The Cellular Redox Status is Altered in Menkes Disease Fibroblasts

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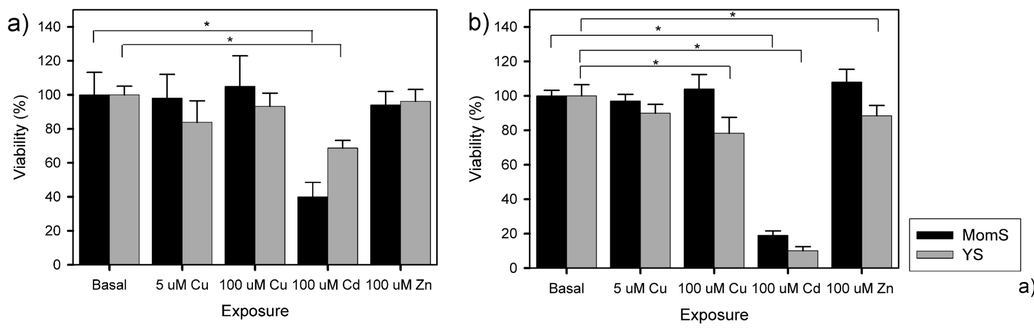
Abstract

Copper is an essential trace element important in numerous biological processes. Its small electrochemical half cell potential makes it a flexible tool in enzymatic reactions but also creates a potential hazard: unprotected 'free' Cu in any biological matrixes promotes the formation reactive oxygen species (ROS). To prevent buildup of toxic levels of ROS, cells have developed a sophisticated system with tight regulation in which Cu is bound by chaperones or target proteins from the moment of cell entry until secretion. To date, the only known proteins in humans involved in Cu excretion are Atp7a and Atp7b, two Cu transporting P-type ATPases with >40% sequence identity. Most tissues express one or the other with notably exception of brain and kidney. Heptatocytes exclusively express Atp7b and as such Wilson disease (WD, mutations in Atp7b) presents with Cu accumulation in the liver. The central focus of our research is to further elucidate the specific role of Cu in WD and here we present our recent results showing how Cu exposure affects the cellular redox state as seen by the GSH/GSSG couple. In our experiments we utilized two immortalized human fibroblast cell lines derived from a Menkes disease patient (lacking Atp7a, YS cells) and the patients mother (controls, MomS cells). Since the YS cells are lacking a copper export system they can serve as a model for WD. Cells were exposed to copper under conditions representing acute and chronic copper overload. As expected, we found an increase in total GSH under 'chronic' copper exposure for both YS and MomS cells. This was further confirmed by RT-PCR analysis of the mRNA levels of glutamate cysteine ligase. For short term, low copper exposure, however, we observed a drop in GSH levels for YS cells with no apparent effect in MomS cells. To our surprise, RT-PCR data did not suggest any changes in GSH levels for YS cells. This apparent discrepancy could not be explained by differences in protein-bound GSH which remained ~ 5% of total GSH for all conditions. The implications of these results as well as others such as glutaredoxin-1 activity and copper measurements will be discussed in this contribution further outlining the complex role of copper in WD.

X-ray Fluorescence Microscopy

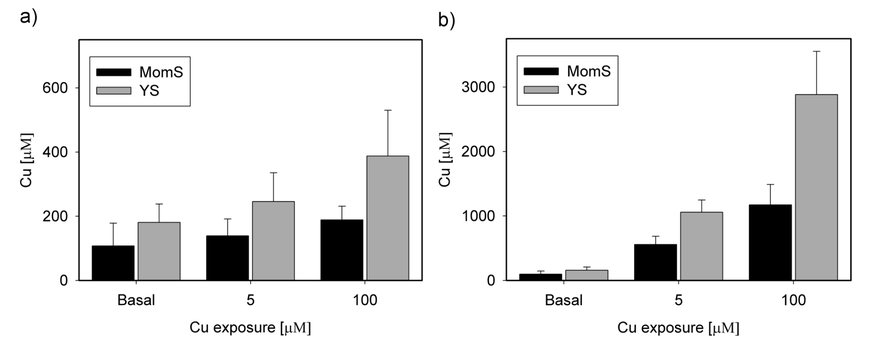
Cellular Viability

Viability assays for MomS (black) and YS cells (grey) relative to basal conditions.
MomS and YS percent cell viabilities at **(a)** 4 hrs and **(b)** 48 hrs are normalized by their respective viabilities under basal conditions. Values are means ±SDs of at least three replicates. Significance is indicated between pairs by *. Both cell lines show high viability even for prolonged high copper exposures.

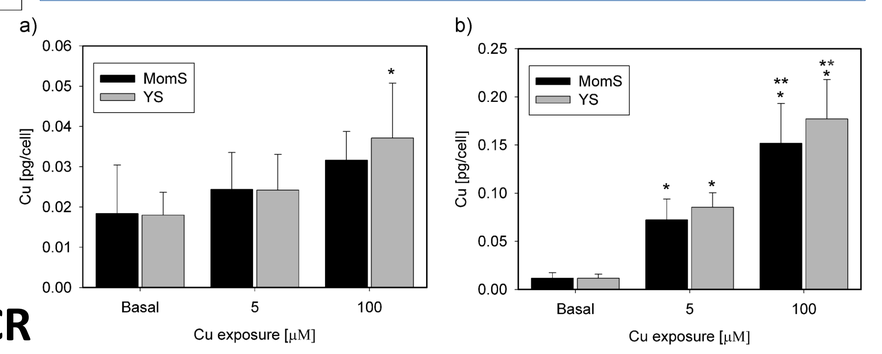


Cellular Copper

Cellular copper concentrations for MomS (black) and YS (grey), increasing media copper for (a) 4 hrs and (b) 48 hrs.
Cellular copper concentrations are expressed in µM. Data are means ± SD from at least three biological replicates.

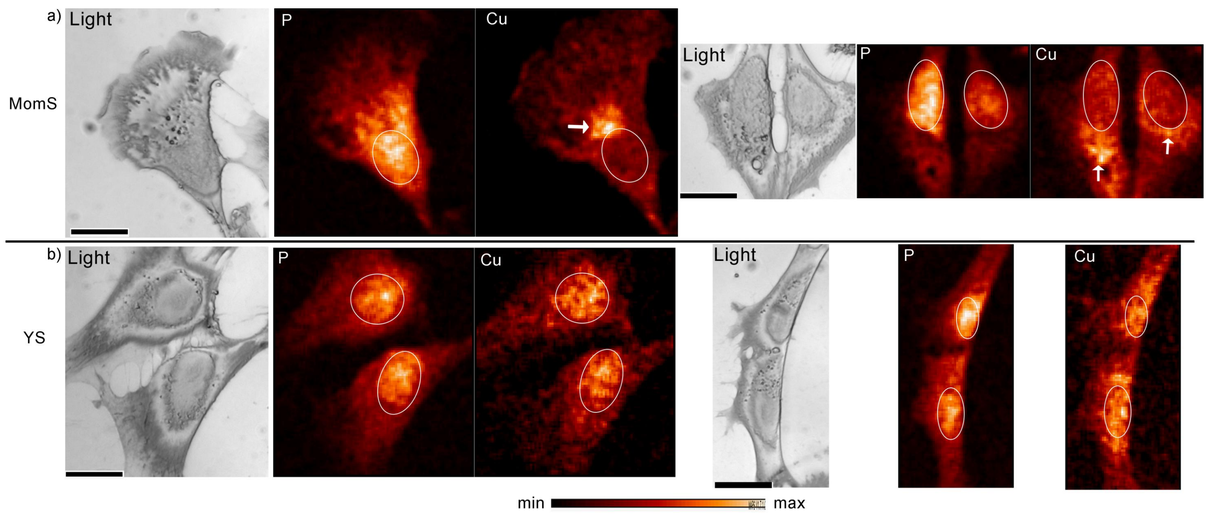


Copper content of MomS (black) and YS (grey) cells with increasing media copper for 4 hrs (a) and 48 hrs (b)
Copper is expressed as pg/cell, yielding more comparable MomS and YS results than copper expressed in µM (see above). Data are means ±SD from at least three biological replicates. Significant differences compared to basal levels are indicated by *, significant differences to 5 µM exposures are indicated by **.



X-ray Fluorescence Microscopy

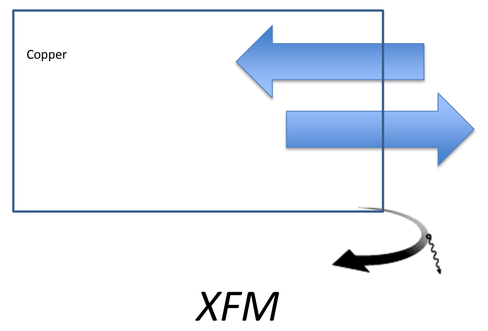
XFM images for MomS and YS cells grown under basal conditions.
Visible light microscope images and elemental maps for phosphorus and copper for MomS **(a)** and YS **(b)**. Two different sets of cells are shown for each cell line (resolution 750 nm/pixel, dwell time 1 sec/pixel). Approximate positions of nuclei are indicated by white circles and white arrows point to Cu in the TGN. All elemental maps were created from full spectra, after applying a 3x3 rectangular smoothing filter on the dataset. The relative elemental concentration is presented as false coloring (red temperature scale bar), the scale bar is 20 nm.



qRT-PCR

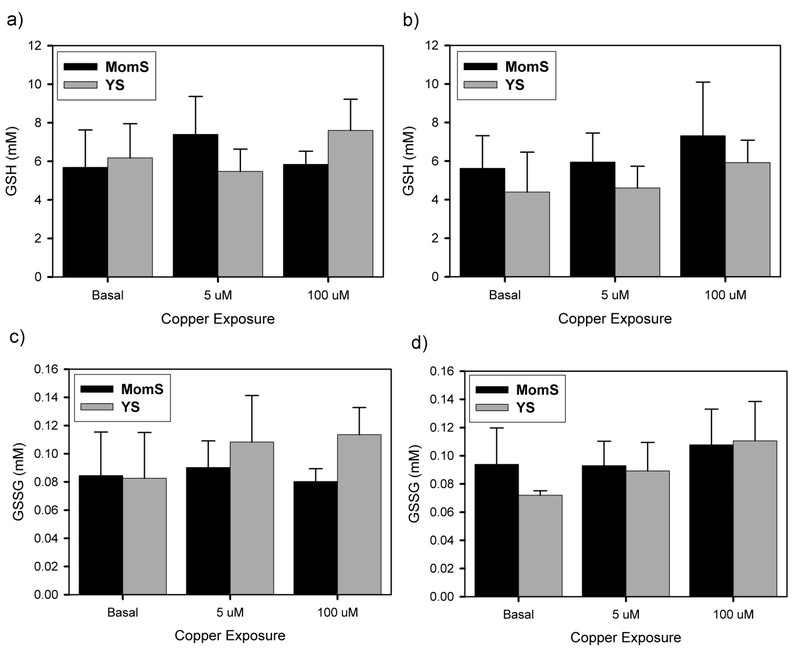
qRT-PCR results for MomS and YS cells
Average fold changes ±SD for each gene (glutamate cysteine ligase subunits GCLC and GCLM and metallothioneins MT1a and MT2a) and condition shown relative to the housekeeping GAPDH. Data from at least 2 biological replicates and 2 independent measurements each. Glutathione synthesis results do not demonstrate the total glutathione dip for YS at 4 hrs, MT increases slightly.

Exposure Cu [µM]	GCLC	GCLM	MT1a	MT2a
		4 hr, MomS		
5	-1.4 ± 0.5	1.2 ± 0.3	1.9 ± 0.73	1.2 ± 0.6
100	1.2 ± 0.4	1.2 ± 0.1	4.5 ± 4.7	2.7 ± 1.6
		4 hr, YS		
5	1.2 ± 0.4	1.1 ± 0.3	2.2 ± 1.7	1.8 ± 0.6
100	1.2 ± 0.7	2.1 ± 1.5	2.0 ± 0.5	2.1 ± 0.7
		48 hr, MomS		
5	-1.5 ± 0.3	1.0 ± 0.1	1.4 ± 0.5	1.5 ± 0.4
100	1.4 ± 0.2	2.0 ± 0.1	1.3 ± 1.1	4.2 ± 0.8
		48 hr, YS		
5	1.6 ± 0.1	1.9 ± 1.0	1.0 ± 0.1	1.2 ± 0.7
100	2.6 ± 0.5	3.8 ± 1.3	2.9 ± 0.5	7.7 ± 1.6

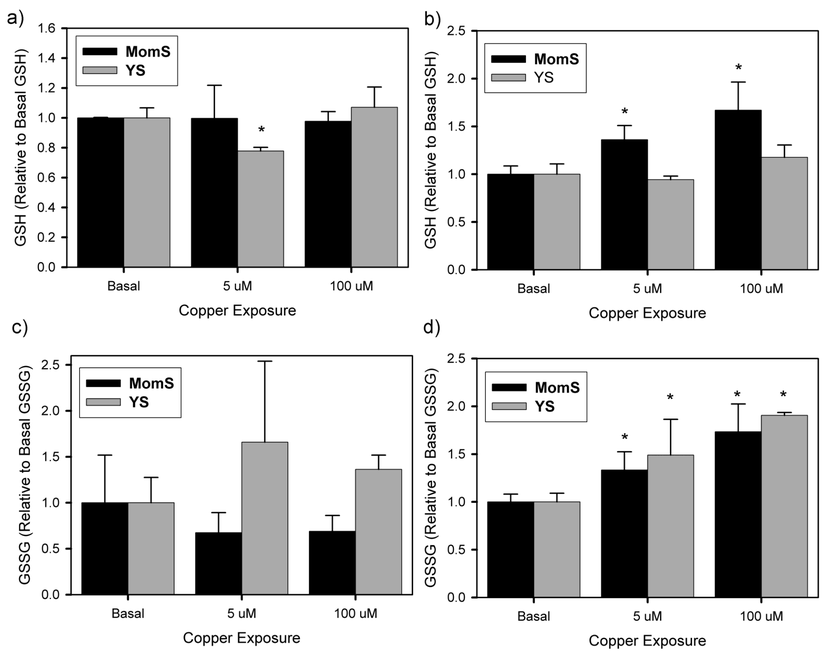


Glutathione

Total GSH and GSSG for MomS (black) and YS (grey) cells as a response to copper exposure at 4 and 48 hr
(a) GSH levels in mM for acute (4 hr) copper exposure at low (5 µM) and high (100 µM) copper for MomS and YS, **(b)** GSH levels for chronic (48 hr) exposures, **(c)** GSSG levels in mM for the same conditions as in a), **(d)** GSSG levels in mM for the same conditions as in b). All data represent means of at least three biological replicates ±SD.

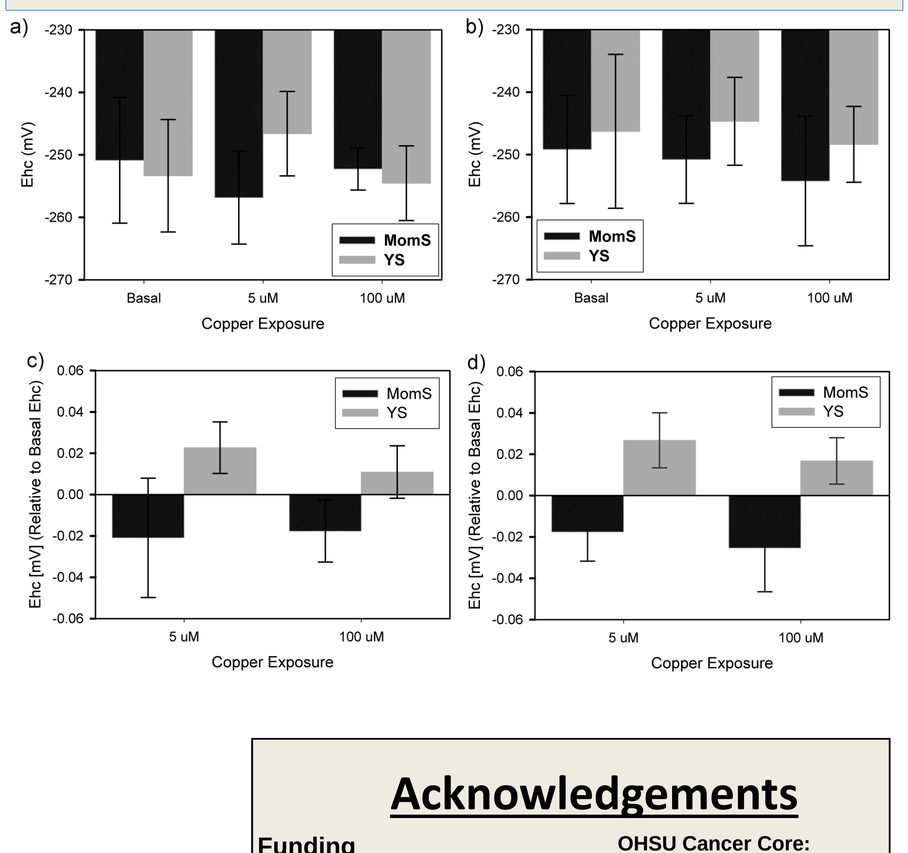


Normalized total GSH and GSSG levels for MomS (black) and YS (grey) from single split experiments
(a) single split GSH levels for acute, 4 hr, copper exposures for MomS and YS. For better visualization, GSH levels were normalized to basal levels. Values are means of duplicated measurements from the same experiment. **(b)** GSH levels for chronic, 48 hr, exposures and GSSG concentrations for acute **(c)** and chronic **(d)** exposures, all single splits, normalized to basal levels. Data are means ±SD from at least three biological replicates. Significant differences in levels or ratios compared to basal conditions are indicated by an *.



Redox

GSH/GSSG redox potentials for MomS (black) and YS (grey) cells determined from respective concentrations
Redox potentials (E_{hc}) were calculated from GSH and GSSG concentrations for MomS and YS cells under acute **(a)** and chronic **(b)** copper exposures at pH 7.4. Values (in mV) are means ±SDs of three sets of triplicate samples. Single split results for acute **(c)** and (**d**) chronic exposures, results are normalized by the respective reduction potential values for basal copper exposures. The values for the single splits are means ±SDs of a duplicated single split replicate.



Protein S-glutathionylation results for YS cells (4 hr exposures)
Percentages are calculated as fractions of total cellular GSH. Changes are too small to completely account for glutathione differences.

Copper exposure	*Protein-SSG (range)*
Basal	2.5 - 4.5%
5 µM $CuCl_2$	2.9 - 3.3%
100 µM $CuCl_2$	4.1 - 4.3%

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