Cofactors of Mitochondrial Enzymes Attenuate Copper-Induced Death In Vitro and In Vivo

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Copper toxicity contributes to neuronal death in Wilson's disease and has been speculatively linked to the pathogenesis of Alzheimer's and prion diseases. We examined copper-induced neuronal death with the goal of developing neuroprotective strategies. Copper catalyzed an increase in hydroxyl radical generation in solution, and the addition of 20μM copper for 22 hours to murine neocortical cell cultures induced a decrease in ATP levels and neuronal death without glial death. This selective neuronal death was associated with activation of caspase-3 and was reduced by free radical scavengers and Z-Val-Ala-Asp fluoromethylketone, consistent with free radical–mediated injury leading to apoptosis. Pyruvate dehydrogenase is especially vulnerable to inhibition by oxygen free radicals, and the upstream metabolites, pyruvate, phosphoenolpyruvate, and 2-phosphoglycerate were elevated in cortical cells after toxic exposure to copper. One approach to protecting pyruvate dehydrogenase from oxidative attack might be to enhance binding to cofactors. Addition of thiamine, dihydrolipoic acid, or pyruvate reduced copper-induced neuronal death. To test efficacy in vivo, we added 1% thiamine to the drinking water of Long Evans Cinnamon rats, an animal model of Wilson's disease. This thiamine therapy markedly extended life span from 6.0 ± 1.6 months to greater than 16 months.

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Copper is an essential trace element, required for the proper function of several enzymes including cytochrome *c* oxidase, copper-zinc superoxide dismutase, and ceruloplasmin. If unbound, its high-redox properties can lead to oxidative attacks,[1] in particular, facilitating the formation of hydroxyl free radicals via the Fenton reaction and redox cycling.[2,3] Disturbances in copper homeostasis and resultant cellular toxicity induce liver and central nervous system damage in Wilson's disease, a rare autosomal recessive disorder (affecting approximately 1 in 50,000 people) associated with loss of function mutations in a P-type copper ATPase, *ATP7B*, predominantly expressed in liver and responsible for moving copper into Golgi for incorporation into ceruloplasmin or bile excretion. On liver failure, copper overflows into other tissues, damaging, in particular, basal ganglia, as well as cerebral cortex, white matter, and thalamus.[4] Copper toxicity also may contribute to the pathogenesis of other neurodegenerative disorders,[5] including a familial form of amyotrophic lateral sclerosis associated with mutations in the superoxide dismutase-1 (*SOD-1*) gene.[6–8] In addition, copper also may participate in the pathogenesis of prion disorders,[9–11] and β-amyloid toxicity in Alzheimer's disease.[12,13]

The primary treatment for Wilson's disease is oral chelation therapy with D-penicillamine, which reduces intestinal copper absorption and tissue copper levels. However, this treatment is slow (improvement can take weeks to months) and can worsen symptoms in 20 to 30% of patients because of copper redistribution to the brain, even causing death in rare cases. Furthermore, allergic reactions preclude its use in 20% of patients, necessitating the use of other less desirable chelators, or the less effective absorption inhibitor, $ZnSO_4$ (reviewed in Cuthbert[14]). Development of alternative therapeutic strategies hence is desirable. One logical approach would be to target copper-induced free radicals with scavenger drugs. Hepatocytes isolated from copper-overloaded rats showed increased lipid peroxidation and decreased survival, and both changes were blocked by administration of vitamin E.[15] Alternatively, one might try to identify specific biochemical

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pathways preferentially damaged by copper/free radical toxicity.

Copper overload induces increased free radical generation, lipid peroxidation marked by formation of 4-hydroxy nonenal (HNE), and mitochondrial dysfunction in the livers of people affected by Wilson's disease.[16–18] Similar effects are seen in Long Evans Cinnamon (LEC) rats, which have a spontaneous autosomal recessive mutation in *atp7b* and develop hepatic failure caused by copper overload.[19,20] Copper chelation with D-penicillamine reverses the morphological changes seen in Wilson's disease mitochondria.[21,22] HNE inhibits pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGDH), perhaps by covalently modifying the lipoic acid moiety of these enzymes.[23,24] PDH also is preferentially sensitive to other insults that induce oxygen free radicals such as ischemia[25,26] or exposure to superoxide radicals or hydrogen peroxide.[27]

The purpose of this study was to test the hypothesis that neurons exposed to excess extracellular copper would develop impairment of energy metabolism associated with inhibition of PDH, and, if so, whether the administration of the PDH (and KGDH) cofactors, thiamine or dihydrolipoic acid,[28] might be neuroprotective. Sheline and colleagues discussed this in an abstract.[29]

Materials and Methods

Cell Culture and Toxicity Studies

Mixed neuronal and glial cultures were prepared from E15 embryonic mouse cortices as previously described.[30] Briefly, dissociated cortical neurons from E15 Swiss-Webster mice were plated in Eagles minimal essential medium (Earle's salts, glutamine-free) containing 21mM glucose, 5% fetal bovine serum, and 5% horse serum at three to four hemispheres per plate onto glial cultures. Chronic toxicity studies were initiated by washing cultures four times with Eagles minimal essential medium containing 21mM glucose, followed by exposure to copper chloride in the same media (in the absence of serum). Compounds were tested against 20μM copper chloride, as was addition delayed by 1–8 hours. Cell death was estimated by phase-contrast microscopy and by measuring lactate dehydrogenase (LDH) efflux[31] and comparing it to the 100% neuronal death induced by treatment with 300μM *N*-methyl-D-aspartate (NMDA) for 24 hours.

Determination of Dihydroxyacetone Phosphate, 2-Phosphoglycerate, Nicotinamide Adenine Dinucleotide, and ATP Levels

Mixed neuronal cultures (13-14 days in vitro) were used for dihydroxyacetone phosphate (DHAP), nicotinamide adenine dinucleotide (NAD^+), and ATP measurements. For ATP measurements, cultures were lysed by addition of 0.1M NaOH per 1mM EDTA, the supernatant was neutralized, and protein was precipitated by addition of perchloric acid. ATP was measured by the luciferin/luciferase luminescence assay.[32] For DHAP and pyruvate measurements, cultures were lysed by addition of 6% perchloric acid after exposure to 20μM copper for 6 to 8 hours or to 100nM staurosporine for 6 to 8 hours (conditions determined to be sublethal), and the supernatant was neutralized and protein was precipitated by addition of potassium carbonate. DHAP was measured by its enzymatic conversion to glycerol-3-phosphate, and the concomitant oxidation of hydrogenated NAD (NADH) was measured spectrophotometrically. Glyceraldehyde-3-phosphate and fructose bisphosphate subsequently were measured in linked enzymatic reactions.[33] 2-Phosphoglycerate, phosphoenolpyruvate, and pyruvate were measured by linked enzymatic reactions ending with the conversion of pyruvate to lactate and the concomitant oxidation of NADH. Citrate, glucose-6-phosphate, fructose-6-phosphate, and isocitrate were measured by linked enzymatic reactions ending with the oxidation of NADH and dinucleotide phosphate NAD (NADPH), respectively. For the NAD^+ measurements, cultures were lysed by addition of 75% ethanol/0.05M K2HPO4 after a 6 to 8 hour 20μM copper exposure, protein was precipitated, and the supernatant was assayed for NAD^+ levels by its enzymatic conversion to NADH, resulting in an increase in fluorescence between 400 and 600nm after an excitation at 340nm.[34]

Microscopy, Histology, and Immunohistochemistry

Mixed neuronal cultures were treated with 20μM copper for 20 hours as indicated and stained with 0.01% Trypan for 60 minutes at 37°C, and phase-contrast and bright field photomicrographs were taken. For the active caspase-3 staining, mixed neuronal cultures were treated with 20μM copper for 6 hours, washed, fixed, permeabilized, blocked, and incubated in 1:5,000 rabbit antiactivated caspase-3 (CM1; Idun, San Diego, CA). Fluorescence photomicrographs were taken after secondary anti-rabbit Alexa 488 (Molecular Probes, Eugene, OR) development and visualization using excitation at 485nm and emission at 530nm.

Long Evans Cinnamon Rat Thiamine Trial

LEC rats (Charles River, Hino, Japan) were inbred to build up the colony; at 5 to 8 weeks, the animals were split into two groups for a blinded placebo-controlled trial, one group receiving water, and the other receiving 1% thiamine in the water for the remainder of the experiment. Both groups drank normally in each case corresponding to approximately 75ml/kg/day. At the time of death, animals were anesthetized with halothane and decapitated, and their livers were removed, dried for copper content, or postfixed in 10% formalin for more than 36 hours before paraffin embedding, sectioning at 7μm, and hematoxylin and eosin staining. Hepatocyte counts were performed on random fields of multiple sections in a blinded manner. Animals were handled in accordance with a protocol approved by our institutional animal care committee. All efforts were made to minimize animal suffering and the number of animals used.

Reagents

Unless otherwise stated, all reagents were from Sigma (St. Louis, MO); Trypan blue was from Gibco (Gaithersburg, MD).

Results

Copper-induced Neuronal Death Had Features Characteristic of Apoptosis

Extracellular addition of 10 to 100μM copper chloride (in Eagles minimal essential medium + 21mM glucose) to mixed cell cultures resulted in progressive neuronal cell body shrinkage and death over the next 10 to 24 hours, associated with Trypan blue staining, and the release of LDH to the bathing medium (Figs 1 and 2B). Glial cells were unaffected by these levels of copper. Copper-induced neuronal death was blocked by concurrent addition of the chelator, histidine (1mM; Table 1).

Immunohistochemistry using an antibody against the active form of caspase-3 showed a large increase in the number of neuronal cell bodies staining before neuronal degeneration and LDH release (Fig 3). Copper-induced neuronal death was inhibited approximately 50% by protein synthesis inhibition (1μg/ml cycloheximide) and caspase inhibition (100μM Z-Val-Ala-Asp-fluoromethylketone; see Table 1).

Copper Increased Hydroxyl Radical Formation, and Free Radical Scavengers Attenuated Copper-induced Death

To verify that toxic copper exposure induced free radical formation, we obtained solution electron paramagnetic resonance spectra by using 5′,5′-dimethyl-pyrroline-*N*-oxide (DMPO) in the presence of 60μM H_2O_2 (a concentration consistent with levels in normal brain tissue[35]). Addition of 25μM $CuCl_2$ increased four peaks separated by 14.9 gauss, indicative of an increase in hydroxyl radicals[36] (Fig 4A). Addition of vitamin E or its analog, Trolox, attenuated copper-induced neuronal death (see Fig 4B).

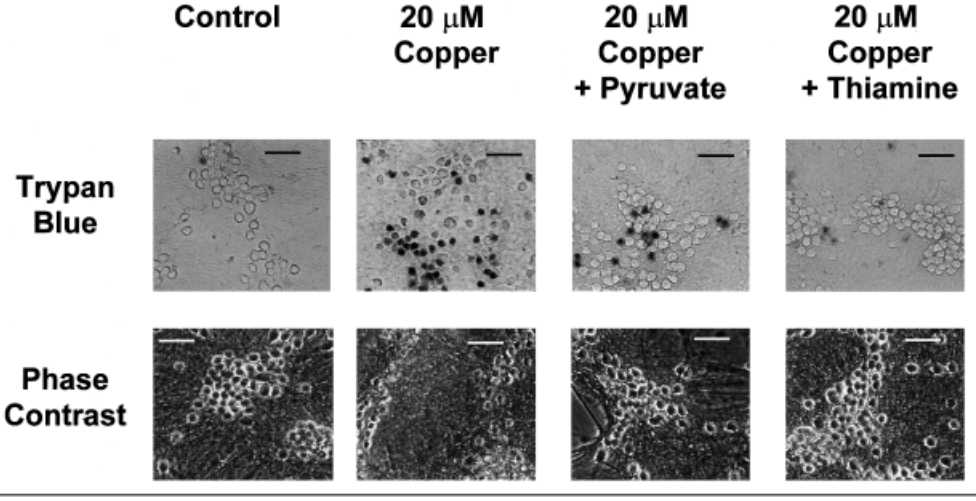
Copper-induced Neurochemical Changes

Exposure to 20μM copper produced a progressive decrease in culture ATP levels that reached significance after 8 hours, whereas the increase in cell death reached significance after 12 hours (Fig 5A and B). Copper specifically induced increases in pyruvate, phosphoenolpyruvate, and 2-P-glycerate, without affecting glucose-6-phosphate or fructose-6-phosphate, consistent with an inhibitory effect at the PDH complex (Table 2). Copper caused a trend toward increased levels of citrate and isocitrate (data not shown) consistent with a lesser inhibitory effect at the KGDH complex. Levels of DHAP and fructose bisphosphate were mildly elevated when assayed 6 hours after copper addition, whereas no significant change occurred after exposure to 100nM staurosporine (see Table 2). Glyceraldehyde-3-phosphate is present at low levels and converted to DHAP preferentially, making it difficult to measure.[28]

Pharmacology of Copper-induced Neuronal Death

Concurrent addition of 4mM thiamine reduced the copper-induced decrease in ATP levels, as well as neuronal degeneration and LDH release (see Table 1, Figs 1 and 5). Both thiamine and dihydrolipoic acid remained neuroprotective when added up to 4 hours after initiating copper exposure (Fig 6). The cell-

Fig 1. Light microscopy of thiamine and pyruvate protection against copper-induced death. Phase-contrast and brightfield Trypan blue fields from mixed neuronal cultures treated 22 hours with 20μM copper in the presence or absence of the indicated drug (4mM each). Bars = 50 μm.



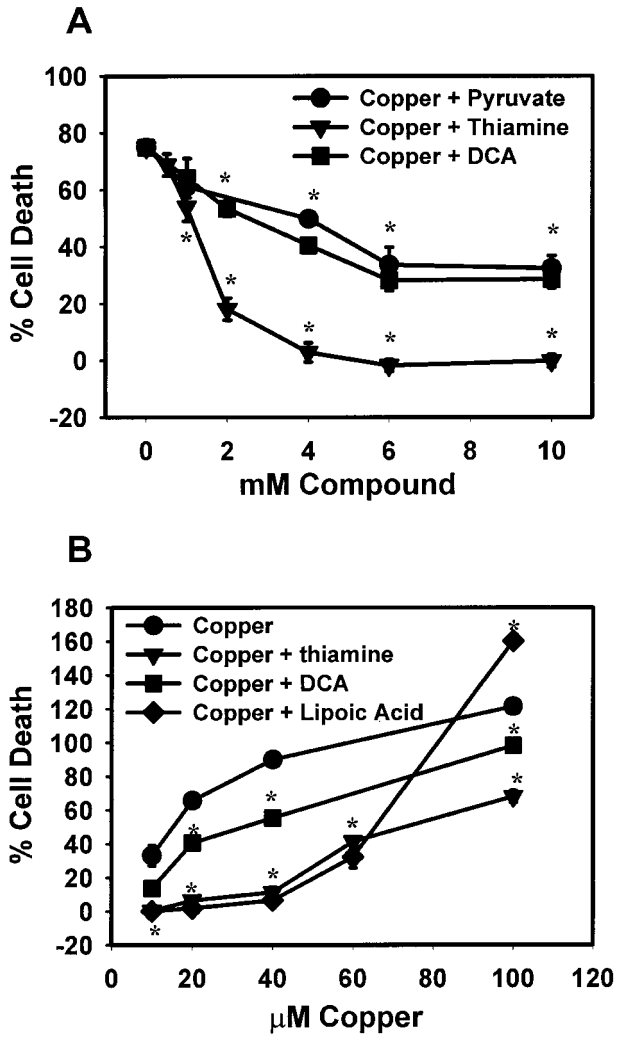


Fig 2. Thiamine, pyruvate, dichloroacetate (DCA), and dihydrolipoic acid reduce copper-induced neuronal death. (A) Mixed neuronal cortical cultures were exposed to 20μM copper for 22 hours in the presence of the indicated concentrations of thiamine, DCA, or pyruvate. (B) Cultures were exposed to the indicated concentrations of copper for 22 hours in the presence or absence of 4mM thiamine, 4mM DCA, or 50μM dihydrolipoic acid. Cell death was determined by lactate dehydrogenase release (three experiments, mean ± SEM, n = 9–12 cultures/condition), expressed relative to the near-maximal neuronal death determined in sister cultures treated for 22 hours with 300μM NMDA (=100). (asterisks) *Difference from copper only at* $p < 0.05$ *by one-way analysis of variance followed by a Bonferroni test.*

impermeant thiamine analog, thiamine pyrophosphate (TPP), was ineffective at attenuating copper neurotoxicity, and the thiamine transport antagonist, pyrithiamine[37] potentiated copper neurotoxicity (see Table 1).

Analogs of dihydrolipoic acid also reduced copper-induced neuronal death (50μM lipoamide or 250μM oxidized α-lipoic acid). Copper-induced neuronal death also was inhibited by 4mM dichloroacetate (DCA), which activates PDH by blocking an inhibitory kinase,[38] but does not serve as an energy substrate in neurons[39] (see Table 1).

Other energy substrates of cortical neurons (α-keto glutarate, β-hydroxybutyrate, or acetylcarnitine[39]), were not neuroprotective against Cu^{2+} toxicity. However, both pyruvate and α-keto butyrate, which can promote conversion of hydrogen peroxide to oxygen and water,[40] were partially neuroprotective (see Table 1; Fig 2).

Thiamine Extends Life Span of Long Evans Cinnamon Rats

The efficacy of thiamine treatment against copper toxicity in vivo was tested using the LEC rat model of Wilson's disease. A group of eight animals were supplied with 1% thiamine in their drinking water (ad lib) from age 5 to 8 weeks on. Water ingestion did not differ from that of seven controls receiving tap water (75ml/kg/day = approximately 750mg thiamine/kg/day). The animals receiving water alone died on average at 6.0 ± 1.6 months (n = 7), whereas those receiving thiamine lived for at least 15 months (at which time some were killed for liver histology, which was different from those receiving water alone at p =

Table 1. Effect of PDH Cofactors, Free Radical Scavengers, Alternate Energy Substrates, and Antiapoptotic Agents on Copper-Induced Neuronal Death

Exposure	% Cell Death
10μM copper	33.1 ± 0.6
10μM copper + pyrithiamine	59.0 ± 1.2[a]
20μM copper	82.4 ± 3.5
20μM copper + 4mM thiamine	1.9 ± 0.9[a]
20μM copper + 4mM TPP	84.6 ± 3.4
20μM copper + 4mM pyruvate	46.8 ± 2.6[a]
20μM copper + 4mM DCA	38.6 ± 6.5[a]
20μM copper + 50μM dihydrolipoic acid	2.3 ± 1.1[a]
20μM copper + 50μM lipoamide	3.3 ± 1.6[a]
20μM copper + 250μM lipoic acid (oxidized)	4.1 ± 0.6[a]
20μM copper + 4mM acetylcarnitine	87.1 ± 3.6
20μM copper + 4mM α-ketoglutarate	73.4 ± 4.2
20μM copper + 4mM α-ketobutyrate	35.6 ± 3.9[a]
20μM copper + 4mM histidine	2.2 ± 0.8[a]
20μM copper + 1μg/ml CHX	43.6 ± 3.8[a]
20μM copper + 100μM ZVAD	42.5 ± 5.2[a]

Cultures were exposed to 20μM copper in the presence of the indicated compounds for 22 hours, and neuronal death was assessed by LDH efflux (three experiments; n = 9–12).

[a]Treated cultures differ significantly from appropriate untreated control at $p < 0.05$.

CHX = cydoheximide; ZVAD = Z-Val-Ala-Asp-fluoromethylketone; TPP = thiamine pyrophosphate; DCA = dichloroacetate.

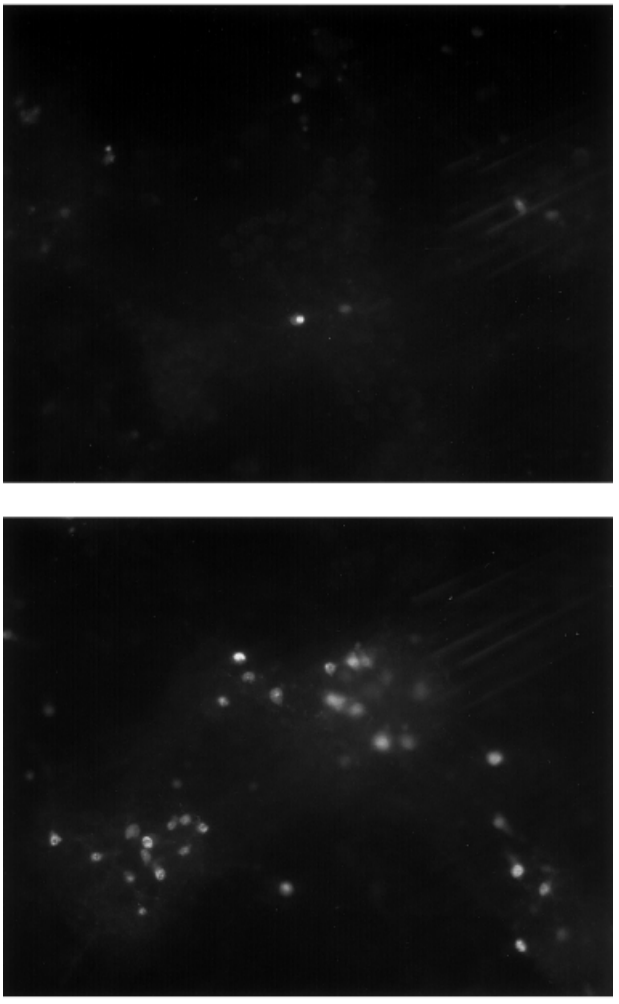


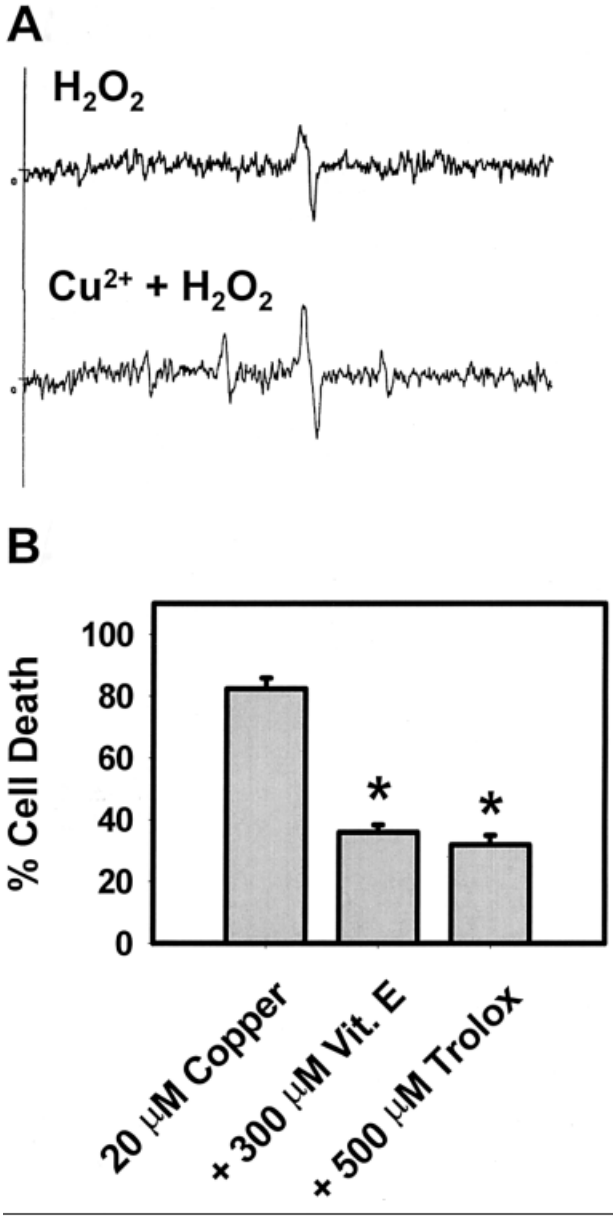
Fig 3. Copper-induced neuronal caspase-3 activation. Mixed cortical neuronal cultures were exposed to sham wash (top) or to 20μM copper (bottom) for 6 hours; then, cultures were fixed and stained with CM1 antibody against the activated form of caspase-3.

0.00031, one-way analysis of variance (ANOVA) followed by a Bonferroni test).

The livers of two untreated and two treated animals were examined, the latter killed at 15 months, and the former euthanized in a moribund state at 5 months showing widespread nuclear swelling, vacuolization, and degeneration of hepatocytes (Fig 7).[41–43] Liver sections from the thiamine-treated animals were also grossly abnormal but showed less cellular vacuolization and degeneration (see Fig 7). Untreated LEC rat liver had 2,722 ± 355 hepatocytes/mm^2, whereas thiamine-treated LEC rat liver had 5,266 ± 485 hepatocytes/mm^2 (different from water alone at $p = 0.00174$, one-way ANOVA followed by a Bonferroni test).

Copper content of the liver was determined to be 772 ± 271μg/gm dry weight in thiamine-treated animals. This value is within the published range for copper content of the liver in untreated LEC rats, from 325 to 2,126μg/gm liver dry weight (mean, 930).[44]

*Fig 4. Copper enhances hydrogen peroxide–induced hydroxyl radical formation. (A) Electron paramagnetic resonance spectra using 5′,5′-dimethylpryrroline-*N*-oxide as a spin-trapping agent. (top spectra) Media stock 22 hours after addition of 60μM H_2O_2; (bottom spectra) same but with 25μM copper also added. (B) Mixed neuronal cultures were exposed to 20μM copper for 22 hours in the presence of the indicated compounds. Cell death was determined by lactate dehydrogenase release (mean ± SEM; n = 9–12 from three experiments), expressed relative to cultures treated for 22 hours with 300μM* N*-methyl-D-aspartate (=100).* (asterisks) *Difference from copper alone at* $p < 0.05$ *by one-way analysis of variance followed by a Bonferroni test.*



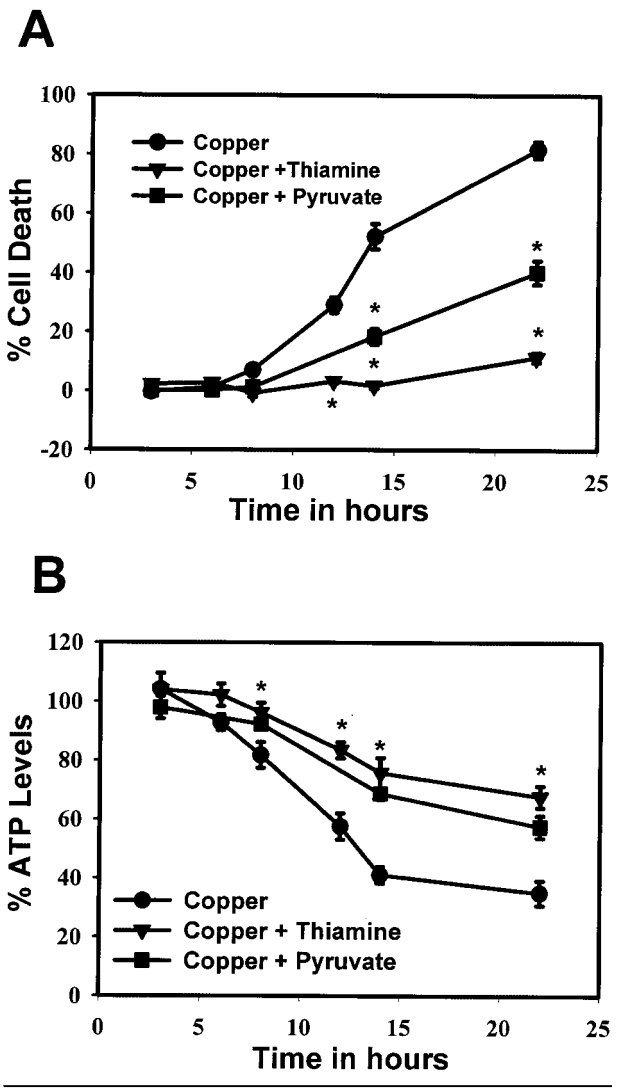


Fig 5. Progressive copper-induced neuronal death and ATP loss were attenuated by thiamine or pyruvate. Cultures were exposed to 20μM copper in the presence or absence of 4mM thiamine or pyruvate. (A) Cell death was determined by lactate dehydrogenase (mean ± SEM; n = 9–12 cultures per condition). (B) The percentage of ATP lost is plotted as determined from sham wash controls from three experiments (n = 9–12). (asterisks) *Treated cultures differ significantly from copper treatment only at* $p < 0.05$.

Table 2. Pyruvate, PEP, and 2-Phosphoglycerate Levels Increase Moderately after 6 Hours in the Presence of 20μM Copper

Metabolite measured	Sham Wash (nmol/plate)	Copper (nmol/plate)	Staurosporine (nmol/plate)
Glucose-6-phosphate	3.1 ± 0.3	3.2 ± 0.3	NP
Fructose-6-phosphate	2.0 ± 0.2	2.5 ± 0.2	NP
FBP	9.4 ± 1.8	17.8 ± 2.6[a]	13.0 ± 2.9
DHAP	7.0 ± 1.4	15.6 ± 2.2[a]	10.0 ± 4.0
Pyruvate	22.6 ± 1.4	32.2 ± 1.2[a]	NP
PEP	7.8 ± 1.4	15.4 ± 1.0[a]	NP
2-P-glycerate	10.6 ± 0.6	14.6 ± 0.4[a]	NP
NAD^+	8.2 ± 0.1	8.0 ± 0.6	NP

Mixed cortical neuronal cultures were treated with 100nM staurosporine for 4 hours, or with 20μM copper for 6 hours, after which the cells were harvested and assayed for levels of the listed glycolytic intermediates or nicotinamide adenine dinucleotide (oxidized) (results are from three separate experiments, n = 6–9).

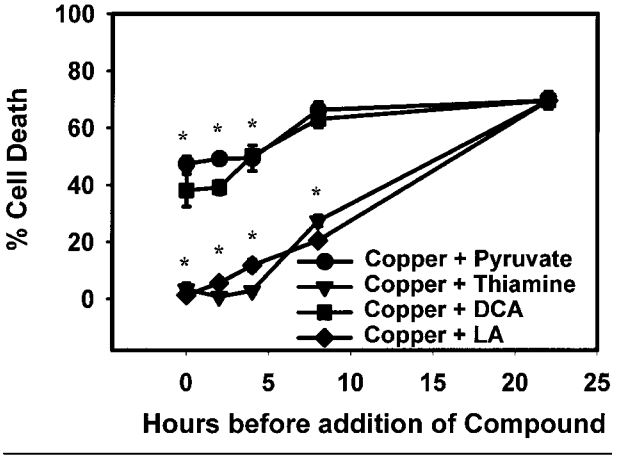
[a]Difference from sham wash controls at $p < 0.05$ by one-way ANOVA followed by a Bonferroni test.

NP = not performed; PEP = phosphoenolpyruvate; FBP = fructose biphosphate; DHAP = dihydroxyacetone phosphate; NAD = nicotinamide adenine dinucleotide.

Discussion

Exposure to low concentrations of copper was selectively toxic to neocortical neurons, inducing neuronal apoptosis at exposure levels that did not kill astrocytes. Expectedly, this neurotoxicity was accompanied by the production of hydroxyl radicals and attenuated by free radical scavengers. Furthermore, we show here (1) that copper neurotoxicity was associated with a reduction in ATP levels and a buildup of pyruvate, phosphoenolpyruvate, and 2-P-glycerate, the substrates upstream of PDH, an enzyme known to be sensitive to free radical attack (see above); and (2) that the PDH cofactors thiamine or dihydrolipoic acid, or the PDH activator dichloroacetate, reduced copper neurotoxicity in vitro. Finally, we demonstrate that oral thiamine prolonged the life span and slowed the pathogenesis of liver damage in the LEC rat model of Wilson's disease, despite

Fig 6. Neuroprotection induced by delayed addition of thiamine, pyruvate, dichloroacetate (DCA), or dihydrolipoic acid. Mixed neuronal cultures were exposed to 20μM copper at time 0; at the indicated later times, thiamine, pyruvate, or DCA were added (4mM each). Cell death was determined by lactate dehydrogenase release from three experiments (n = 9–12). (asterisks) *Difference from copper alone at* $p < 0.05$ *by one-way analysis of variance followed by a Bonferroni test. LA = dihydrolipoic acid.*



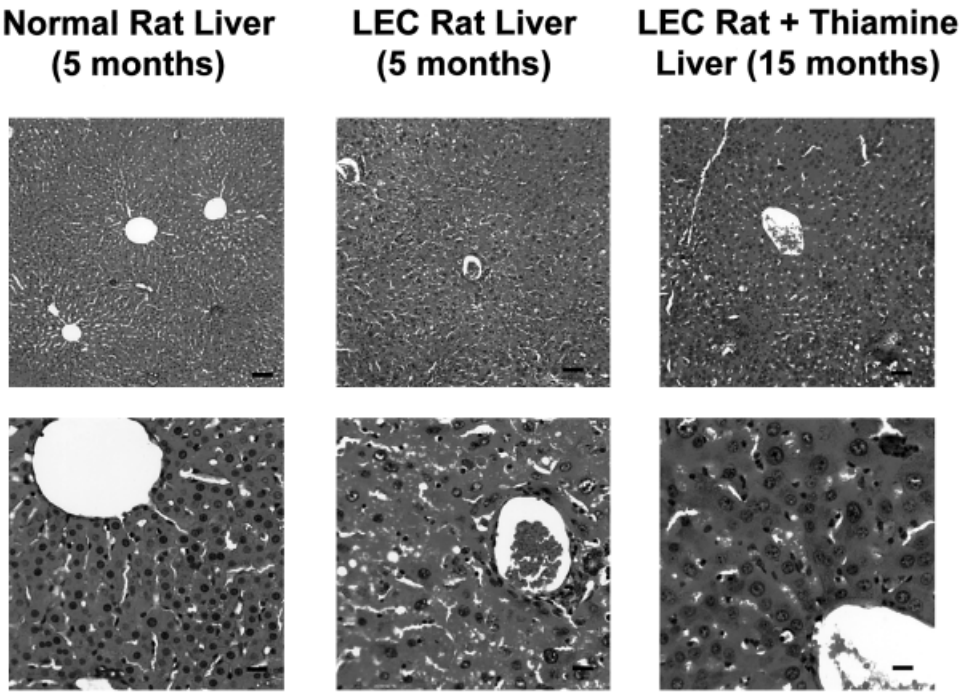


Fig 7. Thiamine treatment attenuated liver degeneration in Long Evans Cinnamon (LEC) rats. Representative hematoxylin and eosin–stained liver sections from a normal rat (aged 5 months, left column), LEC rat (aged 5 months, middle column), and LEC rat treated with oral thiamine supplements (age 15 months, right column). Photomicrographs were taken at ×100 (top panels; bars = 40μm) and at ×400 (bottom panels; bars = 10μm).

persistent hepatic copper accumulation, consistent with a primary cytoprotective effect.

Previous studies have examined copper toxicity, including in immature (3–6 days in vitro) cultures of cortical, hippocampal, glial and cerebellar granule neurons, and the concentration of copper chloride determined to kill 50% of cells was approximately 200, 200, 500, and 150μM, respectively.[45] The neurotoxic levels required were substantially higher than those reported here, perhaps because of the difference in developmental age. Copper (1μM) recently was reported to potentiate cysteine toxicity (100μM) through hydrogen peroxide generation, and pyruvate attenuated the hydrogen peroxide generation and toxicity.[46] In our studies, copper was demonstrated to be a potent neurotoxin, with a neuronal LC_{50} lower than that for zinc, or iron.[45,47]

We have shown previously that zinc neurotoxicity is accompanied by a sharp decrease in NAD^+ levels, likely then inhibiting glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[39] Copper exposure also was accompanied by a slight increase in metabolites upstream of GAPDH (DHAP, fructose bisphosphate), but this increase was much smaller than after toxic zinc exposure, and NAD^+ levels were unaffected.

Taken together, the ability of thiamine, pyruvate, DCA, and dihydrolipoic acid to attenuate copper-induced neurotoxicity is most consistent with action on the PDH complex, a hypothesis also supported as noted above by copper-induced increases in upstream glycolytic intermediates. Direct measurements of PDH activity after Cu^{2+} exposure are ongoing and will be reported subsequently. Thiamine, through its biologically active form, TPP, enhances PDH activity,[48] perhaps by sterically limiting oxidation of critical histidine residues.[49] TPP also inhibits PDH kinase,[50] which phosphorylates and inhibits PDH. Thiamine can show some benefits in treating several disorders associated with deficiencies in PDH, such as mitochondrial encephalomyopathy, West syndrome, lactic acidemia, and some forms of Leigh syndrome.[51–54] Pyruvate and dichloroacetate also can produce some benefits in these disorders through similar mechanisms.[51,55] Dihydrolipoic acid attenuates neuronal death induced by β-amyloid, H_2O_2, glutamate, ischemia, and iron (reviewed in Packer and colleagues[56]).

Copper can cause thiamine to break down into thiochrome and oxodihydrothiochrome with unspecified effects on the copper ion.[57] For this reason, we demonstrated that Cu^{1+} and Cu^{2+} were similarly toxic in cultured cortical neurons (Cu^{1+}, LC_{50} approximately 30μM; C.T.S. and D.W.C., unpublished observation).

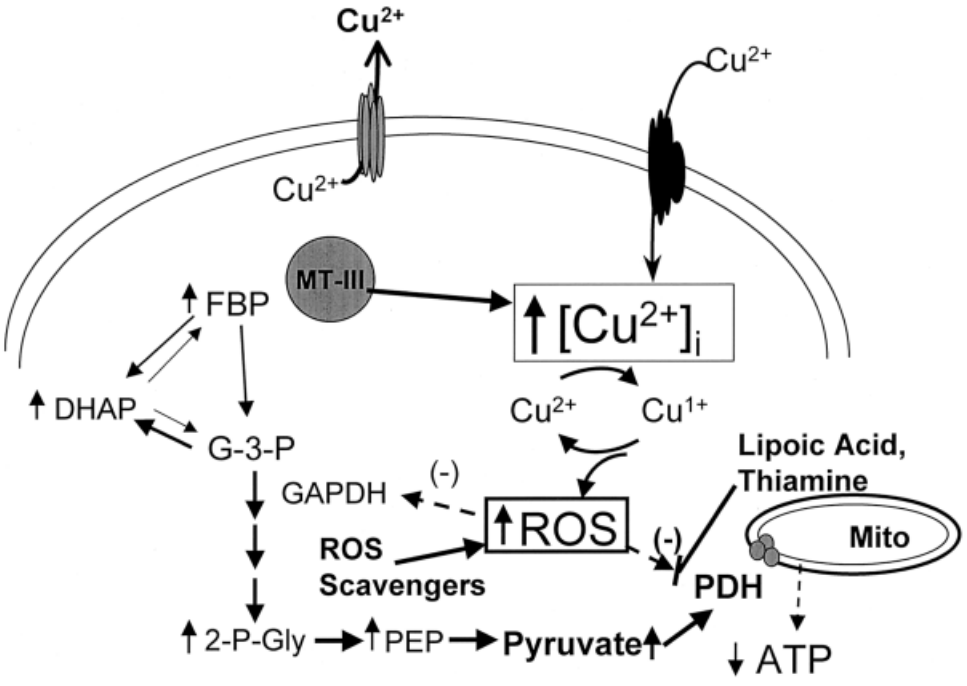


Fig 8. Working model for copper-induced toxicity. DHAP = dihydroxyacetone phosphate; FBP = fructose bisphosphate; G-3-P = glyceraldehyde-3-phosphate; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; Mito = mitochondrion; MT-III = metallothionein-III; 2-P-gly = 2-phosphoglycerate; PDH = pyruvate dehydrogenase; PEP = phosphoenolpyruvate; ROS = reactive oxygen species. Gray shading indicates processes that attenuate copper neurotoxicity. Black indicates processes that promote copper neurotoxicity.

Also, TPP, the biologically active but cell-impermeant cofactor for PDH and KGDH, had no effect on copper toxicity suggesting that thiamine acts intracellularly, and not by changing the redox potential or entry of copper. The observed potentiating effect of the thiamine transport inhibitor, pyrithiamine[37] upon copper neurotoxicity (see Table 1), suggests that baseline neuronal vulnerability to copper toxicity already is limited by endogenous thiamine stores.

α-Lipoic acid is the shuttling hinge that moves pyruvate and acetyl CoA back and forth in the active site of PDH[28] and has been shown previously to reduce reactive oxygen species inhibition of PDH (reviewed in Packer and colleagues[56]). As noted above, one of the major products of lipid peroxidation, HNE, may specifically inhibit PDH and KGDH by modifying the lipoic acid moiety of these enzymes[24]; both of these enzymes require TPP and lipoic acid as cofactors. Excess unconjugated dihydrolipoic acid could act as a sink to keep HNE from inactivating these enzymes; other enhancing or inhibitory effects on PDH activity also have been suggested.[58,59]

In summary, we propose as a working model that copper neurotoxicity is substantially mediated by hydroxyl radical inhibition of the active site of the PDH complex, perhaps through HNE adduction to the lipoic acid moiety of PDH (Fig 8). Compounds that are ligands for or activate PDH or that scavenge HNE, oxygen radicals, or hydrogen peroxide attenuate copper toxicity by ameliorating this inhibition. In particular, the marked increase in life span and reduction in liver pathology observed here in LEC rats supplemented with thiamine suggests that this compound might be a useful adjunct to chelator therapy in humans with Wilson's disease. In addition, it is conceivable that thiamine also might be of therapeutic benefit in aceruloplasminemia or other diseases speculatively linked to copper homeostasis or toxicity such as spongiform encephalopathies or Parkinson's or Alzheimer's diseases.

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