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COPPER ACTIVATION OF NF- κ B SIGNALING IN HEPG2 CELLS

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SUMMARY

Copper is a persistent environmental contaminant, and exposure to elevated levels of this transition metal can result in a variety of pathologies. To protect against metal-induced pathologies, copper affects the transcription of multiple defense and repair genes. To better understand the mechanisms by which copper affects the transcription of stress-responsive genes, HepG2 cells were treated with copper under multiple conditions and microarray analyses were previously performed. Analysis of the microarray data indicated that copper modulates multiple signal transduction pathways, including those mediated by NF- κ B. Luciferase assays, quantitative RT-PCR and chemical inhibition in HepG2 cells validated the microarray results and confirmed that NF- κ B was activated by stress-inducible concentrations of copper. In addition, two novel NF- κ B-regulated genes, SRXN1 and ZFAND2A were identified. Our results indicate that the activation of NF- κ B may be important for survival under elevated concentrations of copper.

INTRODUCTION

Copper is an essential nutrient that is critical for adequate growth, brain development, iron transport, and a variety of metabolic functions ¹. The hepatic copper concentrations in normal adults ranges from 18–45 μ g/g (dry weight) ². In humans, copper deficiency, generally resulting from poor nutrition or malabsorption, leads to hematological defects, seizures, cerebral atrophy and sepsis ³. Likewise in rodents, nutritional deficits in copper can cause birth defects (e.g., gross structural abnormalities, central nervous system defects) and cardiovascular deficits (e.g., anemia, heart enlargement, impaired angiogenesis) ⁴. Menkes disease is a rare, X-linked disorder caused by mutations in ATP7A, a P-type ATPase copper transporter that results in severe copper deficiency in affected individuals. The deficiency results from an inability of intestinal epithelial cells to export copper that is absorbed from the digestive tract ⁵.

While copper is an essential nutrient, elevated levels of copper can induce a variety of pathologies including motor function deficits and liver failure ⁶. Wilson's disease is a recessive disorder caused by mutations in ATP7B, which is also P-type ATPase copper transporter ⁷. Mutations in ATP7B prevent liver excretion of copper into bile ⁸. This eventually produces liver damage, subsequently releasing copper into the blood stream leading to copper accumulation and damage in other organs ⁶. Hepatic copper concentrations as high as 1,500

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µg/g (dry weight) have been recorded in Wilson's disease patients⁹. While Wilson's disease is rare (1 in 30,000 individuals), approximately 1% of the human population carries a mutant allele of ATP7B. Animal studies suggests that heterozygous individuals are at an increased risk for copper toxicosis¹⁰. Indian childhood cirrhosis and idiopathic copper toxicosis have been proposed to be eco-genetic diseases that result from a combination of an undetermined genetic susceptibility and exposure to elevated levels of dietary copper¹¹. Hepatic copper concentrations as high as 6,654 µg/g (dry weight) have been reported in cases of Indian Childhood Cirrhosis¹². Humans can be exposed to high levels of copper through occupational exposures and tap water that uses copper plumbing¹³. A survey conducted from 1981–1983 by the National Institute of Occupational Safety and Health found that over 500,000 U.S. workers were occupationally exposed to copper¹⁴. To maintain intracellular copper homeostasis and to defend against copper toxicity, cells can activate transcription of a variety of copper-responsive genes, including genes which encode metal chelating and repair proteins¹⁵.

A toxicogenomics approach was used to investigate the effects of multiple concentrations of copper (100 µM to 600 µM) over different exposure times (4 h to 24 h) on the transcriptome of the HepG2 human hepatocarcinoma cell line¹⁶. Exposure to low levels of copper (100 µM and 200 µM) induced physiological/adaptive transcriptional responses. In contrast, exposure to higher levels of copper (400 µM and 600 µM) induced toxicological/stress responses. Analysis of the microarray data using principal components analysis, K-means clustering and protein interaction networks suggested that copper affects NF-κB signaling at 400 and 600 µM exposures¹⁶.

NF-κB is a family of homo- and hetero-dimeric transcription factors composed of five proteins; p50, p52, RelA, RelB and c-Rel; which recognize similar DNA sequence motifs. Only RelA, RelB and c-Rel are capable of activating transcription. Under unstimulated conditions, NF-κB is sequestered in the cytoplasm by members of the IκB (Inhibitors of κB) family of proteins, the most common of which is IκBα. The IκB proteins function by binding to the NF-κB dimer and blocking its nuclear localization. In the canonical pathway of NF-κB activation, the IKK complex (Inhibitor of κB Kinase) becomes active and phosphorylates IκBα. The phosphorylated IκBα is ubiquitinated and subsequently degraded by the 26S proteasome. Removal of IκBα exposes the nuclear localization signal of NF-κB, which results in the translocation of the NF-κB dimer to the nucleus and subsequent activation of transcription¹⁷.

NF-κB regulates the transcription of a number of genes under conditions of infection, inflammation, and DNA damage¹⁸. There is contradictory information on the effect of metals on NF-κB activation. Metals have been shown to up-regulate, down-regulate, and have no effect on NF-κB activity¹⁹. The effect of copper on NF-κB activation is also unresolved. Some groups have reported activation of NF-κB by copper^{20; 21; 22}, whereas others report that copper reduces or has no effect on NF-κB activity^{23; 24; 25; 26; 27}. In the analysis of the copper-treated HepG2 cell transcriptome, the NF-κB signaling pathway was identified as one that was activated in response to copper¹⁶. In the present report, the ability of elevated concentrations of copper to increase NF-κB activity in HepG2 cells was confirmed. The activation of NF-κB is considered to be a survival mechanism. Therefore, these results suggest that NF-κB activation may be important for survival in the presence of elevated concentrations of this metal.

RESULTS

Bioinformatics identified activation of NF- κ B by copper

The NF- κ B pathway was previously identified as being activated by copper using Cytoscape jActiveModules. To further investigate the activation of NF- κ B, IPA was used to analyze the differentially expressed genes in each of the microarray conditions for functional networks. Using this approach, IPA found 23 functional networks that included NF- κ B, all of which were associated with exposure to 400 or 600 μ M copper. The functional network with the highest score contained genes that were differentially expressed after 4 h exposure to 400 μ M copper (Fig. 1).

The web-based tool oPOSSUM²⁸ was also used to search for NF- κ B binding sites in the promoter regions of copper-responsive genes. An analysis of genes that were up-regulated after a 4 or 8 hour exposure to 400 μ M copper found a significant enrichment of NF- κ B binding sites (Z-score = 7.66). These results provided additional evidence that NF- κ B is activated by copper in HepG2 cells.

Copper activated NF- κ B-mediated transcription

To confirm that copper activated NF- κ B-mediated transcription, luciferase assays using an NF- κ B reporter were performed. Hydrogen peroxide was used as a positive control for NF- κ B activity²⁹. Both copper and H₂O₂ activated NF- κ B-mediated transcription, however, the kinetics and magnitude of their activation were unique (Fig. 2). Hydrogen peroxide significantly increased NF- κ B transcription 1.3-fold after a 4 h exposure ($p < 0.04$), followed by a decrease in transcriptional activity. In contrast, significant copper-induced increases were continuous: 4 h, 1.5-fold ($p < 0.002$); 6 h, 1.6-fold ($p < 0.001$); and 8 h, 1.8-fold ($p < 0.001$). These results confirmed that copper activated NF- κ B signaling.

Inhibitor of IKK-2 decrease copper-induced NF- κ B activity

In the canonical NF- κ B pathway, the phosphorylation of I κ B α by IKK-2 is required for NF- κ B activation. To assess copper ability to activate NF- κ B through the canonical pathway, the ability of the IKK-2 inhibitor IMD-3054 to inhibit copper-induced activation of NF- κ B-mediated transcription was determined. IMD-3054 decreased NF- κ B-mediated luciferase activity in both copper-treated and untreated cells (Fig. 3). In cells treated with copper and IMD-3054, there was an 11.2 fold decrease ($p < 0.001$) in luciferase activity relative to cells treated with copper alone. This indicated that copper activated NF- κ B through a pathway dependent on activation of the IKK-2 subunit. In cells treated only with IMD-3054, there was a 2.3-fold decrease ($p < 0.05$) in luciferase activity relative to untreated cells, suggesting that NF- κ B was constitutively active in HepG2 cells.

IMD-3054 reduced mRNA levels of copper-induced NF- κ B-regulated genes

IMD-3054 effectively blocked the copper-induced increase in NF- κ B transcriptional activity. Therefore the effect of IMD-3054 treatment on the steady state mRNA levels of four NF- κ B-regulated copper-inducible genes was determined. Heme oxygenase 1 (HMOX1), γ -glutamylcysteine synthetase (GCLC), immediate early response 3 (IER3), and interleukin 8 (IL8) are NF- κ B target genes that were all found to be significantly up-regulated by 400 μ M copper in the microarray study in at least 3 out of the 4 time points examined¹⁶. Steady-state mRNA levels of these genes were measured following 3 h and 6 h exposures to 400 μ M copper alone, IMD-3054 alone, copper plus IMD-3054, or untreated control. Exposure to 400 μ M for 3 or 6 h caused 3- to 49-fold increases in the steady-state levels of the four target genes (Table 2). The steady-state levels of HMOX1, GCLC, IER3 and IL8 mRNAs in cells treated with copper plus IMD-3054 significantly decreased from 2- to 13-fold ($p < 0.001$) following both

3 and 6 h exposures, relative to cells treated with copper alone (Table 2). These results provided further evidence that copper activated NF- κ B through the canonical pathway.

RelA siRNA lowered mRNA levels of copper-responsive, NF- κ B-target genes

Further support of the ability of NF- κ B to mediate copper-inducible transcription was obtained by knocking down RelA expression with siRNA and then measuring the steady-state levels of mRNAs of copper-responsive NF- κ B target genes. Following siRNA transfection, RelA mRNA levels were reduced to 12% of that measured in the non-sense siRNA transfected non-copper treated cells and to 7% of the level measured in non-sense siRNA transfected copper treated cells ($p < 0.001$) (Table 3). In copper treated siRNA transfected cells, mRNA levels of HMOX1, GCLC, IER3 and IL8 mRNAs significantly decreased 2- to 6-fold ($p < 0.001$), relative to cells treated with copper and non-sense siRNA (Table 3). There was also a significant decrease in mRNA levels of all four genes in cells transfected with RelA siRNA alone, relative to cells transfected with non-sense siRNA ($p < 0.001$) (Table 3). These data further support the role of NF- κ B in regulating copper-inducible transcription, and that NF- κ B is constitutively active in HepG2 cells.

Novel copper-responsive NF- κ B-target genes

Putative novel NF- κ B target genes were identified by analyzing the genes up-regulated after exposure to 400 μ M copper for 4 or 8 h with the web-based tool PAINT (Table 4). SRXN1 (sulfiredoxin 1 homolog) and ZFAND2A (zinc-finger, AN1-type domain 2A) were both up-regulated by copper (Table 4). To assess the role of NF- κ B on regulation of these two genes, the effects of copper exposure and NF- κ B inhibition, using IMD-3054 and RelA siRNA, on cognate mRNA levels were determined. Treatment with copper caused a significant increase in SRXN1 and ZFAND2A mRNA levels (Table 2 and Table 3). Co-exposure with IMD-3054 significantly reduced copper-induced expression after both 3 and 6 h copper exposures ($p < 0.0001$) (Table 2). RelA siRNA was also effective in abrogating copper-induced increases in mRNA levels of these two genes ($p < 0.0001$) (Table 3). In addition, treatment with RelA siRNA alone significantly reduced levels of both SRXN1 and ZFAND2A in the absence of copper ($p < 0.001$). These results confirmed that the expression of SRXN1 and ZFAND2A were regulated by NF- κ B under both stressed and unstressed conditions in HepG2 cells.

DISCUSSION

Copper is both an essential nutrient and a persistent environmental toxicant. It is critical to understand the transcriptional response to copper in the cells that regulate copper homeostasis. Signaling pathways involved in the physiological and toxicological responses of cells to copper were identified¹⁶. A bioinformatics approach was used to further characterize the NF- κ B signaling pathway as a potential copper-activated regulatory pathway in HepG2 cells. Previous studies examining the activation of NF- κ B by copper in cell culture found either no significant effect or a decrease in activation. In Jurkat T-cells treated with metals and then with TNF α , TPA, or hydrogen peroxide to stimulate NF- κ B activity, copper inhibited NF- κ B DNA binding²³. Similarly, exposure to 300 or 500 μ M copper inhibited binding of NF- κ B to DNA in BA/F3 β cells²⁴. Likewise in THP-1 cells, no significant change was observed in DNA binding by NF- κ B after exposure to 50, 200 or 400 μ M copper^{25; 26}. No change in the nuclear localization of NF- κ B was observed using primary human peripheral blood lymphocytes treated with 500 μ M copper²⁷. It is important to note that these studies were performed using lymphocyte derived cells, which are not responsible for maintaining copper homeostasis at the organismal level.

The data presented in this report indicates that copper activates NF- κ B in the human hepatocarcinoma HepG2 cell line. Similarly, exposure to 400 μ M copper increased NF- κ B

transcriptional activity in HEK293 cells²². In Sprague-Dawley rats, an intravenous bolus dose of copper increased NF- κ B activity in the liver after 1 h, although by 3 h activity returned to normal levels²¹. In addition, supplementing the food of Sprague-Dawley rats with 1,200 ppm copper caused an increase in NF- κ B activity in the liver after 9 weeks²⁰. These results suggest that the ability of copper to affect NF- κ B activity is cell type specific. In cells involved in adaptive immunity, copper seems to have either no effect or an inhibitory effect on NF- κ B. In contrast, in cells involved in metal detoxification (e.g., liver, kidney) copper seems to activate NF- κ B.

Studies employing genetic models of copper overload also suggest that excess copper activates NF- κ B in hepatocytes. The Jackson toxic milk mouse (tx^J)³⁰ has a phenotype similar to Wilson's disease. In tx^J mice, NF- κ B protein levels are elevated at 3 and 4 months, but decline over the next two months³¹. Fibroblasts derived from Atp7a^{Mobr} and Atp7a^{Modap} mice have high cellular copper concentrations when maintained in normal culture media and or media supplemented with 25 μ M copper³². Our oPOSSUM analysis of the transcriptome from C57BL/6-Atp7a^{Mobr} and C57BL/6-Atp7a^{Modap} derived cells showed a significant enrichment of NF- κ B binding sites (Z-score = 9.42) in genes up-regulated in one or both cell lines. When transcription profiles of livers at different stages of disease from Long-Evans cinnamon rats, which contain a mutation in the Atp7b gene, were compared to wild type Long-Evans agouti rats³³, NF- κ B signaling was mapped as a significantly affected canonical pathway when analyzing genes up-regulated in livers with either mild jaundice ($p < 0.0001$) or severe jaundice ($p < 0.002$). Pathway mapping analysis showed that NF- κ B signaling was not a significantly affected canonical pathway in non-diseased ($p = 0.19$) or slightly diseased ($p = 0.19$) livers. It is possible that copper does not activate NF- κ B until a pathological state is reached.

The present study was performed using a concentration of copper that invokes a stress response in HepG2 cells. Exposure of HepG2 cells to 100 and 200 μ M copper for 4, 8, 12 and 24 h resulted in an adaptive response, while exposure to 400 and 600 μ M copper resulted in a stress response¹⁶. We examined the steady state mRNA levels of HMOX1, GCLC, IER3, IL8, SRXN1, and ZFAND2A in all 16 exposure conditions (4 time points $\times$ 4 concentrations). Under the adaptive response conditions (100 and 200 μ M copper) the expression of HMOX1 significantly increased under two exposure conditions (>2 -fold change, $p < 0.001$) (Table 5). In contrast, under stress-inducible conditions (400 and 600 μ M copper) the expression of the six NF- κ B-responsive genes significantly increased in 45 out of 48 cases (Table 5). This suggests that copper activates NF- κ B as part of a stress response, but not an adaptive response. Activation of NF- κ B is a survival mechanism in hepatocytes (reviewed in³⁴ and³⁵), thus it is possible that activation of NF- κ B is one mechanism hepatocytes use to manage copper toxicity.

SRXN1 and ZFAND2A were identified and validated as NF- κ B-regulated genes. SRXN1, a homolog of yeast sulfiredoxin 1, is responsible for the reduction of peroxiredoxin, which catalyzes the reduction of hydroperoxides³⁶. Thus, it is consistent that SRXN1 transcription would be induced by copper, which is known to induce oxidative stress. ZFAND2A has not been characterized, therefore it is not possible to predict why copper and NF- κ B regulate its transcription.

While activation of NF- κ B is likely protective in this system, activation of NF- κ B is also associated with carcinogenesis³⁷. In particular, hepatitis C-induced hepatocellular carcinomas show increased NF- κ B activity, which can make these cells resistant to apoptosis³⁸. What is particularly interesting is that copper levels increased with increasing histological differentiation in hepatitis C-induced hepatocellular carcinomas³⁹. While it is not known why there are higher copper levels in hepatitis C-induced hepatocellular carcinomas, it is possible the elevated copper contributes to increased NF- κ B activity, eventually leading to cancer.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

Human hepatocellular carcinoma cells (HepG2) were grown in Minimum Essential Media (GIBCO® Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate and 100 μ M MEM non-essential amino acids. The concentration of copper in fetal bovine serum is reported to be 2.5 μ M⁴⁰. Cells were grown in a humidified incubator at 37°C under 5% CO₂ atmosphere.

To measure NF- κ B transcriptional activity, cells were transfected with the NF- κ B reporter plasmid, pNF- κ B-Luc (BD Biosciences, San Jose, CA,) and the control plasmid, pSV β -Galactosidase (Promega, Madison, WI, USA) in a 4:1 ratio. Transfections were performed using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen). Briefly, cells were plated into 24 well plates at 40–50% confluency. The following day, cells were transfected with 800 ng total DNA/well for 8–12 h. Following this incubation, media was replaced and cells incubated for an additional 10–12 h. Cells were then treated with either 400 μ M copper sulfate or 500 μ M H₂O₂ (Sigma-Aldrich, St Louis, MO) for 0, 0.5, 1, 2, 4, 6 or 8 h. Exposure to 400 μ M copper for 8 h corresponds to a LC₂₀ in HepG2 cells, while a 4 h exposure is not cytotoxic⁴¹. After treatment, cells were rinsed with PBS and then lysed in passive lysis buffer (Promega). Luciferase and β -galactosidase activities were measured using Luciferase or β -Galactosidase Enzyme Assay Systems, respectively, following manufacturer's instructions (Promega). The fold-induction of treated samples, relative to the untreated samples, was calculated from luciferase levels that were normalized to β -galactosidase activity.

Inhibition of NF- κ B Pathway Components

IKK-2 Inhibition—HepG2 cells were transfected as described above. Cells were then treated with either 1 μ M IMD-3054 (IKK-2 Inhibitor V; Calbiochem, San Diego, CA)⁴² dissolved in DMSO or DMSO alone 30 min prior to the addition of copper. The final concentration of DMSO in cell culture medium was 0.1%. Cells were then incubated in the presence of copper and IMD-3054 for 6 h, and then lysed. Luciferase and β -galactosidase activities were measured as described above. Total RNA was also collected and quantitative reverse transcription-real time-PCR (qRT-PCR) performed.

siRNA—RelA expression was knocked down using RNA interference. RelA is the dominant NF- κ B trans-activating factor in HepG2 cells, making it an optimal choice for siRNA knock-down⁴³. HepG2 cells were transfected with RelA siRNA (Qiagen, Valencia, CA) (Sense; r (GAGUCAGAUCAGCUCCUAA)dTdT, anti-sense; r(UUAGGAGCUGAUCUGACUC)dAdG) using Lipofectamine 2000. Briefly, cells were plated into 24-well cell culture plates containing Lipofectamine 2000 and 50 nM RelA siRNA (final concentration). As a control, cells were transfected with 50 nM non-specific siRNA. Following 45 h incubation, cells were treated with 400 μ M copper for 3 h. Total RNA was then collected and qRT-PCR performed.

Quantitative Reverse Transcription-Real Time-PCR

qRT-PCR was used to determine the effects of copper and IKK-2 inhibition or RelA siRNA on the steady state mRNA levels of heme oxygenase (HMOX1), γ -glutamylcysteine synthetase (GCLC), immediate early response gene 3 (IER3), interleukin 8 (IL8), sulfiredoxin 1 (SRXN1), and zinc finger, AN1-type domain 2A (ZFAND2A) (see below). These genes were selected because their steady state levels of expression increased after 4 and 8 h exposures to 400 μ M copper in the microarray study. Primers were designed using the open source Primer3 program⁴⁴ and were purchased from Integrated DNA Technologies (Coralville, IA) (Table 1). RelA primers were purchased from Qiagen. HepG2 cells were treated with 400 μ M copper for either 3 or 6 h, after pre-treatment with either IKK-2 inhibitor or RelA siRNA. Cells were then

rinsed with PBS and total RNA was isolated using the RNeasy Mini kit according to manufacturer's instructions (Qiagen). cDNAs were generated using the SuperScript® First-Strand Synthesis System for RT-PCR from 2 µg total RNA according to manufacturer's instructions (Invitrogen). cDNAs were subsequently used in qRT-PCR using Power SYBR Green RT-PCR kits according to manufacturer's instructions (Applied Biosystems, Foster City, CA). Quantitative PCR was performed in an ABI 7900 HT Fast Real-Time System and (Applied Biosystems) and fold changes in mRNA levels were calculated using the $\Delta\Delta C_t$ method⁴⁵ using β -actin as reference mRNA.

Identification of putative NF- κ B target genes

The genes that were up-regulated (>2-fold, p-value <0.001) in the microarray experiment¹⁶ (NCBI Gene Expression Omnibus Series accession number GSE9539) after exposure to 400 µM copper for 4 or 8 h were loaded into the web-based program PAINT. PAINT uses the TRANSFAC Public database to find transcription factor binding sites on DNA sequences⁴⁶. TRANSFAC is a database of eukaryotic transcription factors, their binding sites and other factors involved in gene regulation⁴⁷. oPOSSUM is a web-based program that searches for over-representation of transcription factors in a set of genes²⁸. Enrichment of NF- κ B binding sites was searched for in the 2000 bp upstream of the start site.

Data analysis

Statistical analysis was performed using StatView software (JMP software, Cary, NC). Results are presented as the mean $\pm$ standard mean error. The significance of mean differences was determined by analysis of variance (ANOVA) of log-transformed data followed by Fisher's Protected Least Squares Difference post hoc test for individual comparisons. The criterion for statistical significance was set at $p < 0.05$.

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REFERENCES

1. Olivares M, Uauy R. Copper as an essential nutrient. *Am J Clin Nutr* 1996;63:791S–796S. [PubMed: 8615366]
2. Barceloux DG. Copper. *J Toxicol Clin Toxicol* 1999;37:217–230. [PubMed: 10382557]
3. Madsen E, Gitlin JD. Copper deficiency. *Curr Opin Gastroenterol* 2007;23:187–192. [PubMed: 17268249]
4. Keen CL, Hanna LA, Lanoue L, Uriu-Adams JY, Rucker RB, Clegg MS. Developmental consequences of trace mineral deficiencies in rodents: acute and long-term effects. *J Nutr* 2003;133:1477S–1480S. [PubMed: 12730447]
5. Menkes JH. Menkes disease and Wilson disease: two sides of the same copper coin. Part I: Menkes disease. *Eur J Paediatr Neurol* 1999;3:147–158. [PubMed: 10476363]
6. Menkes JH. Menkes disease and Wilson disease: two sides of the same copper coin. Part II: Wilson disease. *Eur J Paediatr Neurol* 1999;3:245–253. [PubMed: 10595669]
7. Ferenci P. Wilson's Disease. *Clin Gastroenterol Hepatol* 2005;3:726–733. [PubMed: 16233999]
8. DiDonato M, Sarkar B. Copper transport and its alterations in Menkes and Wilson diseases. *Biochim Biophys Acta* 1997;1360:3–16. [PubMed: 9061035]
9. Scheinberg IH, Sternlieb I. Wilson disease and idiopathic copper toxicosis. *Am J Clin Nutr* 1996;63:842S–845S. [PubMed: 8615372]
10. Cheah DM, Deal YJ, Wright PF, Buck NE, Chow CW, Mercer JF, Allen KJ. Heterozygous tx mice have an increased sensitivity to copper loading: implications for Wilson's disease carriers. *Biometals* 2007;20:751–757. [PubMed: 17136311]

11. Pankit AN, Bhawe SA. Copper metabolic defects and liver disease: environmental aspects. *J Gastroenterol Hepatol* 2002;17:S403–S407. [PubMed: 12472971]
12. Pandit A, Bhawe S. Present interpretation of the role of copper in Indian childhood cirrhosis. *Am J Clin Nutr* 1996;63:830S–835S. [PubMed: 8615370]
13. Water, C. o. C. i. D., Toxicology, B. o. E. S. a., Sciences, C. o. L. & Council, N. R. (2000). *Copper in Drinking Water*, National Academy Press, Washington, D.C.
14. NIOSH. National Occupational Exposure Survey Conducted from 1981–1983. 1988. (Health, N. I. f. O. S. a., ed.).
15. Zhu Z, Thiele DJ. Toxic metal-responsive gene transcription. *Exs* 1996;77:307–320. [PubMed: 8856982]
16. Song MO, Li J, Freedman JH. Physiological and Toxicological Transcriptome Changes in HepG2 Cells Exposed to Copper. *Physiol Genomics*. 2009In press
17. Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF- κ B activity. *Annu Rev Immunol* 2000;18:621–663. [PubMed: 10837071]
18. Hayden MS, Ghosh S. Signaling to NF- κ B. *Genes Dev* 2004;18:2195–2224. [PubMed: 15371334]
19. Chen F, Ding M, Castranova V, Shi X. Carcinogenic metals and NF- κ B activation. *Mol Cell Biochem* 2001;222:159–171. [PubMed: 11678598]
20. Cisternas FA, Tapia G, Arredondo M, Cartier-Ugarte D, Romanque P, Sierralta WD, Vial MT, Videla LA, Araya M. Early histological and functional effects of chronic copper exposure in rat liver. *Biomaterials* 2005;18:541–551. [PubMed: 16333755]
21. Persichini T, Percario Z, Mazzon E, Colasanti M, Cuzzocrea S, Musci G. Copper activates the NF- κ B pathway *in vivo*. *Antioxid Redox Signal* 2006;8:1897–1904. [PubMed: 16987042]
22. Muller P, van Bakel H, van de Sluis B, Holstege F, Wijmenga C, Klomp LW. Gene expression profiling of liver cells after copper overload *in vivo* and *in vitro* reveals new copper-regulated genes. *J Biol Inorg Chem* 2007;12:495–507. [PubMed: 17211630]
23. Satake H, Suzuki K, Aoki T, Otsuka M, Sugiura Y, Yamamoto T, Inoue J. Cupric ion blocks NF- κ B activation through inhibiting the signal-induced phosphorylation of I κ B α . *Biochem Biophys Res Commun* 1995;216:568–573. [PubMed: 7488149]
24. Zhai Q, Ji H, Zheng Z, Yu X, Sun L, Liu X. Copper induces apoptosis in BA/F3 β cells: Bax, reactive oxygen species, and NF- κ B are involved. *J Cell Physiol* 2000;184:161–170. [PubMed: 10867640]
25. Lewis JB, Randol TM, Lockwood PE, Wataha JC. Effect of subtoxic concentrations of metal ions on NF- κ B activation in THP-1 human monocytes. *J Biomed Mater Res A* 2003;64:217–224. [PubMed: 12522807]
26. Lewis JB, Wataha JC, Randol TM, McCloud VV, Lockwood PE. Metal ions alter lipopolysaccharide-induced NF- κ B binding in monocytes. *J Biomed Mater Res A* 2003;67:868–875. [PubMed: 14613235]
27. Jimenez Del Rio M, Velez-Pardo C. Transition metal-induced apoptosis in lymphocytes via hydroxyl radical generation, mitochondria dysfunction, and caspase-3 activation: an *in vitro* model for neurodegeneration. *Arch Med Res* 2004;35:185–193. [PubMed: 15163458]
28. Ho Sui SJ, Mortimer JR, Arenillas DJ, Brumm J, Walsh CJ, Kennedy BP, Wasserman WW. oPOSSUM: identification of over-represented transcription factor binding sites in co-expressed genes. *Nucleic Acids Res* 2005;33:3154–3164. [PubMed: 15933209]
29. Musonda CA, Chipman JK. Quercetin inhibits hydrogen peroxide (H₂O₂)-induced NF- κ B DNA binding activity and DNA damage in HepG2 cells. *Carcinogenesis* 1998;19:1583–1589. [PubMed: 9771928]
30. Coronado V, Nanji M, Cox DW. The Jackson toxic milk mouse as a model for copper loading. *Mamm Genome* 2001;12:793–795. [PubMed: 11668395]
31. Roberts EA, Lau CH, da Silveira TR, Yang S. Developmental expression of *Commd1* in the liver of the Jackson toxic milk mouse. *Biochem Biophys Res Commun* 2007;363:921–925. [PubMed: 17910951]
32. Armendariz AD, Gonzalez M, Loguinov AV, Vulpe CD. Gene expression profiling in chronic copper overload reveals upregulation of *Prnp* and *App*. *Physiol Genomics* 2004;20:45–54. [PubMed: 15467011]

33. Klein D, Lichtmanegger J, Finckh M, Summer KH. Gene expression in the liver of Long-Evans cinnamon rats during the development of hepatitis. *Arch Toxicol* 2003;77:568–575. [PubMed: 14574444]
34. Luedde T, Trautwein C. Intracellular survival pathways in the liver. *Liver Int* 2006;26:1163–1174. [PubMed: 17105581]
35. Schoemaker MH, Moshage H. Defying death: the hepatocyte's survival kit. *Clin Sci (Lond)* 2004;107:13–25. [PubMed: 15104533]
36. Jeong W, Park SJ, Chang TS, Lee DY, Rhee SG. Molecular mechanism of the reduction of cysteine sulfinic acid of peroxiredoxin to cysteine by mammalian sulfiredoxin. *J Biol Chem* 2006;281:14400–14407. [PubMed: 16565085]
37. Maeda S, Omata M. Inflammation and cancer: role of nuclear factor- κ B activation. *Cancer Sci* 2008;99:836–842. [PubMed: 18294278]
38. Tai DI, Tsai SL, Chen YM, Chuang YL, Peng CY, Sheen IS, Yeh CT, Chang KS, Huang SN, Kuo GC, Liaw YF. Activation of nuclear factor κ B in hepatitis C virus infection: implications for pathogenesis and hepatocarcinogenesis. *Hepatology* 2000;31:656–664. [PubMed: 10706556]
39. Ebara M, Fukuda H, Hatano R, Yoshikawa M, Sugiura N, Saisho H, Kondo F, Yukawa M. Metal contents in the liver of patients with chronic liver disease caused by hepatitis C virus. Reference to hepatocellular carcinoma. *Oncology* 2003;65:323–330. [PubMed: 14707452]
40. Freedman JH, Weiner RJ, Peisach J. Resistance to copper toxicity of cultured hepatoma cells. Characterization of resistant cell lines. *J Biol Chem* 1986;261:11840–11848. [PubMed: 3745169]
41. Song MO, Freedman JH. Expression of copper-responsive genes in HepG2 cells. *Mol Cell Biochem* 2005;279:141–147. [PubMed: 16283523]
42. Onai Y, Suzuki J, Kakuta T, Maejima Y, Haraguchi G, Fukasawa H, Muto S, Itai A, Isobe M. Inhibition of I κ B phosphorylation in cardiomyocytes attenuates myocardial ischemia/reperfusion injury. *Cardiovasc Res* 2004;63:51–59. [PubMed: 15194461]
43. Chiao PJ, Na R, Niu J, Sclabas GM, Dong Q, Curley SA. Role of Rel/NF- κ B transcription factors in apoptosis of human hepatocellular carcinoma cells. *Cancer* 2002;95:1696–1705. [PubMed: 12365017]
44. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000;132:365–386. [PubMed: 10547847]
45. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–408. [PubMed: 11846609]
46. Vadigepalli R, Chakravarthula P, Zak DE, Schwaber JS, Gonye GE. PAINT: a promoter analysis and interaction network generation tool for gene regulatory network identification. *Omics* 2003;7:235–252. [PubMed: 14583114]
47. Matys V, Kel-Margoulis OV, Fricke E, Liebich I, Land S, Barre-Dirrie A, Reuter I, Chekmenev D, Krull M, Hornischer K, Voss N, Stegmaier P, Lewicki-Potapov B, Saxel H, Kel AE, Wingender E. TRANSFAC and its module TRANSCOMP: transcriptional gene regulation in eukaryotes. *Nucleic Acids Res* 2006;34:D108–D110. [PubMed: 16381825]

Figure 1. IPA pathway mapping of copper-regulated genes

Pathway generated by Ingenuity Pathways Analysis software genes differentially regulated by 400 μ M copper in HepG2 cells. Genes that were up-regulated are in red. Genes that were down regulated are in green. Genes whose expression levels were not significantly changed are in pink. Light blue lines indicate known interactions of NFKB1. NFKB1 is outlined in blue. Gene abbreviations can be found via their Gene Cards (<http://www.genecards.org>)

Figure 2. Effects of copper and hydrogen peroxide on NF- κ B activation

The effects of 500 μ M hydrogen peroxide (*open bars*) and 400 μ M copper (*closed bars*) on NF- κ B activity was assessed. NF- κ B transcriptional activity was determined by co-transfecting HepG2 cells with an NF- κ B-driven luciferase promoter plasmid and a β -galactosidase control plasmid. The fold change in NF- κ B activity was determined by comparing β -galactosidase-normalized luciferase levels of treated cells to untreated cells. Error bars indicate standard error from four experiments. Asterisks indicate conditions that are significantly different from untreated control.

Figure 3. Effect of IKK-2 inhibitor on NF- κ B activation

HepG2 cells were treated with 1 μ M of the IKK-2 inhibitor IMD-3054 in the presence (*closed bars*) or absence (*open bars*) of 400 μ M copper. NF- κ B transcriptional activity was determined by co-transfecting HepG2 cells with an NF- κ B-driven luciferase promoter plasmid and a β -galactosidase control plasmid. The fold change in NF- κ B activity was determined by comparing β -galactosidase-normalized luciferase levels of treated cells to untreated cells. Error bars indicate standard error from 3 experiments. Asterisks indicate condition is significantly different from untreated control.

TABLE 1Primers used in the qRT-PCR analysis of copper-responsive NF- κ B-regulated genes

Gene	Forward (5'→3')	Reverse (5'→3')
β -Actin	GATATCGCTGCGCTGGTCGTC	ACGCAGCTCATTGTAGAAGGTGTGG
GCLC	GTGGATGTGGACACCAGATG	GCG ATAAACTCCCTCATCCA
IER3	AGGAGAAGAAATGGGGAGGA	CGCCGAAGTCTCACACAGTA
IL8	CAAACCTTTCCACCCCAAAT	CTCTGCACCCAGTTTTCTT
SRXN1	GGGCTGGACCCACTGTAGTA	CCAGGGACTCTTGGTTTCA
ZFAND2A	GGGGAAGCATTGTTTCAGAAA	ATCCTTCTGGAATGCAAACG

TABLE 2

Effect of IMD-3054 and copper on NF-κB-target genes

Gene	3hr			6hr		
	Copper	Copper plus IMD-3054	IMD-3054	Copper	Copper plus IMD-3054	IMD-3054
HMBOX1	39 ± 9	4.7 ± 1 *	0.87 ± 0.08	52 ± 23	8.4 ± 4 *	0.79 ± 0.05
GCLC	2.9 ± 0.4	0.86 ± 0.02 *	0.61 ± 0.09 ##	5.2 ± 0.6	0.97 ± 0.06 *	1.0 ± 0.1
IER3	4.8 ± 0.2	2.3 ± 0.2 *	2.0 ± 0.3 ##	3.1 ± 0.1	1.5 ± 0.2 *	0.92 ± 0.2
IL8	15 ± 1	4.8 ± 0.4 *	2.4 ± 0.4 ##	38 ± 4	7.2 ± 0.7 *	1.9 ± 0.3 ##
SRXN1	5.8 ± 0.5	0.94 ± 0.08 *	0.79 ± 0.01	13 ± 2	1.0 ± 0.06 *	1.5 ± 0.2 ##
ZFAND2A	9.9 ± 0.5	4.2 ± 0.4 *	0.82 ± 0.2	33 ± 5	7.7 ± 0.3 *	0.76 ± 0.1

Numbers indicate fold-change ± SEM relative to no copper, no IMD-3054 control.

* indicates copper plus IMD-3054-treated cells are significantly different from copper-only treated cells.

indicates IMD-3054 treated cells are significantly different from no copper, no IMD-3054 control cells.

TABLE 3

Effect of RelA siRNA and copper on NF-κB-regulated genes

Gene	Copper plus non-sense siRNA	Copper plus RelA siRNA	RelA siRNA
HMOX1	24 ± 3	11 ± 1 *	0.62 ± 0.2
GCLC	2.9 ± 0.2	1.1 ± 0.1 *	0.48 ± 0.03
IER3	5.7 ± 1	1.8 ± 0.2 *	0.39 ± 0.03
IL8	31 ± 7	5.4 ± 1 *	0.22 ± 0.02
SRXN1	7.6 ± 0.8	1.6 ± 0.4 *	0.58 ± 0.06
ZFAND2A	9.7 ± 0.8	3.7 ± 0.3 *	0.62 ± 0.04
RelA	0.64 ± 0.07	0.07 ± 0.01 *	0.12 ± 0.01

Numbers indicate fold-change ± SEM relative to non-sense siRNA control.

* indicates significantly different from copper plus non-sense siRNA treated control.

TABLE 4Putative NF- κ B-target genes identified by TRANSFAC analysis that are regulated by copper

Common Name	Genbank Accession	Fold Change	
		4 h	8 h
ATF3	BC006322	8.7	3.5
NCF2	NM_000433	7.1	12
MT1X	NM_005952	6.3	4.9
NPPB	NM_002521	5.1	5.2
SRXN1	NM_080725	5.0	4.1
ZFAND2A	NM_182491	4.8	7.3
MXD1	NM_002357	3.6	2.2
AMOTL2	NM_016201	3.5	1.7
MT2A	BC007034	3.3	2.2
GCLC	BC039894	3.1	3.8
PALLD	NM_016081	2.8	4.9
KLF5	NM_001730	2.7	3.1
IER3	NM_003897	2.6	1.8
GCNT3	BC017032	2.5	3.0
GDF15	NM_004864	2.4	1.6
UBC	NM_021009	2.4	1.9
CAMTA2	NM_015099	2.4	3.3
SLC38A2	NM_018976	2.3	2.0
CCL20	NM_004591	2.3	2.2
UNC5B	NM_170744	2.2	1.6
ANKRD11	NM_013275	2.1	2.2
CXCL3	NM_002090	2.1	1.2
DUSP5	NM_004419	1.6	3.0
SLC40A1	NM_014585	1.7	2.6
SWAP70	NM_015055	1.4	2.3
HIST2H2AA3	NM_003516	1.4	2.3
RRAD	BC011645	1.2	2.2
RHOF	AF239923	1.8	2.1
SPIB	NM_003121	1.5	2.1
C20orf111	NM_016470	1.9	2.1

TABLE 5

Fold change in NF- κ B-responsive genes following copper exposure

Gene	100 μ M				200 μ M			
	4 hr	8 hr	12 hr	24 hr	4 hr	8 hr	12 hr	24 hr
HMOX1	1.9	3.1	2.0	1.3	2.4*	3.8*	2.8	1.7
GCLC	1.2	1.4	1.3	1.3	1.3	1.4	1.3	1.4
IER3	1.2	1.0	1.2	1.1	1.4	1.2	1.5	1.3
IL8	1.2	1.2	1.3	1.0	1.5	1.2	1.5	1.0
SRXN1	1.3	1.4	1.3	1.5	1.5	1.5	1.4	1.8
ZFAND2A	1.0	1.4	1.1	0.96	1.1	1.4	1.4	0.92
Gene	400 μ M				600 μ M			
	4 hr	8 hr	12 hr	24 hr	4 hr	8 hr	12 hr	24 hr
HMOX1	16*	18*	14*	7.9*	15*	19*	28*	9.5*
GCLC	2.9*	3.7*	2.0*	2.0*	5.0*	6.8*	4.2*	1.8
IER3	2.6*	1.8	3.4*	2.4*	4.2*	2.8*	4.0*	3.1*
IL8	4.6*	5.5*	5.3*	2.1*	13*	22*	22*	3.8*
SRXN1	5.0*	4.1*	2.5*	3.2*	5.5*	8.0*	5.4*	3.5*
ZFAND2A	4.8	7.3	4.1	1.5	9.7*	15	12	3.0

Significantly up-regulated genes (2-fold, $p < 0.001$) are indicated with an asterisk.