Copper Alters the Conformation and Transcriptional Activity of the Tumor Suppressor Protein p53 in Human Hep G2 Cells

NADINE M. TASSABEHJI, JACOB W. VANLANDINGHAM, AND CATHY W. LEVENSON[1]

Department of Nutrition, Food & Exercise Sciences, Florida State University, Tallahassee, Florida 32306-4340

The tumor suppressor protein p53 plays a role in the molecular response to DNA damage by acting as a DNA-binding transcription factor that regulates specific target genes to arrest the cell cycle, induce repair mechanisms, and initiate apoptotic cell death. To test the effect of copper on the transcriptional activity of p53, Hep G2 cells were transiently transfected with a luciferase reporter gene downstream from multiple p53 response elements. Co-transfection with the *p53* gene resulted in a 6-fold increase in luciferase activity, showing that p53 acts as a transcription factor in this system. However, in the presence of copper, luciferase activity was significantly reduced. Oligonucleotide arrays representing 145 known p53-associated genes were hybridized with biotinylated cDNAs from mRNA extracted from control and copper-treated Hep G2 cells. Among the genes that were differentially regulated were *fos*, *RB1*, *glutathione peroxidase*, *TGF-β*, and *15-lipoxygenase*, a gene known to be activated by mutant p53. Although control Hep G2 cells synthesize wild-type p53, immunocytochemistry identified not only wild type, but also mutant p53 in the presence of copper and other agents that induce oxidative damage. Thus, this report not only identifies genes that may play a role in copper-mediated apoptosis, but also suggests that copper-induced oxidative processes result in the synthesis of mutant p53 with altered transcriptional properties. Exp Biol Med 230:699–708, 2005

Key words: apoptosis; liver; oxidation; Wilson disease; Cu

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[1]To whom correspondence should be addressed at Florida State University, 237 Biomedical Research Facility, Tallahassee, FL 32306-4340. E-mail: levenson@neuro.fsu.edu

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Introduction

The tumor suppressor protein, p53, plays a key role in monitoring the molecular response to cellular damage. Specifically, DNA damage triggers p53 transcription, translation, and nuclear translocation. In the nucleus, p53 acts as a DNA-binding transcription factor that regulates downstream genes responsible for cell cycle arrest and apoptotic cell death. This p53-mediated apoptosis is a key mechanism in the elimination of cells with genomic damage that might otherwise become cancerous (1). Thus, mutations that result in the deletion of p53, or the formation of a mutant conformation that cannot function as a transcription factor, result in abnormal cellular proliferation. In fact, approximately 50% of all human malignant cells carry mutations in the *p53* gene, making it the single most frequently mutated gene in human cancer cells (2, 3).

A variety of cellular stressors, such as UV damage (4), oxidation (5), hypoxia (6), heavy metal toxicity (7, 8), and other genotoxic agents (9) have been shown to result in DNA damage, *p53* induction, and apoptosis. For example, copper accumulation causes apoptosis that seems to be mediated by DNA damage and subsequent *p53* activation (10, 11). Although there has recently been considerable concern about the health effects of environmental copper contamination (12, 13), dangerously elevated levels of copper are most frequently seen in the autosomal recessive disorder, Wilson's disease (WD), and in other copper-related disorders, such as Indian childhood cirrhosis and idiopathic copper toxicosis (14). Wilson's disease is caused by a mutation in the gene that codes for the P-type copper-transporting ATPase, *ATP7B* (15), resulting in abnormal hepatic copper export and accumulation that leads to liver disease, including hepatocyte death, hepatitis, cirrhosis, fibrosis, and liver failure (16). Long term patients with WD are also at risk for developing a variety of aggressive malignant tumors (17, 18).

The mechanisms responsible for copper-mediated DNA damage and apoptosis are not fully understood, particularly at the molecular level. Although copper overload induces the

formation of reactive oxygen species (ROS), it seems that not all copper-mediated DNA damage is caused by ROS, because both Cu^{+} and Cu^{2+} are mutagenic (19). In fact, much of the copper-induced damage is likely the result of copper binding to specific sites on double-stranded DNA, resulting in strand scission (19, 20). The affinity of copper for these sites is higher than other metals, including nickel, zinc, magnesium, cadmium, silver, manganese, and chromium (20).

We have previously shown that excess copper induces both p53 and apoptosis in the human hepatoma cell line Hep G2 (10) that express wild-type p53 (21). Furthermore, we now know that copper-mediated hepatocyte apoptosis is dependent on p53 (11). However, the molecular targets for p53 action in these cells are not known. Thus, this work was designed to identify the genes that function downstream of *p53* and participate in copper-mediated hepatocyte apoptosis.

Second, this work used a p53-induced reporter gene and conformation-specific antibodies to test the hypothesis that copper accumulation alters the normal conformation and molecular function of p53. Two lines of evidence support this hypothesis. First, *in vitro* work has shown that copper can displace zinc from its normal binding site on p53, resulting in abnormal protein folding and disruption of p53 function (22). Second, despite the induction of the tumor suppressor, *p53*, hepatic copper accumulation, both in humans (17, 18) and in Long Evans Cinnamon rats (23, 24), carries a significant risk of developing cancerous neoplasms, suggesting that p53 function may be impaired.

Materials and Methods

Cell Culture. Human hepatoma cells (Hep G2) isolated by liver biopsy (25), were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in a humidified incubator with 5% CO_2 and 95% air at 37°C, and maintained in Minimum Essential Medium (αMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% calf serum (Cosmic Calf Serum; Hyclone Laboratories, Logan, UT), 0.5 μg/ml of gentamicin (GIBCO BRL, Rockville, MD), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B (Sigma Chemical).

Annexin V Apoptosis Marker. Hep G2 cells were grown on glass coverslips in 35-mm dishes at approximately 60%–70% confluence, and allowed to attach for 24 hrs. Cells were then treated with 200 μ*M* copper as cupric sulfate ($n = 3$) for 6 hrs, as previously described (10). After copper treatment, cells were incubated with Annexin V (BD Biosciences, San Jose, CA) specifically designed for the fluorescent detection of the early stages of apoptosis. The manufacturer's suggested protocol, which included incubation of copper-treated cells with Annexin V and propidium iodide for 15 mins, was followed. Coverslips were mounted onto glass microscope slides (Corning, New York, NY), using a commercially available mounting medium formulated to inhibit photobleaching (FluorSave Reagent; Calbiochem-Novabiochem, La Jolla, CA). Cells were viewed using a Nikon microscope (Nikon Microphot Fx; Nikon, Melville, NY) equipped with epifluorescence.

Caspase Inhibitor. Hep G2 cells were grown on glass coverslips in 35-mm dishes at approximately 60%–70% confluence, and allowed to attach for 24 hrs. Cells were first treated with 50 μ*M* caspase-3 inhibitor (Ac-DEVD-CHO; BD Sciences, San Jose, CA; $n = 3$) or the vehicle, dimethyl sulfoxide (DMSO; $n = 3$). After 1 hr, cells were treated with 200 μ*M* copper as cupric sulfate. Eighteen hours later, cells were washed once with phosphate buffered saline (PBS) and fixed with 3.7% formaldehyde for 10 mins. After washing in PBS, cell nuclei were stained for 10 mins with 4', 6-diamidino-2-phenylindole (DAPI, 1:300; Sigma Chemical) and visualized using fluorescent microscopy as described in Annexin V methods.

Transfections and Reporter Gene Assay. Hep G2 cells were transiently transfected in serum-free αMEM using liposome-mediated transfection with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were plated in 6-well plates and transfected with 3 μg of a luciferase reporter gene construct attached downstream from 15 tandem repeats of a known p53-binding site (Stratagene, La Jolla, CA) and 3 μg of pSV40 β-galactosidase to correct for transfection efficiency. Co-transfection studies were performed using an expression plasmid containing wild-type p53 (Stratagene). All transfections were carried out in triplicate in two separate experiments.

After 6 hrs of transfection, serum was added to the media at a final concentration of 10%, and cells were treated with 0, 25, 50, 100, or 200 μ*M* copper as cupric sulfate for 18 hrs, as previously described (10). Cells were washed three times with PBS, lysed using cell lysis buffer (Pharmingen-Becton Dickson Co., San Jose, CA), and immediately assayed for luciferase activity, using an Optococomp 1 luminometer (Hamden, CT) and β-galactosidase, as previously described (26).

p53 Target Gene Expression Profiling. Total cellular RNA was isolated by Trizol extraction (GIBCO/BRL, Life Technologies) from cells treated with 200 μ*M* copper for 18 hrs and from untreated control cells. The intact nature of the RNA was confirmed after quantification by spectrophotometry and ethidium bromide visualization after electrophoresis on a denaturing formaldehyde-agarose gel. Ten micrograms of RNA was reverse transcribed and labeled with biotin-dUTP using sequence-specific primers supplied with the TransSignal p53 Target Gene Arrays kit (Panomics, Inc., Redwood City, CA). Each array kit contains 145 duplicate spots of sense-strand oligonucleotides specific for known human p53 target genes as well as controls. Chemiluminescent arrays were exposed to Hyperfilm (Amersham Pharmacia Biotech, Piscataway, NJ) and Biorad Gel Foc System (Biorad Laboratories, Hercules, CA) was used to compare expression of individual genes normalized to the expression of glyceraldehyde 3' phosphate dehydrogenase (GADPH) mRNA.

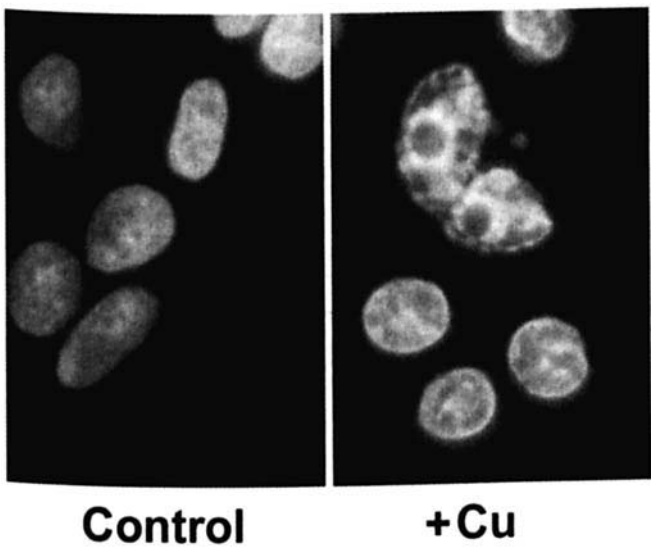

Figure 1. Copper induction of apoptosis in Hep G2 cells. The nuclei of control and copper-treated (+Cu, 200 μ*M* Cu for 18 hrs) Hep G2 cells were stained with DAPI to permit an analysis of nuclear morphology. Copper treatment (+Cu) resulted in chromatin aggregation and nuclear blebbing consistent with apoptosis. Photomicrographs are representative of images from $n = 6$ dishes at original magnification ×100.

Immunocytochemistry. Conformation-specific antibodies and immunocytochemistry were used to examine the relative abundance and intracellular localization of p53 in the wild-type and mutant conformations. Hep G2 cells ($n = 6$, in two separate experiments) were plated on glass coverslips and treated with either 200 μ*M* copper, iron as 15 μ*M* ferrous sulfate, magnesium as 200 μ*M* magnesium chloride, or 100 μ*M* hydrogen peroxide. Cells were fixed for 10 mins with 3.7% formaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in PBS and permeabilized for 5 mins with 0.2% Triton X-100 in PBS at room temperature. After three successive PBS washes, cells were incubated in 10 mg/ml of bovine serum albumin (BSA; Sigma Chemical) for 10 mins. Cells were incubated at 4°C overnight, with either a mouse monoclonal p53 primary antibody p53Ab-5 (1:200 in BSA; PAb1620; Oncogene, San Diego, CA), which recognizes human p53 only in the wild-type conformation; or p53Ab-3 (1:200; PAb240; Oncogene), which has been shown to react only with p53 in the mutant conformation under nondenaturing conditions. Other cells were incubated with an anti-human recombinant 15-lipoxygenase (15-LO) provided by Dr. T.E. Eling at the National Institute for Environmental Health and Safety. This antibody was previously characterized and shown to be specific for this lipoxygenase (27). In a separate experiment, cells were incubated with a rabbit monoclonal caspase-3 antibody (CPP-32; Zymed, San Francisco, CA). These incubations were followed by incubations with an IgG (Fc fragment specific) antibody conjugated to the fluorescent dye, cyanine 3 (Cy3; Jackson ImmunoResearch Laboratories, Westgrove, PA). The secondary antibody was selected for its minimal cross-reactivity with human serum proteins expressed by Hep G2 cells. After washing in PBS, cell nuclei were stained for 10 mins with 1:300 DAPI, and coverslips were mounted onto glass microscope slides using a commercially available mounting medium formulated to inhibit photobleaching.

Western Analysis. Copper-treated and control Hep G2 cells were collected with Nonidet P-40 (NP-40) protein extraction buffer while on ice and were centrifuged at 12,000 *g* for 20 mins. Supernatant was collected for protein analysis by the Bio-Rad protein assay method. Protein (50 μg) was loaded for each condition and separated on a 10% polyacrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred by electrophoresis to a nitrocellulose membrane. The membrane was blocked with 4% nonfat milk and incubated overnight at 4°C with the 15-LO antibody, previously described (26). Membranes were then incubated with a 1:3000 dilution of a horseradish peroxide (HRP)-conjugated goat anti-rabbit secondary antibody (Amersham-Pharmacia, Piscataway, NJ) for 90 mins at room temperature. Enhanced chemiluminescence (ECL; Amersham-Pharmacia) exposure on Kodak X-MAT AR film (Estman Kodak Company, Rochester, NY) was used to visualize protein abundance. Films were analyzed by quantitative photodensitometry using a Bio-Rad Gel Doc system (Bio-Rad) in conjunction with QuantiOne software (Bio-Rad).

Results

Role of p53 in Copper-Induced Hepatocyte Apoptosis. Treatment of Hep G2 cells with 200 μ*M* copper increases cellular copper concentrations approximately 6-fold (10). This treatment resulted in morphologic changes consistent with apoptosis. Staining with DAPI revealed chromatin aggregation and nuclear blebbing (Fig. 1). Additional evidence of apoptosis was provided using Annexin V staining in copper-treated cells, in which the pattern of plasma membrane staining of phosphatidylserine at 6 hrs in unpermeabilized cells was consistent with the early stages of apoptosis (Fig. 2). Copper treatment also increased immunoreactivity for the apoptosis-associated enzyme, caspase-3 (Fig. 3). Furthermore, using a caspase inhibitor (Fig. 4) in copper-treated cells, we were able to abolish morphologic changes consistent with apoptosis, such as chromatin aggregation and nuclear blebbing, seen in copper-treated cells (Fig. 4).

Effect of Copper on p53 Transcriptional Activity. As expected, transfection of untreated Hep G2 cells with the luciferase reporter gene downstream from multiple p53 binding sites resulted in very low basal levels of luciferase activity (Fig. 5). Co-transfection of these cells with a construct designed to permit the overexpression of wild-type p53 in human cells resulted in a >6-fold increase in luciferase activity. The addition of low concentrations of copper (25 μ*M*) increased luciferase activity ($P < 0.05$), whereas increasing concentrations of copper decreased the

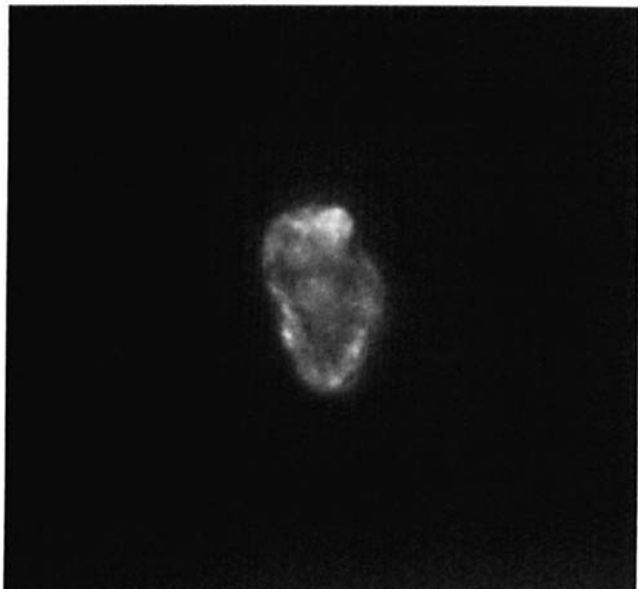

Figure 2. Annexin V staining in copper-treated Hep G2 cells. Copper-treated (+Cu, 200 μ*M* Cu for 6 hrs) Hep G2 cells were stained with Annexin V to detect the position of phosphatidylserine (PS) in the cell membrane. Copper treatment (+Cu) resulted in redistributing PS to the outer layer of the membrane without nuclear propridium iodide staining, consistent with apoptosis. Photomicrographs are representative of images from $n = 3$ dishes at original magnification ×40.

luciferase activity (Fig. 6), such that at 200 μ*M* copper was not significantly different from untreated controls (Figs. 5 and 6). In cells transfected with the *p53* construct and the reporter gene, 200 μ*M* copper abolished the increase in reporter gene activity (Fig. 5).

Copper-Regulation of p53-Responsive Genes. Examination of the oligonucleotide arrays revealed that the Hep G2 cells expressed 21 p53-associated genes. Figure 7 shows representative mRNAs in duplicate for the arrays. The housekeeping gene, *GAPDH*, was not regulated by copper treatment, whereas other genes, such as *15-LO* and *PIG 8* were differentially regulated (Fig. 7). Using the established criteria, 12 genes were differentially regulated in copper-treated cells (Table 1). Of these, three genes were upregulated between 1.9- and 3.2-fold above control. Nine genes were downregulated after 18 hrs of copper treatment (Table 1). Further analysis of 15-LO by immununocytochemistry and Western analysis showed that copper treatment resulted in a 2-fold increase in this enzyme (Fig. 8).

Copper Regulation of p53 Conformation *In Vivo*. Use of conformation-specific antibodies showed that, in untreated cells, there were undetectable levels of both wild-type and mutant p53 (Fig. 9). Copper treatment not only resulted in changes in nuclear morphology, but also increased the cytoplasmic and nuclear abundance of both wild-type and mutant p53 (Fig. 9). All copper-treated cells observed seemed to contain both wild-type and mutant p53. Quantification of pixel density suggested that approximately 60% of the p53 in copper-treated cells was in the mutant conformation, whereas 40% remained in the wild-type conformation. Additionally, iron and hydrogen peroxide–treated cells contained mutant p53 staining. However, mutant p53 was undetectable in magnesium-treated cells (Fig. 10).

Discussion

The data reported here show that copper overload of hepatic cells results in apoptosis. Evidence for the early stages of apoptosis was seen after 6 hrs of copper treatment, at which point, there was Annexin V staining of phosphatidyl serine at the plasma membrane, without nuclear staining with propidium iodide. At 18 hrs of copper treatment, caspase-3, a terminal enzyme in the apoptotic cascade, was induced in Hep G2 cells. This was accompanied by morphologic evidence of apoptosis, including chromatin aggregation and nuclear blebbing.

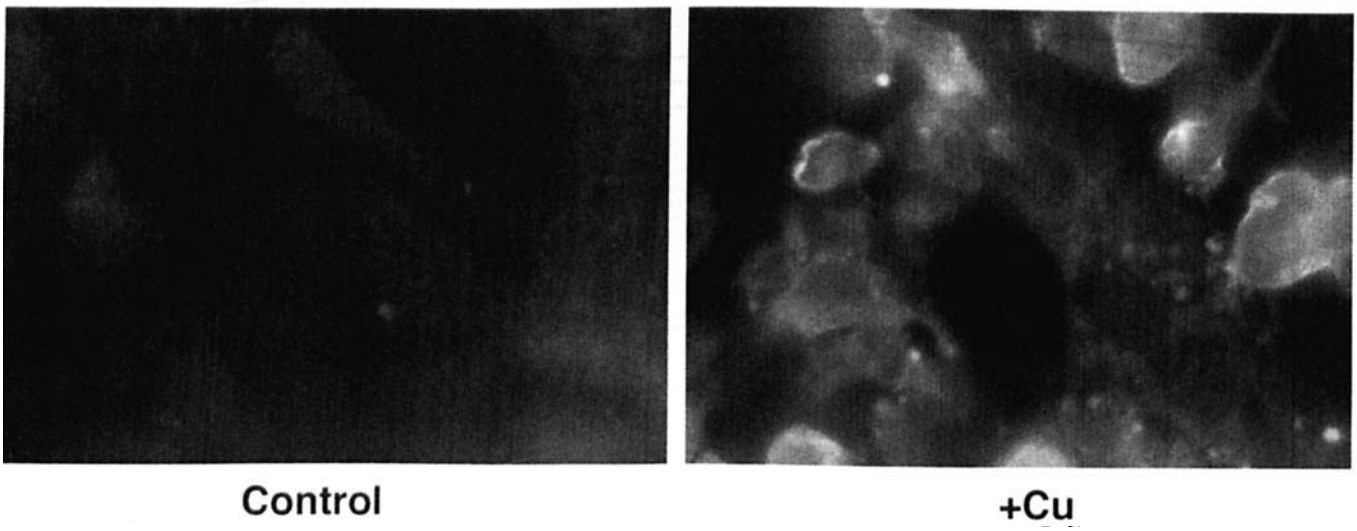

Figure 3. Copper induction of caspase-3 expression in Hep G2 cells. Control and copper-treated (+Cu, 200 μ*M* Cu for 18 hrs) Hep G2 cells were incubated with a caspase-3–specific antibody (CPP-32). Caspase-3 staining was present in copper treated Hep G2 cells (+Cu) but not in control cells. Photomicrographs are representative of images from $n = 6$ dishes at original magnification ×40.

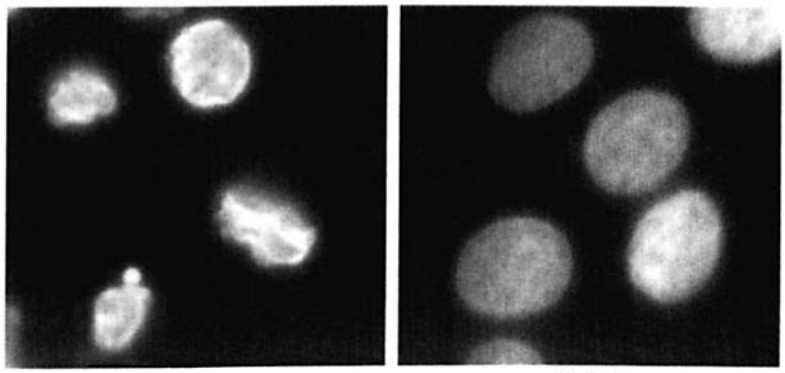

Figure 4. Caspase inhibitor prevents apoptosis in copper-treated Hep G2 cells. Hep G2 cells were pretreated with caspase inhibitor (CI, 50 μ*M*) or vehicle (DMSO) for 1 hr followed by copper treatment (+Cu, 200 μ*M*) for 18 hrs. Cells were fixed and stained with DAPI to permit analysis of nuclear morphology. Photomicrographs are representative of images from $n = 3$ dishes at original magnification ×40.

Further evidence for the apoptotic nature of copper-induced cell death, as opposed to necrotic death, was provided by pretreatment of cells with a caspase-3 inhibitor that prevented copper-induced apoptotic death. Although none of these tests exclude the likely presence of necrosis in addition to apoptosis, they do suggest that apoptosis is a significant factor in copper-mediated hepatocyte death.

Previous work (10) has implicated DNA damage and the tumor suppressor *p53* in copper-induced apoptosis. Damage to DNA induces the tetramerization of *p53* and binding to two copies of the consensus sequence 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by 0–13 base pairs (42). Furthermore, it seems that p53 binding can occur even when several of the base pairs do not conform to the recognized consensus sequence (43). Given the sequence heterogeneity that this provides, it is not surprising that p53 oligomers have been shown to bind to the regulatory regions of a large variety of both proapoptotic and antiapoptotic genes (44).

To test the hypothesis that copper alters the normal transcriptional activity of p53, we used a reporter gene with multiple p53-binding sites attached upstream from the luciferase gene. Given that copper clearly induces p53 expression in both this and in previous work (10), we were surprised to find that that copper treatment inhibited p53 functional activity, as monitored by luciferase activity. Furthermore, copper impaired the ability of p53 to activate the reporter gene in cells co-transfected with the *p53* gene construct.

Caution is warranted when interpreting these results

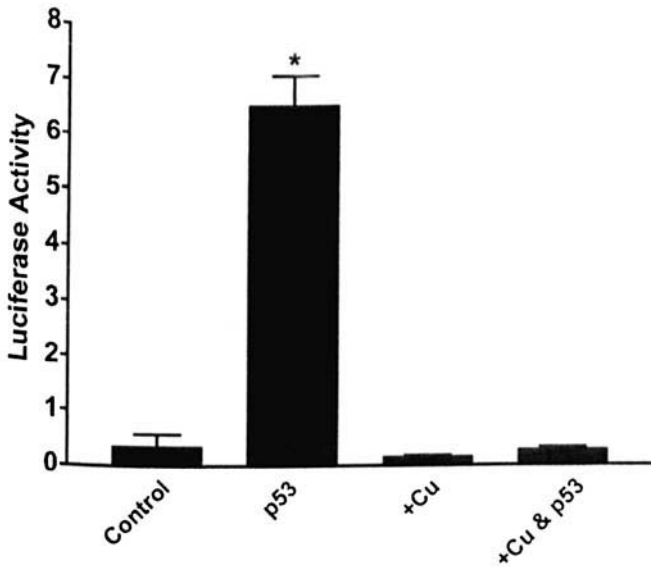

Figure 5. Copper inhibits the transcriptional activity of p53. The effect of copper toxicity on the ability of p53 to activate a luciferase reporter gene with tandem p53-binding sites was measured in Hep G2 cells. Cells transfected with the reporter gene were either co-transfected with a p53 expression vector (p53), treated with 200 μ*M* copper (+Cu), or co-transfected and treated with copper (+Cu & p53). Control cells were transfected with the reporter gene only. Bars represent mean ± SD luciferase activity ($n = 6$ in two separate experiments). *Significantly different from control at $P \leq 0.05$.

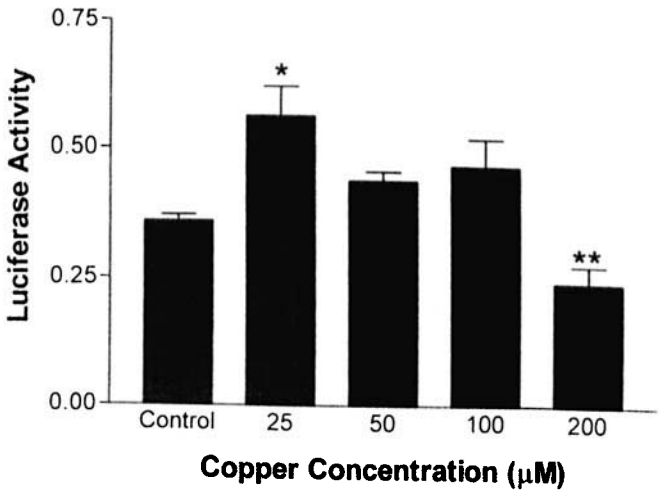

Figure 6. Effect of increasing copper concentrations on p53 transcriptional activity. The effect of increasing media copper concentrations (0, 25, 50, 100, and 200 μ*M*) on the ability of p53 to activate a luciferase reporter gene with tandem p53-binding sites was measured in Hep G2 cells. Control cells were transfected with the reporter gene only. Bars represent mean ± SD luciferase activity ($n = 3$). *Significantly different from control at $P < 0.05$. **Significantly different from 25, 50, and 100 μ*M* copper at $P < 0.05$.

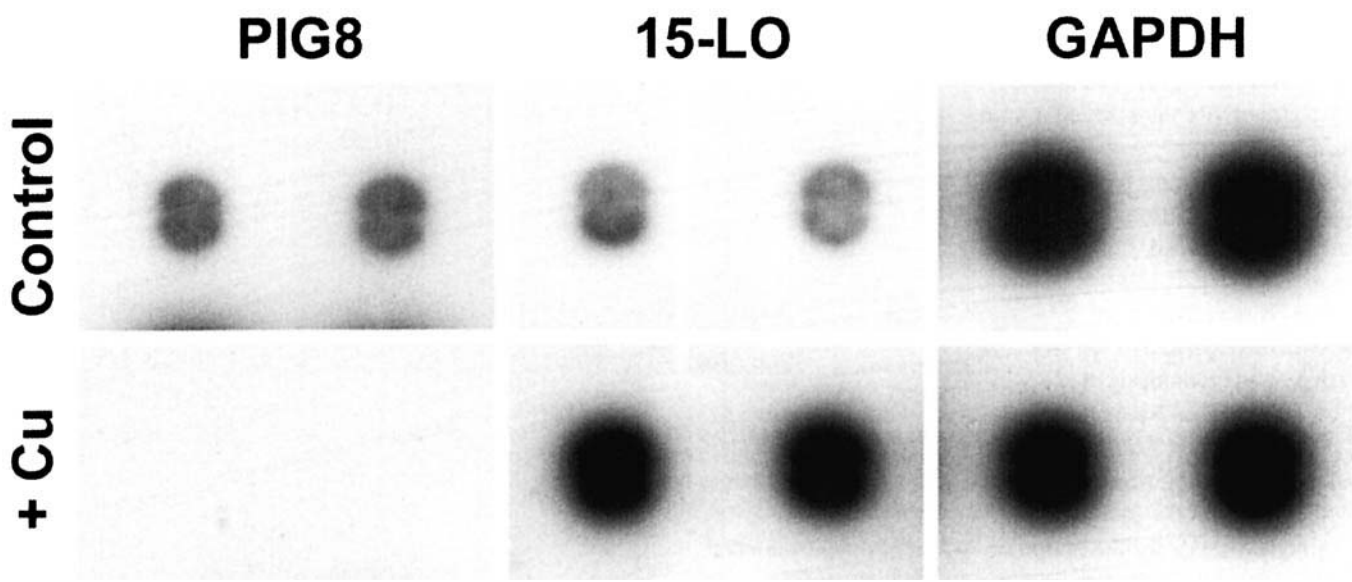

Figure 7. Copper induces alterations in the expression of p53-target genes. Oligonucleotide arrays representing 145 known p53-associated genes were hybridized with biotinylated cDNAs from mRNA extracted from control and copper-treated (200 μ*M* for 18 hrs) Hep G2 cells. Photos show representative mRNAs that were differentially expressed (PIG 8, 15-LO). Glyceraldehyde 3' phosphate dehydrogenase (GADPH) mRNA abundance served as the control.

because it is possible that high concentrations of copper inhibit luciferase activity or induce proteolytic degradation of the luciferase enzyme after it has been synthesized. To eliminate this as a possible explanation, we conducted an *in vitro* experiment in which Hep G2 cell lysates were prepared from cells that expressed luciferase (cells co-transfected with the reporter gene and the *p53* construct). Measurement of luciferase activity in these lysates with and without the addition of 200 μ*M* copper had no effect on luciferase activity (data not shown), suggesting that copper is acting at the level of enzyme synthesis rather than by direct inhibition of enzymatic activity. This finding is consistent with previous work conducted in yeast and glial cells, showing that luciferase enzymatic activity is not impaired by copper (45, 46).

Thus, to understand the mechanism of p53 action in the presence of copper, we used oligonucleotide arrays designed to examine the differential regulation of p53-responsive

Table 1. Copper-Induced Alterations in the Expression of p53-Responsive Genes in Cultured Human Hepatocytes (Hep G2)

Gene	Relative expression[a]	Gene product	Function	Reference
115-LO	3.2 ± 0.2	15-Lipooxygenase	Anti-inflammatory enzyme	28
α-Actin	2.5 ± 0.7	α-Actin	Cytoarchitecture, apoptotic bleb formation	29
RB-1	1.9 ± 0.1	Retinoblastoma susceptibility protein-1	Tumor suppressor, downregulation of fos	30–32
LATS 2	0.7 ± 0.1	Large tumor suppressor-2	Downregulation of Bcl-2, proapoptotic	33
CDKN/p14 ARF	0.6 ± 0.01	Cyclin-dependant kinase inhibitor 2A	Tumor suppressor, regulates p53 stability	34
Fos	0.6 ± 0.2	c-fos	Proto-oncogene, AP-1 binding	35
TGF-β	0.4 ± 0.01	Transforming growth factor-β	Growth factor, proapoptotic	36
LRDD/ PIDD	0.4 ± 0.02	Leucine-rich death domain–containing protein	Regulates cellular growth; proapoptotic	37
Ker 15	0.3 ± 0.04	Keratin 15	Cytoarchitecture	38
MAP 4	0.1 ± 0.08	Microtubule-associated protein 4	Cytoarchitecture, antiapoptotic	39
GPX	0.1 ± 0.05	Glutathione peroxidase	Antioxidant, antiapoptotic	40
PIG 8	0.02	Etoposide-induced protein	Redox regulator, proapoptotic	41

[a] Relative mRNA abundance determined by photodensitometry of duplicates after chemiluminescent detection and normalization to GAPDH mRNA abundance.

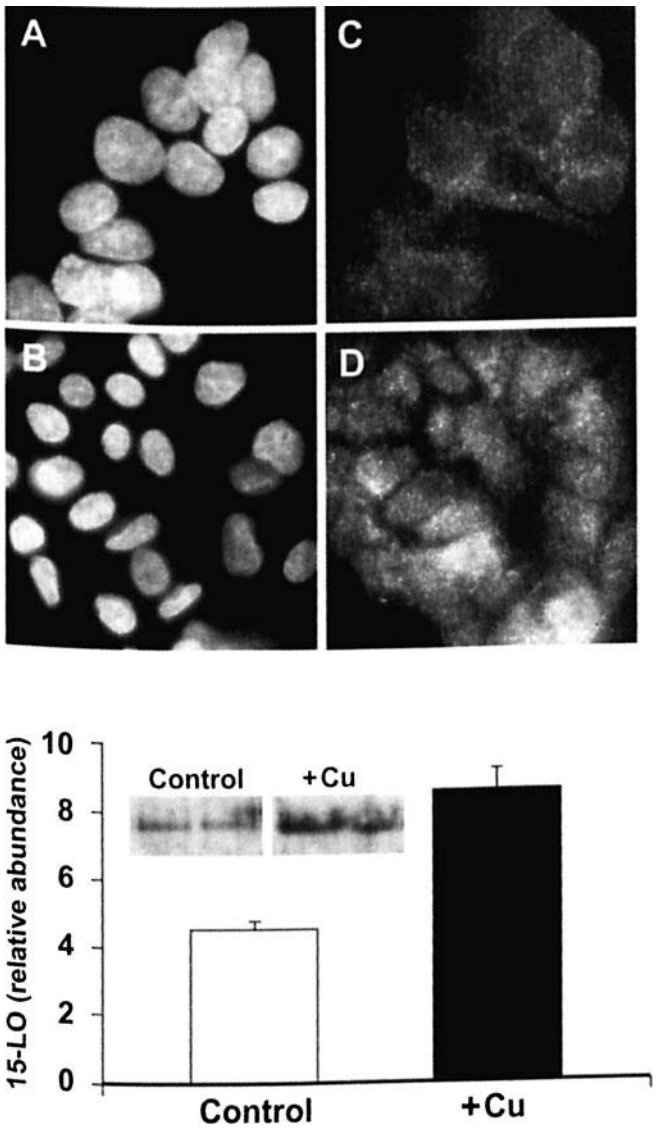

Figure 8. Copper treatment increases immunodetectable 15-LO in Hep G2 cells. (A) Nuclear morphology of untreated control Hep G2 cells as seen after DAPI staining. (B) DAPI-stained nuclei of Hep G2 cells after copper-treatment (200 μM for 18 hrs) showing nuclear condensation. (C) Immunodetectable 15-LO in control Hep G2 cells. (D) Immunodetectable 15-LO in copper-treated cells. Photomicrographs are representative of images from $n=6$ dishes at original magnification ×100. Bottom panel shows Western blot analysis of 15-LO in control and copper-treated (+Cu) Hep G2 cells. Relative abundance of 15-LO was quantified by densitometry ($n=4$ separate dishes).

genes in the presence of high copper. After copper treatment, a variety of proapoptotic and antiapoptotic genes was found to be regulated. For example, the finding that the retinoblastoma-1 gene (*RB-1*) was expressed in Hep G2 cells is, to our knowledge, the first report of *RB-1* expression in a cell of hepatic origin. The *RB-1* gene product is a tumor suppressor protein that plays an important role in the cell cycle and apoptosis by governing the passage of cells through the G_1 phase-restriction point, promoting terminal differentiation, and preventing cell cycle re-entry (30, 31). Furthermore, our finding that *fos* expression is downregulated in copper-treated cells is consistent with the increase in RB-1 expression, because *RB-1* can reduce the transcriptional activity of the *c-fos* genomic promoter (32).

In addition to the upregulation of the proapoptotic *RB-1* gene, the gene that codes for the antiapoptotic enzyme glutathione peroxidase (*GPX*) was downregulated in copper-treated cells. A reduction in GPX, an enzyme that participates in the antioxidant process by converting hydrogen peroxide into water, renders cells vulnerable to free radicals and ROS, further exacerbating copper-mediated DNA damage.

By far the most striking finding was the increase in *15-LO* mRNA abundance. This gene codes for an enzyme responsible for the oxidative metabolism of linoleic and arachidonic acid to a variety of metabolites involved in anti-inflammatory mechanisms, including 12-*S*-hydroxyoctadecadienoic acid (HODE), lipoxins, and 15-*S*-hydroxyeicosatetraenoic acid (27). Interestingly, *15-LO* gene expression has been shown to be upregulated by a mutant conformation of p53 (MTp53; Ref. 47). Although the exact biologic significance of *15-LO* in cancer cells is not known (27), several reports have suggested that HODE (the main metabolite of linoleic acid) inhibits cellular proliferation (48) and induces apoptosis (28). Thus, the upregulation of *15-LO* in copper-treated Hep G2 cells is not only consistent with copper-mediated apoptosis, but also supports the hypothesis generated by the reporter gene assay, namely that high levels of cellular copper can result in alterations in p53.

These findings led to the direct testing of the hypothesis that copper induces the formation of a conformational mutant form of p53 (MTp53). Immunocytochemistry confirmed the presence of both MTp53 and WTp53 in copper-treated Hep G2 cells. There are several possible causes for the observation. First, this is consistent with recent work showing an increase in p53 mutations, such as G:C to T:A transversions in liver samples from WD patients (18). Thus, although not tested in this work, it is possible that copper treatment results in genomic mutations in p53. Second, although there is *in vitro* evidence that copper may result in the displacement of zinc in the p53 structure and that this can be responsible for protein misfolding and the formation of a mutant conformation (22), it is not clear whether this occurs *in vivo* or under the concentrations of copper used in this work to mimic WD.

Finally, there is evidence that the products of lipid peroxidation, such as 4-hydroxynonenal, may cause p53 mutations (49). Thus, we tested the possibility that copper-induced ROS, rather than the interaction of copper with p53, is responsible for the observed induction in MT p53. We show here that addition of either iron or hydrogen peroxide, both of which are powerful oxidants, like copper, induce MTp53. In contrast, addition of the nonoxidative metal, magnesium, produced undetectable levels of MTp53. Together, these data suggest that oxidative processes may be responsible for alterations in p53 conformation observed in the present study. Because the Hep G2 cell line is a hepatoma cell, it is not known whether these same

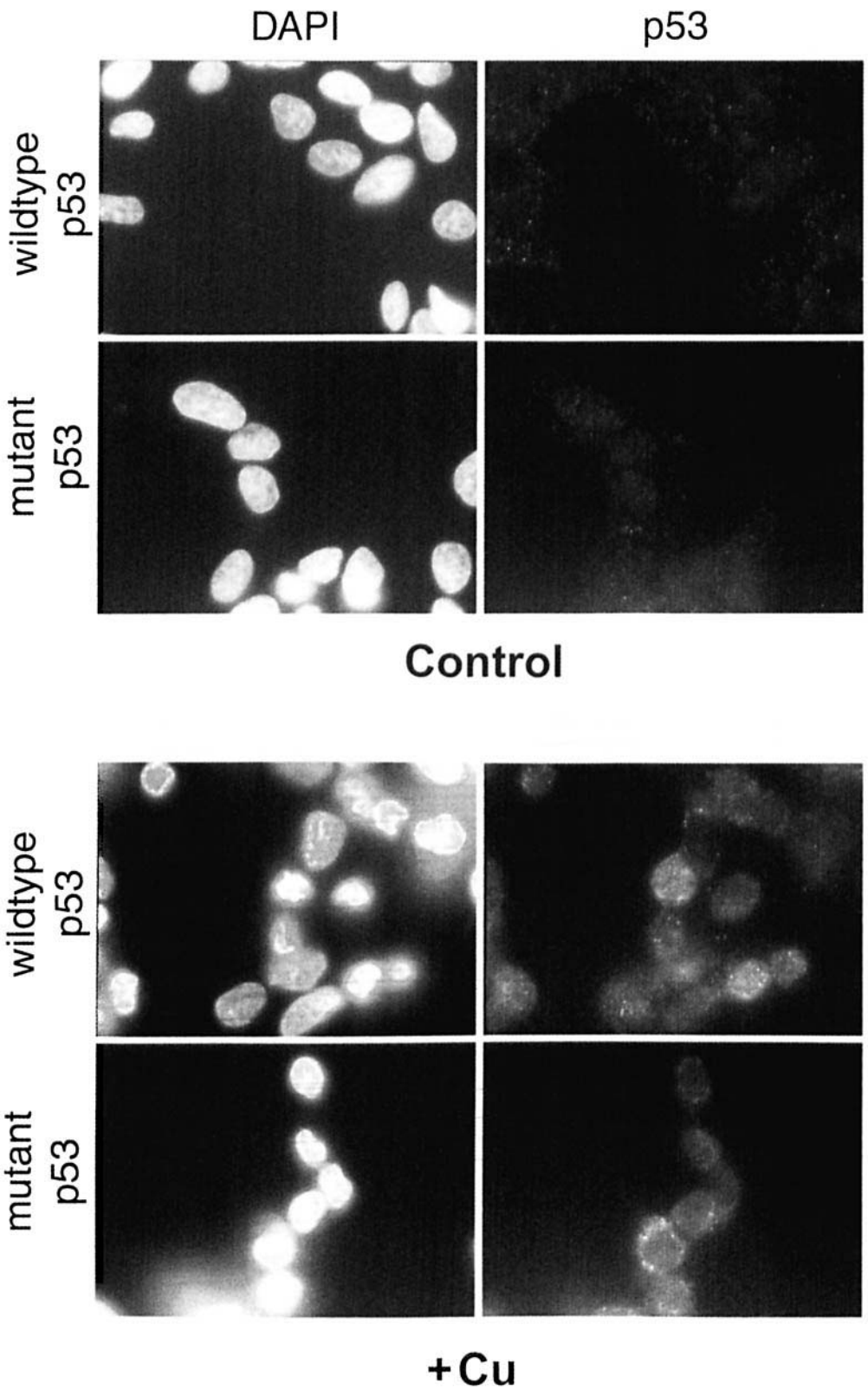

Figure 9. Copper treatment results in the nuclear localization of wild-type and mutant p53. The top panels show the immunocytochemical localization of wild-type and mutant p53 in untreated control cells. Nuclear staining with DAPI was used to confirm the presence of Hep G2 cells in the absence of *p53*. DAPI staining of copper-treated (200 μM for 18 hrs) Hep G2 cells confirmed the induction of apoptosis. Use of conformation-specific antibodies for p53 revealed the presence of both wild-type and mutant p53 in copper-treated cells. Photomicrographs are representative of images from $n = 6$ dishes at original magnification $\times 100$.

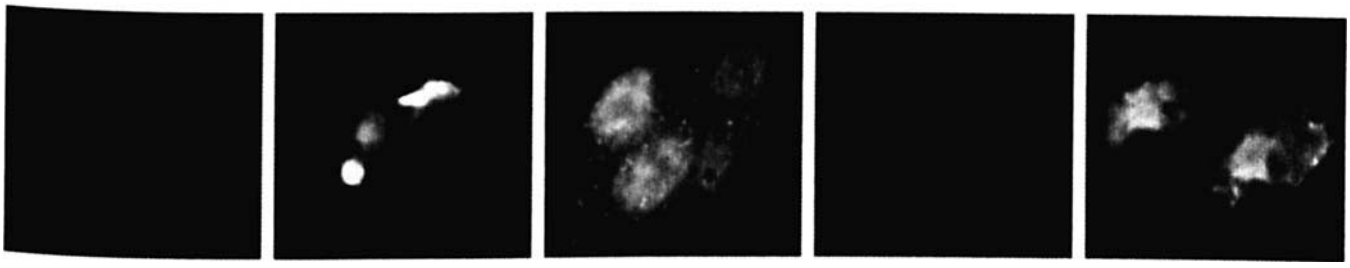

Figure 10. Metal treatment results in mutant p53 confirmation. Hep G2 cells were treated with copper (+Cu), iron (+Fe), magnesium (+Mg), or hydrogen peroxide (+H_2O_2) for 18 hrs and immunostained using a conformation-specific antibody for mutant p53. Photomicrographs are representative of images from $n = 2$ dishes at original magnification ×40.

mechanisms would be at work in noncancerous hepatocytes. However, Hep G2 cells are a good model for the study of *p53* and its mechanism of action because it has been shown that, unlike many other cancer cell lines, these cells synthesize WTp53.

Given the results of the reporter gene assays showing that copper essentially eliminated p53 transcriptional activity, the finding of WTp53 in copper-treated cells was initially surprising. However, these data are, in fact, consistent with several studies showing that MTp53 can act to disrupt the activity of WTp53 (37, 38) and can act as a dominant-negative suppressor of WTp53 activity (50). It should also be noted that, as discussed previously, the consensus binding site provides a significant amount of heterogeneity. The construct used in our reporter gene represents only one of the possible binding sites for p53 and does not represent all of the possible DNA sequences that may bind wild-type or mutant p53. Furthermore, it is important to note that type of mutation, whether conformational or genomic, plays a significant role in the functionality of the p53. Thus, future work will be needed to explore the role of copper in the formation of conformational mutants of p53.

This report suggests that the sequence of events leading to hepatic copper toxicity and hepatocyte apoptosis begins with copper induction of ROS. This not only results in DNA damage, but also results in the appearance of a conformational mutant form of the tumor suppressor protein, p53, with altered functional properties. Under these conditions, we have now identified the downstream genes that are subsequently induced, and seem to be, at least in part, responsible for the apoptotic cell death associated with hepatic copper overload. Future work will be needed to determine the exact mechanisms responsible for the regulation of these proapoptotic and antiapoptotic genes. Specifically, the next stage of this research will need to determine which of these genes are regulated by copper, which are regulated by ROS and oxidative products, and which are transcriptionally regulated by copper-induced p53.

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