitis C virus RNA on lipid metabolism in the liver.[21]

Huh 7.5 human hepatoma cells were plated into borosilicate chambers and incubated for 12 h. At this time, Cu solutions were added to final concentrations of 10 and 100 μM. After 12 and 24 h treatments, cells were rinsed with PBS, fixed, and imaged by CARS microscopy (Figure 1). At 12 h, the cells treated with $Cu(his)_2$ and Cu(EDTA) clearly show an increase in size, quantity, and total volume of lipid droplets (Figure 1) as measured by voxel analysis,[17] compared with untreated cells while those treated with $CuCl_2$ show a much smaller change in number but also an increase in size of lipid bodies. This suggests that the complexed Cu is leading to a 300% increase in lipid density through either increased lipid storage or decreased lipid export.[20] The effect of $Cu(his)_2$ and Cu(EDTA) was observed at both 10 and 100 μM after 12 h. At 24 h, the samples treated with $Cu(his)_2$ and Cu(EDTA) show less of an increase in size and number of lipid bodies (14−19% average lipid volume), suggesting that the misregulation of lipid metabolism by Cu is a transient phenomenon and cells that have functioning

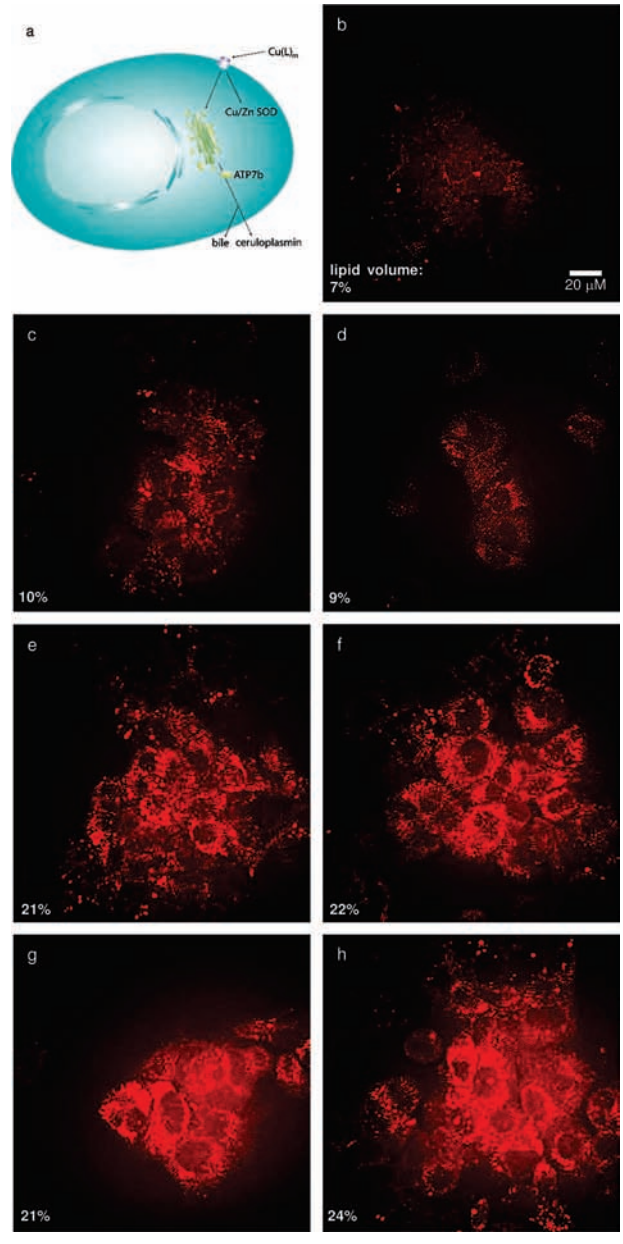

Figure 1. (a) Schematic of Cu transport in hepatic cells. (b−h) CARS images of Huh 7.5 cells treated with (b) media (control) for 12 h and solutions of the following Cu species in media for 12 h: (c) 10 μM $CuCl_2$, (d) 100 μM $CuCl_2$, (e) 10 μM $Cu(his)_2$, (f) 100 μM $Cu(his)_2$, (g) 10 μM Cu(EDTA), and (h) 100 μM Cu(EDTA). Percent lipid by volume per cell (average of 20 cells per sample, error ± 2%) shown in bottom left corner of each image.

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ATP7B quickly undergo homeostasis. The lipid bodies also appeared to be clustering together as larger lipid aggregates at the 100 μM concentrations for $Cu(his)_2$ and Cu(EDTA). This increase is consistent with the observations in ATP7B knockout mice where lipid and cholesterol biosynthesis is down regulated.[13] Similar results were seen by CARS microscopy upon PPARα antagonism, which resulted in the down-regulation of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) that is the rate controlling enzyme of the mevalonate pathway that produces cholesterol.[20]

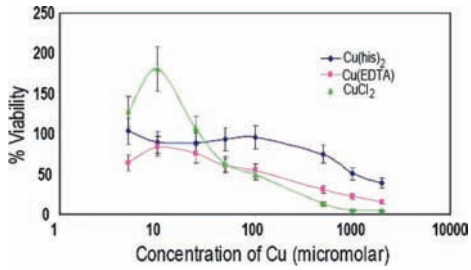

Figure 2. MTT curves of Huh 7.5 cells treated with $CuCl_2$, Cu(EDTA), and $Cu(his)_2$ in concentrations ranging from 5 μM to 2 mM.

The 100 μM $CuCl_2$ treated samples were observed to be much less confluent during microscopy implying that the treatments were toxic; therefore we determined the toxicity profile of $Cu(his)_2$, Cu(EDTA), and $CuCl_2$ using a colorimetric assay that measures mitochondrial activity based on the metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The IC_{50} value for cells treated with $CuCl_2$ was ~100 μM confirming the previous observation. At 10 μM, $CuCl_2$ significantly increased the proliferation of cells (Figure 2, 180% compared to mock cells). This result agrees with observations that under conditions of slight Cu excess, genes promoting cell division are upregulated.[13] Upon repeating the assay with a shorter incubation (24 vs 72 h) the increase in cell proliferation at low $CuCl_2$ concentration was considerably less (114% at 10 μM), indicating that cell division is markedly increased between 24 and 72 h. This suggests a second response to Cu accumulation that is specific to uncomplexed Cu and occurs only after intracellular Cu reaches a given threshold. For $Cu(his)_2$, the IC_{50} value was found to be ~1 mM, and low doses did not stimulate cell division. For Cu(EDTA) the IC_{50} was ~150 μM, and again, no accelerated cell growth was observed. None of the samples appear toxic at 10 μM; however, $CuCl_2$ clearly is affecting the rate of mitosis. At 100 μM, the $CuCl_2$ and Cu(EDTA) samples both show significant toxicity but very different lipid profiles.

To ascertain if toxicity and/or lipid body storage was directly linked to intracellular Cu concentration, Cu accumulation of cells harvested at 12 and 24 h was performed using inductively coupled plasma mass spectrometry (ICP-MS) (Supporting Information, Table S3). These data show a difference in uptake pattern for complexed versus uncomplexed Cu species. At 12 h, the concentration of Cu inside the cells treated with 100 μM $Cu(his)_2$ or Cu(EDTA) is 50% greater than those treated with 100 μM $CuCl_2$ and ~7 times greater than that of the control. At 24 h, the concentration of Cu in cells treated with a 10 μM concentration of all three Cu species is the same and only slightly higher at 100 μM for the two complexed species. This implies that the increase in lipid storage is not simply dependent on the uptake of Cu but on the ligand environment of the Cu. We also observe that Cu toxicity is not dependent on Cu uptake into the cells. The Cu content of cells treated with both 10 and 100 μM $CuCl_2$ are the same, whereas the latter treatment was considerably more toxic to cells than the former. This implies that the 10-fold increase in Cu in the extracellular environment is responsible for cytotoxicity and not solely due to an increase in intracellular Cu. There is a difference in the rate of Cu uptake with Cu(EDTA) and $Cu(his)_2$. Here a 10-fold increase in extracellular Cu results in a 50% increase in intracellular Cu at 12 h, but at 24 h, the difference in uptake between 10 and 100 μM samples is ~20%.

The coordination state of Cu plays an important role in its cellular uptake and transport,[15] and we find that only an excess of complexed Cu results in significant increases in lipid content in hepatic cells in contrast to treatment with ionic Cu. A link between Cu and lipid metabolism in Wilson's disease has been previously established, and we show here for the first time that a Cu overload from complexed bioavailable sources results in a rapid and dramatic increase in lipid body formation that dissipates slowly as Cu toxicity sets in from Cu overload. We also show that toxicity from $CuCl_2$ is not directly related to Cu accumulation inside the cell, and thus it may arise from oxidation at the cell surface. CARS microscopy, in combination with other techniques, should allow for detailed studies of the effects of incubation time, ligand and oxidation state, and the study of proteins involved in Cu homeostasis in a variety of cell types. These studies are currently underway.

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Supporting Information Available: Additional CARS images are available as are complete experimental procedures, additional data tables, and explanations of how the data were analyzed. This information is available free of charge via the Internet at http://pubs.acs.org/.

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