

Original Article

Copper Induces Apoptosis of Neuroblastoma Cells Via Post-translational Regulation of the Expression of Bcl-2-family Proteins and the tx^J Mouse is a Better Model of Hepatic than Brain Cu Toxicity

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Received October 19, 2007; accepted November 16, 2007; available online January 20, 2008

Abstract: The basic mechanism(s) by which altered Cu homeostasis is toxic to hepatocytes and neurons, the two major cell types affected in copper storage diseases such as Wilson's disease (WD), remain unclear. Using human M17 neuroblastoma cells as a model to examine Cu toxicity, we found that there was a time- and concentration-dependent induction of neuronal death, such that at 24 h there was a ~50 % reduction in viability with 25 μ M Cu-glycine₂. Cu-glycine₂ (25:50 μ M) treatment for 24 h significantly altered the expression of 296 genes, including 8 genes involved with apoptosis (BCL2-associated athanogene 3, BCL2/adenovirus E1B 19kDa interacting protein, caspase 5, regulator of Fas-induced apoptosis, V-jun sarcoma virus 17 oncogene homology, claudin 5, prostaglandin E receptor 3 and protein tyrosine phosphatase, non-receptor type 6). Surprisingly, changes in the expression of more 'traditional' apoptotic genes (Bcl-2, Bax, Bak and Bad) did not vary more than 20 %. To test whether the induction of apoptosis in neuroblastoma cells was via posttranslational mechanisms, we measured the protein expression of these apoptotic markers in M17 neuroblastoma cells treated with Cu-glycine₂ (0-100 μ M) for 24-48 h. Compared with glycine treated cells, Cu-glycine₂ reduced Bcl-2 expression by 50 %, but increased Bax and Bak expression by 130% and 400 %, respectively. To assess whether Cu also induced apoptotic cell death in a mouse model of WD, we measured the expression of these apoptotic markers in the liver and brain of mice expressing a ATP7b gene mutation (tx^J mice) at 10 months of age (near the end of their lives when overt liver pathology is displayed). Changes in the liver expression of these apoptotic markers in tx^J mice compared to background mice mirrored those of Cu treated neuroblastoma cells. In contrast, few changes in apoptotic protein expression were detected in the brain between tx^J and background mice, indicating the tx^J mouse is a good model of hepatic, but not brain, Cu toxicity. Our results indicate that Cu-induction of neuronal apoptosis does not require de novo synthesis or degradation of apoptotic genes, and that Cu accumulation in the aged tx^J mouse brain is insufficient to induce apoptosis.

Key Words: copper, glycine, neuron, apoptosis, gene expression, protein expression, toxic milk mice, Bcl-2, Bax, Bak, Bad, metal ion, Wilson's disease

1. Introduction

Copper is a trace metal required as a catalytic cofactor in many enzymes including Cu/Zn superoxide dismutase, lysyl oxidase, cytochrome c oxidase, ceruloplasmin, and

tyrosinase. Although Cu is an essential nutrient, this metal can be toxic if allowed to accumulate in excess of cellular needs. Cellular Cu homeostasis is therefore tightly regulated [18,34] allowing sufficient Cu for normal cellular functions, while preventing

excess Cu accumulation beyond the normal cellular storage capacity.

The incidence of Cu toxicosis in the general population is low [8,30]; acute Cu poisoning is usually the consequence of the consumption of contaminated foods or deliberate ingestion of high quantities of Cu salts [8,30]; chronic Cu toxicosis occurs in Wilson's disease (WD), Indian childhood cirrhosis, endemic Tyrolean infantile cirrhosis, and idiopathic Cu toxicosis. These diseases result in abnormal hepatic Cu accumulation that can result in hepatic failure and neurological disease [28]. WD is an autosomal recessive disorder that affects 1 in 200,000 live births and is caused by mutations in the P-type ATPase, ATP7b [9,11,43]. ATP7b is expressed predominantly in the liver and is responsible for transporting Cu into the hepatocytic-biliary secretory pathway and hence the removal of Cu from the circulation [2]. Mutations in this gene inhibit biliary Cu excretion leading to the accumulation of Cu in the liver and brain [44,51]. Individuals carrying an ATP7b mutation most commonly present with liver cirrhosis (45%), central nervous system involvement (35%) or psychiatric illness (10%) [21]. Symptoms of liver disease present nearly a decade earlier than neurological symptoms [20,52], and in the few cases that present with neither of the 3 aforementioned symptoms, further investigations have revealed evidence of liver dysfunction [21].

The decreased excretion of biliary Cu in WD results in a ~2-fold increase in serum Cu levels (~10 μ M). This increased serum Cu leads to the accumulation of Cu in the liver and brain, and results in hepatic cirrhosis, and neuropathological and neurological changes, respectively. In the brain, Cu accumulates mainly in the basal ganglia, subthalamic nuclei and grey and white matter [15,42], those areas affected by neurodegeneration in WD. Neuropathological changes include changes in the basal ganglia, shrunken corpus striatum, increased size and number of astrocytes, neuronal loss in the putamen and caudate, and perivascular thickening [44]. Although acute and chronic Cu accumulation induce liver and brain injury, the molecular mechanisms by which excess Cu induces cellular toxicity have not been fully elucidated. Determining the mechanisms responsible for neuron death and/or dysfunction that lead to the neurological changes observed in WD has

been hampered by the lack of an appropriate animal model. While both the current animal models of WD, the Long Evans Cinnamon rat and the toxic milk (tx^l) mouse [13,45] display clinicopathological changes consistent with chronic hepatitis due to increased hepatic Cu concentrations, these rodent models do not appear to display neuropathological or neurological changes [10,19].

To examine how neurons respond to increasing Cu load, and to determine the mechanism by which Cu induces neuron death, we cultured human M17 neuroblastoma cells with increasing concentrations of Cu for varying lengths of time. Cu induced a concentration- and time-dependent increase in apoptotic cell death via a mechanism that did not require degradation of Bcl-2 or synthesis of Bax, Bak and Bad mRNA. Cu decreased the expression of Bcl-2 protein, but increased Bax and Bak protein expression. Cu induced similar changes in apoptotic protein expression in the liver, but not in the brain, of tx^l mice, indicating the tx^l mouse to be a good model of hepatic, but not of brain, Cu toxicity.

Materials and Methods

Reagents and Preparation

CuCl₂ (99.999 % grade), glycine and crystal violet were purchased from Sigma Chemical Co., (St Louis, MO) and Aldrich Chemical Co. (Milwaukee, WI). Since metal ions added as simple salts may hydrolyze in a pH-dependent manner to form metal-hydroxy and oxy polymers, which may either bind non-specifically to proteins or become biologically inert [7,40], we used metal:chelator complexes to maintain Cu solubility and approximate the in vivo exchange of a metal ion from one protein or ligand to another [27]. A Cu-glycine₂ stock solution (50:100 mM) was prepared in doubly deionized water and allowed to come to equilibrium for 24 h at room temperature. The concentration of metal ions in treatment medias was confirmed by inductively coupled plasma - mass spectrometry (ICP-MS) for Cu, Zn, Fe, Al, Mn and Co (data not shown). Prior to use, all buffers and stock solutions of metal ions were filtered through a 0.22 μ m filter (Gelan Sciences, Ann Arbor, MI).

Rabbit IgG polyclonal antibodies against Bcl-2,

Table 1: Description of Cu treatments of human neuroblastoma cells assayed for gene expression

Sample Name	Control/Test	[Cu] (μM)	[Glycine] (μM)	Time (h)
CuGlyT(0,0,0)	Control	0	0	0
*CuGlyT(0,50,24)	Control	0	50	24
CuGlyT(25,50,6)	Copper	25	50	6
CuGlyT(25,50,12)	Copper	25	50	12
CuGlyT(25,50,24)	Copper	25	50	24
CuGlyT(25,50,48)	Copper	25	50	48

*All samples treated with Cu-glycine (CuGlyT(25,50,6-48)) were incubated in media containing 50 μM glycine for 24 h prior to the addition of Cu-glycine. CuGlyT(0,50,24) was incubated in the glycine containing media for 24 h, but was not treated with Cu-glycine. It was hence used as the 0 h control so that changes in gene expression could be attributed to the addition of Cu as opposed to glycine.

Bad, Bak and Bax were from Stressgen (British Columbia, Canada). Anti-rabbit horseradish peroxidase conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, California, USA). A rabbit polyclonal antibody against β -actin was from Santa Cruz Biotechnology (Santa Cruz, California, USA) and molecular size markers were from Bio-Rad Laboratories (Hercules, California, USA).

Experimental Treatment of Neuroblastoma Cells

M17 human neuroblastoma cells were maintained in Opti-Mem Reduced Serum Medium (Gibco, Carlsbad, California, USA) supplemented with 3 % (v/v) DCS (Gibco, Carlsbad, California, USA) and 1 % (v/v) penicillin-streptomycin (Gibco, Carlsbad, California, USA). Cells were maintained in 75 cm² culture flasks (5 % CO₂, 85 % humidity) and trypsinized (1 ml; 0.25 % Trypsin, 1 mM EDTA-4Na) and split at ratios of 1:10 to 1:2 upon reaching confluence.

For experiments analysing the effect of Cu on neuronal viability, M17 neuroblastoma cells were plated at approximately 1×10^5 cells/mL in 96-well culture-treated plates (Falcon, Millville, New Jersey, USA) and incubated overnight prior to treatment. The media was then replaced with 200 μL of media containing Cu-glycine₂ (0:0, 5:10, 10:20, 20:40, 30:60, 40:80, 50:100, 100:200 μM) and incubated for 0, 6, 12, 24, 36 and 48 h. A crystal violet assay was then performed to assess viability. For gene and protein expression experiments, M17 neuroblastoma cells were incubated

overnight in 75 cm² culture flasks before adding 10 mL of media containing Cu-glycine₂ (0:0, 0:50, 0:100, 25:50 and 50:100 μM) and incubated for 0, 6, 12, 24 and 48 h.

Viability Experiments

To avoid problems associated with the oxidoreductive properties of Cu ions, which many commercially available kits (e.g. LDH assay) utilize to determine viability, we employed the crystal violet method that relies on the physical binding of a dye to the

Although commonly used to distinguish gram-positive and gram-negative bacteria, crystal violet has been used previously to determine the viability of neurons [1]. Media was removed from cells cultured in 96-well plates, the cells washed with D-PBS (Gibco, Carlsbad, California, USA), and 50 μL of crystal violet added to each well at room temperature for 10 min. Following this, 200 μL of 33% (v/v) acetic acid was added to the wells, the plate shaken and then read at 570 nm on a Spectramax 190 microplate spectrophotometer reader (Molecular Devices; Sunnyvale, California, USA).

Blank wells containing only media and media containing 100:200 μM Cu-glycine₂ were used to calculate background absorbance. The mean of these background readings was subtracted from the sample means. In order to quantify viability as a percentage of untreated control neurons, the blank adjusted absorbance level of Cu-treated wells was divided by the blank adjusted absorbance of untreated wells at that time point. The control

cells were cultured for the same time interval as the treated cells, but in 200 μ L of media with no added Cu-glycine₂. A two-sample unequal variance (heteroscedastic) t-test was conducted to test the null hypothesis that there was no significant difference between the treated and control cells.

Gene Expression Analysis

M17 neuroblastoma cells were cultured in 75 cm² flasks and incubated overnight (24 h) in 0:50 μ M Cu-glycine₂ media before being treated with 25:50 μ M Cu-glycine₂ media for 6, 12, 24 and 48 h and extracted for total RNA. Total RNA also was extracted from the M17 neuroblastoma cells incubated in 0:50 μ M Cu-gly media as well as M17 neuroblastoma cells incubated in 0:0 μ M Cu-glycine₂ media (controls). Cells from 3 samples at each treatment were pooled prior to RNA extraction using the RNeasy system from Qiagen (California, USA). cDNA strand 1 and strand 2 syntheses from the isolated total RNA was carried out according to the protocol provided for the Gibco SuperScript Choice System (Gibco; Carlsbad, California, USA) with an oligo-dT primer containing a T7 RNA polymerase promoter. Details of each sample including the concentration of Cu, glycine and the time point at which the sample was collected is listed in **Table 1**. cDNA (10 μ l) was then used to synthesize biotin-labeled cRNA as per the protocol described by the Enzo BioArray High Yield RNA Transcript Labeling Kit (Farmingdale, New York, USA). 2 μ l of 5 x fragmentation buffer (200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc; Sigma; St. Louis, Missouri, USA) was then added to the resulting Biotin-labeled cRNA. The final cRNA concentration was quantified by measuring 260:280 absorbance ratios on the resulting samples. Results ranged from 0.5 - 2 μ g/ μ L which was sufficient for microarray analysis (results not shown). The Biotin-labeled cRNA was then hybridized for 16 h to Affymetrix HG-U133 A and B chips (Santa Clara, California, US). Together, these chips contain 22,283 probe sets with each probe set comprising of 16 to 20 oligonucleotide probes designed to correspond to a region in the 3' end of human genes as identified on NCBI gene databases. Subsequently, the arrays were washed and stained with a streptavidin phycoerythrin stain using a GeneChip Fluidics Station 400 (GeneChip; Santa Clara,

California, USA). The microarray chips were then emptied and placed in the Hewlett Packard GeneArray™ (Hewlett Packard; Palo Alto, California, USA) laser scanner and scanned the recommended 2 times before generating values indicating the signal level of each probe. The pixel value was set to 3 μ M and the wavelength to 570 nm. The signal for each probe was stored in a CEL file and exported to Excel file (Microsoft Corporation, Seattle, WA). A linear scaling model implemented by Affymetrix Microarray Suite Software and a non-linear model proposed by Li and Wong, ([26]) were implemented in their respective software environments in order to find the most appropriate Normalization method. These two Normalization methods were implemented using the 'affy' package in the R programming environment using the data created from the image file. The Li and Wong method was deemed the most appropriate of these alternatives, a finding that is consistent with other reports [6]. The array with the median mean expression was the Cu treated cells at 48 h and this array was designated the baseline array which other arrays were normalized to. Expression values were calculated based on the perfect match/mismatch difference model.

Gene Filtering

Filtering represents the first step in the high-level data analysis. Since data on 22 283 probe sets for 5 different time points were generated, the data set was restricted in order to focus on the genes whose expression changed the most. After Normalization of the array data, only those probe sets where the maximum value/minimum value ratio across the time points was greater than 4 were further analyzed. This step decreased the size of the data set from 22283 to 296 sets. This data set was used for downstream analyses such as hierarchical clustering and chromosomal analysis [12].

Cell Lysis and Western Blot Analysis

Following the determination of cell viability using the trypan blue exclusion method, neuroblastoma cultures incubated for 0, 6, 12, 24 and 48 h with various Cu:Glycine₂ treatments (0:0 μ M, 0:50 μ M, 0:100 μ M, 25:50 μ M, 50:100 μ M) were collected into lysis buffer and samples sonicated for 10 sec. (Biologics Model 150V/T Ultrasonic

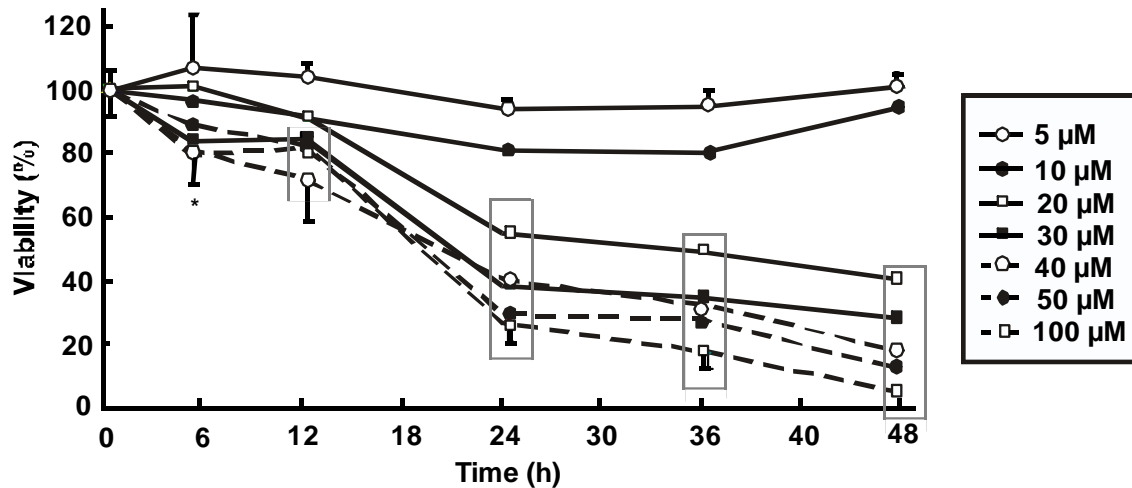


Figure 1: Cu is toxic to human M17 neuroblastoma cells in a concentration- and time-dependent manner. The graph represents the effect of varying concentrations of Cu (5 μ M, 10 μ M, 20 μ M, 40 μ M, 50 μ M and 100 μ M) on the viability of M17 neuroblastoma cells at 6, 12, 24, 36 and 48 h. Viability of treated cells is expressed as a percentage of the viability of untreated cells at each time point. Data is presented as mean \pm SE (n = 6 replicates; * P < 0.05; boxes indicate significance changes of multiple points).

Homogenizer; Gainesville, Virginia, USA). Protein levels in cell lysates (and tissue homogenates) were measured using the BCA Protein Assay Kit (Pierce; Rockford, IL) and samples loaded and run on polyacrylamide gel electrophoresis (Tricine gels, 10-20%; Novex; San Diego, CA), transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories; Hercules, CA), fixed with 1% (v/v) glutaraldehyde, blocked with 10% (w/v) non-fat milk and then probed with primary antibody overnight at 4 °C. The blot was then incubated with horseradish peroxidase (HRP) conjugate secondary antibody (Santa Cruz Biotechnology; Santa Cruz, CA) for 2 h at room temperature, and developed with ECL reagent (1 min; Amersham, Little Chalfont, England) as per the manufacturer's instructions. The chemiluminescent signal was captured on autoradiographs (Eastman Kodak Company, Aurora, IL, USA) and the signal quantified on a UVP BioImaging System using the LabWorks 4.0 Image Acquisition and Analysis Software (UVP, Inc., Upland, CA, USA). Images were captured and the intensity of the autoradiograph signals determined. Control and treatment values were corrected for blank values and the results expressed as a fold change over loading control values.

Toxic Milk Mice

Toxic milk (tx⁻) mice (C3HeB/FeJ-Atp7btx-j; stock # 001576) and control mice (C3HeB/FeJ; stock #, 000658) were obtained from the Jackson Laboratory, ME. Toxic milk (tx⁻) mice contain a glycine to aspartate substitution (Gly712Asp) that renders the ATP7b protein dysfunctional and results in Cu accumulation in a distribution resembling that observed in human cases of WD [13,45]. The mice were maintained in the Animal Resource Centre (ARC) at Case Western Reserve University, Cleveland, OH. Mice were housed in microisolator cages at 21° C with a 12 h light/dark cycle and fed a standard pelleted mouse chow and water ad libitum. Male tx⁻ and control mice were aged to 10 months prior to sacrifice.

Results

Neurotoxicity of Cu

To determine that the concentrations of Cu to be administered to the neuroblastoma cells were accurate, the concentration of Cu in media containing serum, media containing serum and glycine and media containing serum, glycine and Cu were analyzed by ICP-MS for metal ion content. Media containing serum and/or glycine contained less than 1 μ M Cu. In media containing added Cu, the concentrations were within 5 % of intended

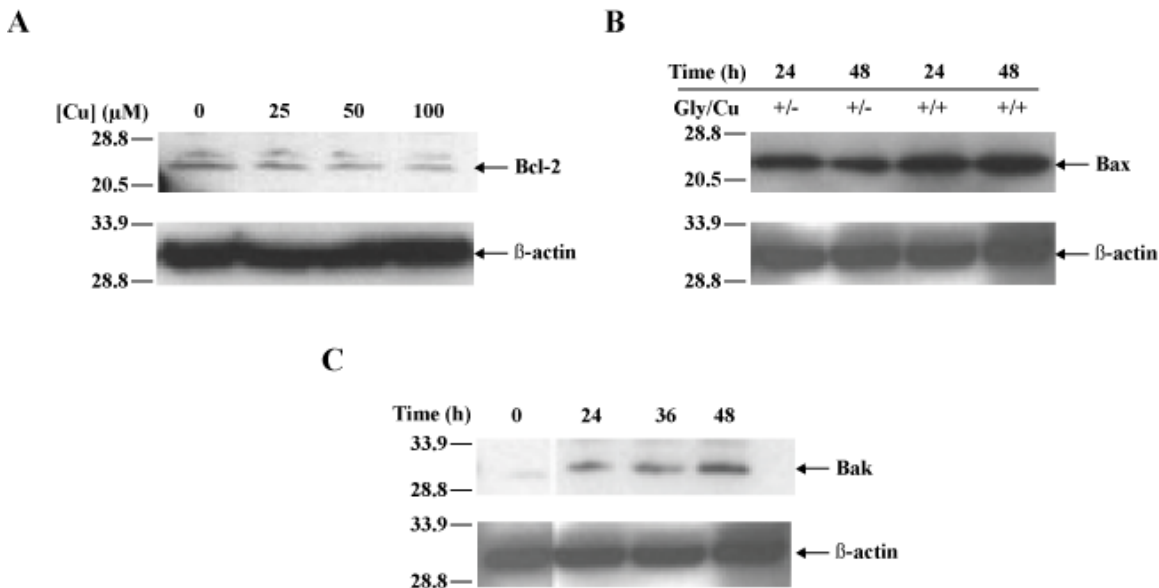


Figure 2: Cu induction of apoptotic protein expression in human M17 neuroblastoma cells. (A) M17 neuroblastoma cells were incubated with 0, 25, 50 and 100 μM Cu-glycine₂ for various times (0, 6, 12, 24 and 48 h) and the expression of Bcl-2 (A), Bax (B) and Bak (C) determined by immunoblot analysis. The expression of β -actin was measured as a loading control. Molecular weight markers are shown on the left. Experiments are representative of 3 independent experiments.

values. Concentrations of other metal ions (Fe, Zn, Al, Mn, Ni and Co) were mostly constant between media, indicating added Cu-glycine₂ did not markedly affect the concentration of these metal ions. These results indicate that concentrations of Cu below 1 μM are sufficient for growth of neuroblastoma cells.

The time- and concentration-dependent induction of neuronal death by excess Cu was characterized for M17 neuroblastoma cells. Cells were incubated with increasing concentrations of Cu-glycine₂ for 6, 12, 24, 48 and 72 h. Neuroblastoma viability decreased at an increasing rate with increasing concentrations of Cu. At 6 h, a Cu concentration of 100 μM induced a significant decrease in cell viability (20.5 % decrease, $p = 0.028$, $n = 6$; **Figure 1**). By 12 h, there was a significant decrease in neuron viability in media containing 30 μM (15.2 %, $p = 0.015$), 40 μM (28.7 %, $p = 0.003$), 50 μM (17.5 %, $p = 0.016$), and 100 μM Cu (18.5 %, $p = 0.012$). After 24, 36 and 48 h incubation, media containing Cu concentrations as low as 20 μM , as well as higher concentrations (30 μM , 40 μM , 50 μM and 100 μM) produced a similar, significant decrease in cell viability such that

at 48 h, viability was respectively 41.0% ($p = 0.021$), 28.0% ($p = 0.007$), 17.7% ($p = 0.003$), 11.6% ($p = 0.002$) and 4.6% ($p = 0.001$) (**Figure 1**). These results indicate that there is both a time- and concentration-dependence to Cu-induced neuronal death. The maintenance of cell viability at lower Cu concentrations until 12-24 h indicates that neurons may be able to respond, at least in the short term, to low concentrations of Cu to prevent death.

Cu-induced Changes in the Expression of Apoptotic Genes

To examine the effects of Cu on gene expression in neurons, RNA extracted from neuroblastoma cells treated as outlined in **Table 1** were analyzed by microarray analyses. After Normalization, the filtered gene set contained 296 genes whose expression was deemed to be significantly up- or down-regulated (Chan, Atwood, unpublished data). Since Cu used at the concentration (25 μM) in the viability experiment induced neurotoxicity, we analyzed the Affymetrix NetAffx database for genes associated with apoptosis. Of the filtered gene set, 8 of the 296 genes were identified as being involved in apoptosis (**Table 2**). Two of the genes were related to

Table 2: Apoptotic genes identified in the filtered data set and their respective fold changes at the indicated time points

Gene	Fold Change at Indicated Time Point			
	6h	12h	24h	48h
caspase 5, apoptosis-related cysteine protease	9.5	-1.2	1.0	11.1
BCL2-associated athanogene 3	3.8	-1.2	-1.4	3.1
regulator of Fas-induced apoptosis	2.9	-1.5	-1.0	2.3
v-jun sarcoma virus 17 oncogene homolog (avian)	-1.8	-4.1	-1.0	-4.5
claudin 5 (transmembrane protein deleted in velocardiiofacial syndrome)	-2.2	-7.0	-1.2	-4.8
prostaglandin E receptor 3 (subtype EP3)	-4.3	-4.0	1.6	-1.9
BCL2/adenovirus E1B 19kDa interacting protein 1	-4.8	-2.2	-4.2	-1.2
protein tyrosine phosphatase, non-receptor type 6	-6.4	-6.8	-1.3	-3.6

decreased Bcl-2 protein function (BCL2-associated athanogene 3, BCL2/adenovirus E1B 19kDa interacting protein 1), one was a regulator of Fas-induced apoptosis and another was caspase 5.

Analysis of all apoptotic genes indicated relatively small changes in gene expression over 48 h with Cu treatment. For example, changes in mRNA expression of more 'traditional' apoptotic genes did not vary more than 20 %; Bax, Bak and Bad displayed a similar expression pattern, with lower expression levels at 6 h and 12 h than that of the control sample, increasing at 24 h and 48 h back to control or slightly higher levels. These results suggest that Cu induction of apoptosis is via translational mechanisms.

Cu-induced Changes in the Expression of Apoptotic Proteins

To determine if Cu-induced cell death was a result of altered translation of apoptosis-related proteins, changes in the expression of the α - and β -isoforms of Bcl-2 (25- and 22-kDa, respectively) were measured in M17 neuroblastoma cells exposed to increasing concentrations (0, 25, 50 and 100 μ M) of Cu:glycine₂ for 24 h (**Figure 2A**). The 50 % decrease in Bcl-2 protein expression at 24 h with 25 μ M Cu-glycine₂ was considerably greater than the decrease in Bcl-2 gene expression (17 %), indicating that while the direction of the change in protein expression reflected that of the gene expression, the magnitude of change was markedly different. These results indicate that either a small decrease in Bcl-2 expression can lead to a large decrease in protein expression (possibly

a reflection of the fast turnover rate of the Bcl-2 mRNA and/or protein), or that excess Cu may directly induce degradation of Bcl-2 mRNA and protein (such as reported for Cu transport proteins - [55]).

We next tested whether the expression of other Bcl-2 apoptosis family members was altered. The immunoblot analysis of Bax protein expression (21-kDa) indicated a 1.3-fold and 2.1-fold increase in protein expression at 24 and 48 h, respectively, following Cu-glycine₂ (25:50 μ M) treatment compared with glycine treatment alone (**Figure 2B**). These results indicate that Cu induces an increase in Bax expression, which like the decrease in Bcl-2, is indicative of apoptosis.

We next examined changes in the expression of Bad, a 22-kDa protein that is thought to activate apoptosis via binding to Bcl-xL, thereby reversing its anti-apoptotic activity [14]. Bad requires the presence of Bax or Bak in order to induce apoptosis (Zong et al., 2001; Cheng et al., 2001). Immunoblot analysis of Bad protein expression in neurons exposed to increasing concentrations (0, 25, 50 and 100 μ M) of Cu:glycine₂ for 24 h indicated a slight decrease in expression with Cu treatment (data not shown). We therefore examined the Bad binding protein, Bak, a 23-kDa homolog of Bcl-2, that mediates its actions by binding to Bad to increase/decrease Bad binding to Bcl-2 and promote apoptosis [14]. Immunoblot analysis of Bak protein expression (23-kDa) indicated a pronounced 3.8-fold increase in protein expression at 24 h and a 3.8-fold and 6.9-fold increase, respectively, at 36 and 48 h compared to neurons treated with 0:0 μ M Cu-glycine₂ (**Figure 2C**). These results indicate

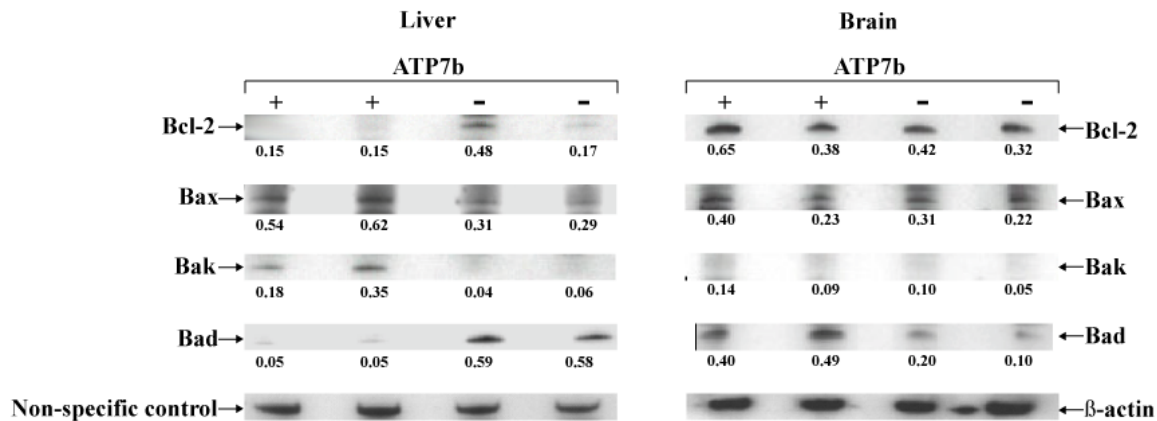


Figure 3: Apoptotic protein expression in the liver and brain of toxic milk mice. The expression of Bcl-2, Bax, Bak and Bad in the liver (A) and brain (B) of 2 tx^l and 2 control mice was determined by immunoblot analysis. Relative band densities (normalized to a non-specific loading control (liver) or β-actin (brain)) are shown below each band. Apoptotic protein expression was observed in the liver but not the brain.

that the increased expression of Bak would increase Bad binding to Bcl-2 thereby inducing apoptosis. These results indicate that high concentrations of Cu induce apoptosis in neuroblastoma cells, and that the measurement of gene expression alone is insufficient to examine the effects of Cu on cellular metabolism.

Liver and Brain Expression of Apoptotic Markers in the Toxic Milk Mouse

Mice expressing a mutation in the ATP7b gene (tx^l mice) accumulate Cu, particularly in the liver and brain, developing liver cirrhosis similar to that observed in the individuals with WD [13]. To generate preliminary data to test whether the Cu-induced changes in protein expression in neuroblastoma cells also

occurred in tx^l mice, we aged mice to 10 months, i.e. near the end of their life (life expectancy of 10-12 months for the tx^l mice) when they are known to display overt liver pathology [10] and then collected their liver and brain for analysis. In tx^l mouse liver, there was a clear decrease in the expression of Bcl-2 and Bad, and a corresponding increase in the expression of Bax and Bak when compared to background mice (Figure 3A). The direction in the expression of these apoptotic markers is very similar to that observed for Cu treated neuroblastoma cells, and indicates a decreased survival (anti-apoptotic) and increased apoptotic (pro-apoptotic) signaling in the tx^l mouse liver. In the brain, there was no difference in the expression of Bcl-2, Bax and Bak between tx^l and background mice, although there was an increase in Bad expression between tx^l and background mice (Figure 3B). These results suggest that unlike the liver, there is less apoptotic signaling in the brains of tx^l mice, and likely a decreased propensity for neuronal cells to undergo apoptosis in this mouse.

Table 3: Relative changes in protein expression in human neuroblastoma cells compared to the liver and brains of toxic milk (tx^l) mice

Gene	In vivo		
	Neuroblastoma Cells	Liver	Brain
Bcl-2	↓	↓	No change
Bax	↑	↑	No change
Bak	↑↑	↑	No change
Bad	↓	↓	↑

Discussion

Cu Neurotoxicity

Cu:glycine₂-induced toxicity of human M17 neuroblastoma cells was mediated via apoptotic mechanisms in a concentration and time-dependent manner (Figures 1-3; Table 3). Although concentrations of Cu:glycine₂ ≤ 10

μM were not toxic to neuroblastoma cells cultured for up to 48 h, $\text{Cu}:\text{glycine}_2$ concentrations above this level ($\geq 20 \mu\text{M}$) had a significant neurotoxic effect after 24 h (**Figure 1**). Contrasted with normal physiological concentrations of Cu within the brain parenchyma ($70 \mu\text{M}$) and elevated levels (of up to $\sim 1300 \mu\text{M}$) in the most affected regions of the WD brain [16], the concentration of Cu that is neurotoxic in vitro to monocultures is substantially lower. This is likely due to the absence of glial support cells such as astrocytes, oligodendrocytes and ependymal cells in vitro; astroglia have been reported to act as important metal depots in the brain and are part of the tight homeostatic system that stabilizes Cu levels within the neuropil, [46]. That neurons are not well equipped to handle excess Cu is supported by the finding that only neurons are susceptible to free-radical mediated apoptosis induced by $20 \mu\text{M}$ Cu in a mixed neuronal and glial E15 embryonic mouse cell culture over 24 h [37]. Astroglia are able to provide neurons with the Cu-binding amino acid, cysteine, as well as the anti-oxidant pyruvate, supporting the role of astroglia, [53] but not necessarily microglia, [37] in providing neuronal protection from metal ion toxicity. Nonetheless, neuron cell death does occur in the WD brain as a direct result of Cu toxicity, indicating that the neuroprotective capacity of glial cells has been surpassed. Thus, although there are regional differences in Cu tolerability and Cu concentration in the brain, the monoculture experimental design utilized in this study allows determination of how neurons would be predicted to respond in vivo to Cu in excess of cellular requirements.

Cu Induced Neuronal Apoptosis – in vitro

The time-dependent increase in Bax/Bak expression and decrease in Bcl-2 expression in M17 neuroblastoma cells treated with $25:50 \mu\text{M}$ $\text{Cu}:\text{glycine}_2$ is a classic Bcl-2 mediated apoptotic response [35] that correlates well with the significant decrease in viability after 24 h (Figure. 2). Our results are consistent with the prevailing theory that an alteration in the ratio of Bcl-2:Bax protein expression dictates apoptotic cell death. The protection mediated by the binding of Bcl-2 and its homologs is lost upon binding of a BH3-only protein (such as Bad; **Figure 3**) to Bcl-2, leading to homo-oligomerization of Bax and Bak, release of cytochrome c from the

mitochondria, activation of Apaf1 which in turn promotes caspase 9 activation [57].

Cu-induced neuronal death exhibiting characteristics of apoptosis has previously been reported in human NT2-N neurons [48] and murine neocortical cells and NTERA-2-N neurons [37,49]. Cu-induced apoptosis via both Bcl-2 and non-Bcl-2 associated pathways has been reported for organs other than the brain [41,56]. Importantly, our results indicate that Cu-induced apoptosis does not appear to require de novo synthesis or degradation of mRNA, and that the increased translation of proteins from pre-existing mRNA is sufficient to induce programmed cell death of neurons following Cu insult (**Figure 2; Table 2**). Our study suggests that caution must be made in extrapolating gene expression results to functional protein changes, at least for apoptotic genes involved in Cu-induced apoptosis [32,33,50].

The toxic effects of Cu on hepatocytes and neurons may be attributed to its role in promoting reactive oxygen species generation via Haber-Weiss chemistry and the subsequent oxidation of biomolecules such as lipids, proteins, and nucleic acids [4,36]. In vivo studies have shown that markers of lipid peroxidation are increased in mitochondria from livers of WD patients [39]. There also is evidence of lipid peroxidation in livers of rats chronically fed very high Cu diets [29,38]. The suspected role of ROS in Cu overload is supported by observations of increased generation of lipid peroxidation products, as well as evidence of DNA damage in hepatocytes reacted with ionic Cu [8,47]. Moreover, BU17 human glioma cells and SH-SY5Y human neuroblastoma cells exposed to media supplemented with Cu (unchelated) in the range $0\text{-}250 \mu\text{M}$ for periods up to 48 h displayed significant increases in radical generation ($p < 0.001$) [54]. However, the induction of oxidative stress may not be required in order to induce toxicity. Chelated forms of Cu (such as $\text{Cu}:\text{glycine}_2$) have been shown to prevent oxidative stress and lipid peroxidation, but not death of F14 cells (N18TG2 mouse neuroblastoma cell fused with a cerebellar cell), and that antioxidants applied to cells in the presence of glycine chelated Cu also did not inhibit Cu's toxic effects [22]. Likewise, treatment of mouse fibroblasts containing mutations of ATP7a (C57BL/6-Atp7aMobr and C57BL/6-

Atp7aModap, which prevent Cu export from the cell) with Cu-His do not display increased gene expression for key antioxidant enzymes despite the accumulation of abnormally high levels of Cu [3]. These results clearly indicate that Cu induction of death can be independent of oxidative stress and is dependent upon the form of chelation. Since cellular Cu is almost invariably bound to ligands [34], the use of Cu-Gly chelates in our experiments provides a physiological means of assessing the mechanism of Cu toxicity independently of oxidative damage.

How then might Cu induce apoptotic cell death? One explanation relates to the antagonistic effects of Cu on the binding of other metal ions to key proteins (e.g. [5,17]). Cu may saturate metal storage proteins such as metallothioneins, displacing other metals that may be more toxic for cells. For example, Predki and Sarkar, [31] showed that Cu can be substituted for Zn in Zn-finger proteins, and that this ablates their function. Another possibility is that Cu alters energy metabolism, particularly glycolysis [25]. Such substitutions in essential metalloproteins would explain the non-oxidative toxicity of Cu. Glycine or other chelators may facilitate these processes since neuron Cu uptake is increased when Cu is chelated [23] and glycine increases Cu toxicity without any apparent oxidative stress or lipid peroxidation [22]. High levels of Cu in cells also could lead to precipitation of cupric salt crystals (as occurs in Wilson's disease) which could damage cell organelles [24].

Cu Induced Apoptosis – in vivo

Apoptotic markers were observed in the liver of aged male tx^l mice (**Figure 3A**), consistent with changes in liver pathology (hepatic nodular transformation) previously observed in aged mice with a homozygous null mutation in the ATP7b gene [10]. The direction in the expression of these apoptotic markers (\downarrow Bcl-2, \uparrow Bax, Bak) in the liver was very similar to that observed for Cu-treated neuroblastoma cells, and indicates that hepatocytes in the tx^l mice are undergoing increased apoptosis (**Table 3**). A previous study has shown CD95-mediated apoptosis in vitro in a human hepatoma cell line (HepG2; [41]), as well as in the liver samples of 4 cases of acute WD associated fulminant hepatic failure (FHF). Together, these results indicate that Cu overload induces hepatic apoptosis.

In contrast to the liver, although an increase in brain Cu has been well characterized in the tx^l and ATP7b null mice as they age [10,13,45], similar apoptotic changes were not observed in the brains of tx^l mice (**Figure 3B**). Therefore, while the tx^l mouse may be a good model of WD liver pathology and biochemical changes, the model may not be as useful for examination of the effects of Cu accumulation in the brain.

Implications for WD

The increase in liver and brain Cu levels in Cu storage diseases occurs over a period of many years, and phenotypic consequences are not reported until Cu levels are extremely elevated. The lack of appreciable pathological changes from ever-increasing Cu levels until late in the disease process suggests that compensatory homeostatic mechanisms must exist. However, our results indicate that at some threshold level of Cu, apoptosis of hepatocytes and neurons leads to pathological and functional changes in the liver and brain. Maintenance of tissue Cu levels below that which induce apoptosis using lipid soluble chelators and Zn acetate in conjunction with inhibitors of apoptosis [58] are therapeutic strategies that can protect hepatocytes.

Acknowledgements

This work is supported by funds from the National Institute of Health (RO1 AG19356) and Alzheimer's Association to CSA and The McCusker Foundation for Alzheimer's Disease Research to RNM. Microarray analyses were performed at the Cancer Center Gene Expression Core Facility at CWRU.

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