Copper excess in liver HepG2 cells interferes with apoptosis and lipid metabolic signaling at the protein level

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ABSTRACT

Background/Aims: Copper is an essential trace element that serves as an important catalytic cofactor for cupro-enzymes, carrying out major biological functions in growth and development. Although Wilson’s disease (WD) is unquestionably caused by mutations in the ATP7B gene and subsequent copper overload, the precise role of copper in inducing pathological changes remains poorly understood.

Materials and Methods: Our study aimed to explore, in HepG2 cells exposed to copper, the cell viability and apoptotic cells was tested by MTT and Hoechst 33342 staining respectively, and the signaling pathways involved in oxidative stress response, apoptosis and lipid metabolism were determined by real time RT-PCR and Western blot analysis.

Results: The results demonstrate dose- and time-dependent cell viability and apoptosis in HepG2 cells following treatment with 10μM, 200μM and 500μM of copper sulfate for 8 and 24 h. Copper overload significantly induced the expression of HSPA1A (heat shock 70kDa protein 1A), an oxidative stress-responsive signal gene, and BAG3 (BCL2 associated athanogene3), an anti-apoptotic gene, while expression of HMGCR (3-hydroxy-3-methylglutaryl-CoA reductase), a lipid biosynthesis and lipid metabolism gene, was inhibited.

Conclusion: These findings provide new insights into possible mechanisms accounting for the development of liver apoptosis and steatosis in the early stages of Wilson’s disease.

Keywords: Wilson’s disease, copper, oxidative stress response, apoptosis, lipid metabolism, steatosis

INTRODUCTION

Wilson’s disease (WD) is a severe genetic disorder of copper metabolism caused by mutations in ATPase Cu²⁺ transporting beta polypeptide (ATP7B), which is also a P-type ATPase copper transporter (1). In WD, the direct link between elevated hepatic copper and development of liver pathology has been firmly established. The loss of ATP7B expression or function disrupts copper homeostasis, particularly in the liver, by greatly diminishing the ability of hepatocytes to export excess copper into bile (2). Copper accumulates to levels as high as 1,500 μg/g (dry weight) in the liver (3), causing severe morphological and functional changes, including hepatitis, cirrhosis and liver failure. Although phenotypic manifestations of WD have been well-described, the detailed mechanisms underlying the hepatodegenerative process in WD needs further elucidation. Generation of reactive oxygen species (ROS) by excess copper as well as the subsequent oxidative changes in lipid oxidation, cell cycle and apoptosis have been involved in the development and progression of WD (4-7). However, Huster et al. failed to find significant changes in protein machinery associated with oxidative stress in Atp7b⁻/⁻ mice (6). Microarray analyses have shown an increase in the mRNA expression of heat shock 70kDa protein 1A (HSPA1A) and BCL2-associated athanogene 3 (BAG3) in HepG2 cells exposed to copper and a decrease in the mRNA expression of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) in Atp7b⁻/⁻ mice (6,8,9). To our knowledge, no further studies confirmed the changes of these three genes at the protein level. The aim of our study was therefore to examine the
responses of liver cells to physiological and toxic levels of copper and determine the involved changes in expression of the HSPA1A, BAG3 and HMGCR genes at the protein level.

MATERIALS AND METHODS

Cell culture and treatment

HepG2 cells (ATCC HB-8065, VA, USA) were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin. Cells were maintained in a humidified incubator at 37°C with 5% CO₂. Copper overload of the control HepG2 cells was achieved by treatment with 10 μM, 200 μM and 500 μM copper sulfate (Sigma, St Louis, MO, USA) in the media for 8h and 24h respectively. These doses were chosen on the basis of reported data both in HepG2 cells (10) and in WD patients (11). During incubation with copper sulfate (Sigma, St Louis, MO, USA) cells were grown in minimum essential medium supplemented with L-glutamine. All experiments were repeated at least three times.

Real time RT-PCR

Total RNA were prepared from the untreated and treated cells with RNeasy kits (Qiagen, Melbourne, Australia). cDNA was synthesized by using the RevertAid First-Strand kit (Invitrogen, Melbourne, Australia) as per the manufacturer’s instructions. Gene-specific primers used for amplification of Homo sapiens HSPA1A, HMGCR and BAG3 cDNA are shown in Table 1. PCR was performed with 1 μg of reverse-transcribed RNA and 100 ng/μL of sense and antisense primers in a total volume of 20 μL. After an initial denaturation at 95°C for 30 s, a two-step cycle procedure was used (denaturation at 95°C for 5 s, annealing and extension at 60°C for 30 s) for 40 cycles. Gene expression levels were normalized according to the level of GAPDH expression. Relative amounts (RQ values) of HSPA1A, BAG3 and HMGCR mRNA in each sample were calculated by the ΔΔ Ct method. Data were presented as mean±standard deviation.

Western blot analysis

Cells were grown to 80% confluence, harvested and lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM NaVO₃, 1mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, and 25 μg/ml apro tin). Proteins (30-50 μg) were heated at 95°C for 5 min, separated by SDS-PAGE on a 6% and 12% gel, and electrophoretically transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). The blots were blocked for 1 h in 5% milk powder in TBST (10 mmol/L Tris (pH 8), 150 mmol/L NaCl, 0.05% Tween 20) and incubated at 4°C overnight with mouse HSPA1A antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), mouse HMGCR antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), rabbit BAG3 antibody (Abcam, Cambridge, MA, USA) and mouse GAPDH antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) at dilutions of 1:500, 1:800, 1:400 and 1:800, respectively. The membrane was washed three times with PBS and then incubated for 1 h with 1000-fold dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, Little Chalfont, Buckinghamshire, England) to detect BAG3 and 1000-fold dilution of horseradish peroxidase-conjugated anti-mouse IgG (Amersham, Little Chalfont, Buckinghamshire, England) to detect HSPA1A and HMGCR. Immuno-reactive proteins were visualized using enhanced chemiluminescence (Millipore Biologicals, Billerica, MA, USA). All membranes were stripped and re-probed with an anti-GAPDH antibody (Sigma-Aldrich, Sydney, Australia) at a dilution of 1:2000 to ensure even loading of proteins.

Analysis of cell viability

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was done as described previously (12). HepG2 cells were treated as mentioned above. The supernatant was aspirated and 200 μL medium mixed with 3 μL MTT reagent (5 mg/mL) was added to each well. After a 3-h incubation at 37°C, 200 μL of DMSO was added to dissolve the formazan crystals and the absorbance was measured at 490 nm using a Digiscan Microplate Reader (Bio-TEK Instruments, USA). Wells only containing 200μl medium were used as blanks and were subtracted as background from each sample. Results were expressed as a percentage of the untreated cells.

Identification and quantification of apoptotic cells

Treated cells were stained for 20 min with 1 μM Hoechst 33342 (Yuntian Bi, China) to assess nuclear morphology and with 1 μM calcein AM (Sigma, St Louis, MO, USA) and 2 μM propidium iodide (PI, Yuntian Bi, China) to evaluate membrane integrity. Approximately 100 cells were examined in each field at 200x magnification and five random fields for each experimental condition were chosen.

Statistical analysis

Values were represented as mean ± standard deviation. Statistical analysis was performed using SPSS17.0 (IBM, USA). Data analysis was performed using one-way ANOVA. A value of p<0.05 was considered statistically significant.
RESULTS
Copper sulfate induces apoptosis and reduces cell viability in HepG2 cells
When evaluating the percentage of apoptotic cells, significant differences were observed between cells exposed to copper and untreated cells. This held true both for the 8-hour timepoint (2.8%, 4.4%, 6.6% and 7.8% apoptosis after treatment with 0 μM, 10 μM, 200 μM and 500 μM concentrations of copper sulfate, respectively) (p<0.05) and for the 24-hour timepoint (3.2%, 6.6%, 16.4% and 23.2% for the same copper concentrations) (p<0.05) (Figure 1). Copper sulfate-induced apoptosis was characterized histologically by condensed and fragmented nuclei and indicated that apoptosis was the primary cause of cell death.

With regard to cell viability, cell exposure to increasing doses of copper sulfate for 8 h reduced the viability by 9.6%, 76.1% and 87.4% as compared with untreated cells (p<0.05) (Figure 2). The decrease was, as expected, more pronounced when copper exposure lasted 24 h (cell viability decreased by 14.6%, 81.7% and 92.4% for 10 μM, 200 μM and 500 μM concentrations of copper sulfate, respectively) (p<0.05) (Figure 2).

Copper increased HSPA1A and BAG3 mRNA expression and decreased HMGCR mRNA expression in a dose-dependent manner
For HSPA1A (Figure 3a), the fold induction was significant at 8 h (6.5 and 30.4 fold with 200 μM and 500 μM, respectively), and at 24 h (4.9 and 25.1 fold for the same concentrations) (p<0.05). There was no significant difference between 8 h and 24 h exposure with 10 μM copper sulfate (p>0.05), while the HSPA1A gene showed a maximal expression after 8 h exposure with 200 μM and 500 μM copper sulfate.

For BAG3 (Figure 3b), the protein expression increase was significant (p<0.05) for all concentrations of copper sulfate (10 μM, 200 μM and 500 μM) at 8 h exposure (5.4, 10.4, and 19.1 fold, respectively) and at 24 h (8.9, 27.7 and 56.3 fold, respectively). BAG3 gene also clearly showed a time-dependent increase in expression for all concentrations of copper sulfate (10 μM, 200 μM, and 500 μM) at 24 h exposure when compared with an 8 h exposure (p<0.05).

For HMGCR (Figure 3c), mRNA expression decreased significantly (p<0.05) by 21.1%, 47.3%, 78.9% when exposed to 10 μM, 200 μM and 500 μM of copper sulfate, respectively, for 8 h and 44.5%, 62.7% and 83.6% for the same concentrations for 24 h exposure. The change in HMGCR gene expression decreased significantly between 8 h and 24 h with 10 μM and 200 μM copper sulfate exposure (p<0.05).

Copper sulfate exposure induces HSPA1A and BAG3 protein expression but decreases HMGCR protein expression
HSPA1A protein expression was significantly increased (p<0.05) by 2.3-, 2.9-, 2.8- and 3.5-fold in HepG2 cells when exposed to 200 μM and 500 μM, respectively, when compared to the nega-
tive control. For both concentrations, protein expression was significantly increased at 24 h as compared with 8 h (p<0.05). In contrast, no significant differences were observed with cellular exposure to 10 μM copper sulfate for both time points (p>0.05) (Figure 4a, b).

For BAG3, protein expression increased significantly (p<0.05) at 8 h by 3.1-fold, 6.4-fold, 9.1-fold (for 10 µM, 200 µM and 500 µM) at 8h), and at 24 h by 11.9-fold, 15.6-fold and 19.6-fold for the same concentrations at 24 h, and for all these concentrations the protein expression was significantly decreased at 24 h as compared to 8 h (p<0.05) (Figure 4a, c).

For HMGCR, a significant decrease was observed, when cells were exposed to 10 µM, 200 µM and 500 µM of copper sulfate. This decrease was 13.5%, 37.5%, 61% at 8 h, and 32.5%, 44.5% and 68% at 24h, as compared to the negative controls (all p<0.05). There were only significant differences between 8 h and 24 h at 10 µM copper sulfate (p<0.05) (Figure 4a, d).

**DISCUSSION**

Data obtained from the MTT assay indicated a strong time- and dose-dependent response relationship with respect to copper toxicity. However, the preserved cell viability at lower copper concentrations until 24 h indicated that liver cells may be able to respond, at least in the short term, to low copper concentrations in order to prevent death.

It has been proposed in some studies that free radical generation by excessive copper and subsequent oxidative changes in hepatocytes account for the development and progression of liver damage in WD patients (13). Copper exposure has also been shown to dramatically alter proteins involved in antioxidant defense in HepG2 cells (14,15). A study of copper in liver samples from WD patients indicated severe dysfunction of enzymes related to mitochondrial energy production (16). The role of oxidative stress remaining, however, somewhat debated, we further explored this mechanism by focusing on the expression of an oxidative stress gene--HSPA1A. The HSPA1A gene showed a dose-dependent increase in expression with a maximal expression after 8 h for the highest copper sulfate concentration. This induction decreased after 24 h, which may be explained by the development of cell membrane damage with subsequent leakage of internal enzymes involved in oxidative damage. These findings strengthen the previous results obtained at the transcriptional level by Song and Freedman (8), since we showed that the enhanced expression was also present at the protein level.

![Figure 3. a-c. Effect of copper sulfate on HSPA1A, BAG3 and HMGCR gene expression. HepG2 cells were exposed to 0, 10 μM, 200 μM and 500 μM copper sulfate for 8 and 24 h. The relative differences in RNA expression in samples with different treatments and exposure times were assessed by the comparative Ct method using the threshold cycle (Ct) values. Data are expressed as mean±standard error. Experiments were repeated at least three times.](image-url)

The progressive loss of viability and increased HSPA1A expression in response to copper overload in HepG2 cells, a well-known causative factor of ROS-dependent damage of important biomolecules, is likely involved in the activation of the programmed cell death pathway, apoptosis (17). Copper-induced neuronal death exhibiting characteristics of apoptosis has previously been reported in M17 neuroblastoma cells, human NT2-N neurons and NTERA-2-N neurons (18-20). Copper-induced apoptosis via both Bcl-2 and non-Bcl-2 associated pathways has also been reported in liver cells and observed in the liver of aged male tx1 mice (7). Our study indicated that there was both a time- and concentration-dependency to copper-induced apoptosis, which is in accordance with the results of other studies (8,10,21). To our knowledge, our study is the first to detect changes of BAG3 expression after copper exposure at the protein level.

Recently, it has been shown that exposure to copper activated cholesterogenic gene expression in macrophages, suggesting a mechanistic link between copper exposure and atherosclerosis (22). A study in Atp7b-/- mice also indicated imbalance of cholesterol metabolism (6). HMGCR, which plays a central role in cholesterol biosynthesis, was down-regulated in Atp7b-/- mice. Further analysis of human HMGCR mRNA by RT-PCR demonstrated that HMGCR transcript levels were markedly down-regulated (3.4-fold) in WD livers compared with the control (6). Our in vitro study also showed that HMGCR mRNA expression was down-regulated in copper-overloaded HepG2 cells, but further indicated that this increased expression was also present at the protein level.
Nagasaka et al. showed that excessive expression of PPARγ (peroxisome proliferator-activated receptors gamma) might contribute to the development and progression of liver steatosis due to enhanced liver lipogenesis or increased lipid uptake of the liver in WD. Moreover, relatively suppressed PPARα leading to the decrease of mitochondrial fatty acid β-oxidation might be responsible for the development and progression of the steatosis in WD (23).

The study by Liggy et al. highlighted significant correlation between copper concentration and hepatic steatosis with an increase in severity as tissue copper content increases (11). This is interesting because different levels of copper accumulation in WD patients would be expected to exhibit a “dose-dependent” relationship with phenotypic severity. It is plausible that patients with higher copper excess have more suppression of signal transduction pathways and therefore more severe clinical defects, favoring a copper accumulation-phenotype correlation in WD.

In summary, the analysis of copper-exposed HepG2 cells identified cytotoxic effects together with significant changes of three copper-responsive genes at both the transcriptional and protein levels. The overall results support the view that free radical generation by excess copper and the subsequent changes in hepatocyte organellar lipids and apoptosis may play a crucial role in the development and progression of steatosis and cell death in WD. However, further studies will be required to elucidate the causality between copper accumulation and the pathological changes of the liver in patients with WD.

Conflict of Interest: No conflict of interest was declared by the authors.

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