Copper-inducible transcription: regulation by metal- and oxidative stress-responsive pathways

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The transition metal copper serves an essential role in biological processes because of its catalytic and structural properties. It is critical for cells to maintain homeostatic concentrations of this metal, because abnormally high or low levels can lead to pathological conditions. Pathologies associated with copper toxicity include gastrointestinal disorders and liver cirrhosis. On a cellular level, the pathological effects associated with copper exposure, cells express metallothioneins (MTs). Metallothioneins are small, cysteine-rich proteins that are ubiquitous among eukaryotes (21, 27). Proposed functions of MTs include maintaining homeostasis for essential metals such as zinc and copper, cellular detoxification, and scavenging free radicals. Elevated concentrations of many transition metals have been shown to elicit rapid induction of MT mRNAs and proteins (21, 23, 27, 35, 42). In addition, a variety of nonmetal stressors such as heat shock, alkylating agents, oxidative stress, and UV radiation induce MT transcription (9, 38, 40, 43, 48). This suggests that MT may function as a general stress-response protein.

Metal-inducible MT transcription is regulated primarily through the interaction between metal response elements (MREs) and metal transcription factor (MTF)-1 (22, 37). MREs are 13- to 15-bp upstream regulatory elements with the core consensus sequence CTNTGCRCNCGG that are found in the promoter region of most metal-inducible MT genes (47). MTF-1 specifically binds to the MRE and has been shown to be essential for the metal-inducible transcription of MT (22, 37). Although MTF-1 is essential for copper-inducible MT transcription, the interaction among MREs, MTF-1, and copper has not been defined.

Additional cis regulatory elements that also regulate inducible MT transcription are the antioxidant response elements (AREs), which have a core consensus sequence TGGAC-NNNGCC (7). AREs regulate oxidative stress-inducible transcription of many genes, including glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase (NQO1), and several MTs (7, 24). The ARE of the GST Ya gene is activated in response to a variety of chemical agents, including metals, β-naphthoflavone, 1-naphthylhydroquinone (tBHQ), butylhydroxyanisole, and hydrogen peroxide (11, 31–33, 49, 52). The ARE-mediated induction of detoxification genes is thought to be a critical mechanism involved in protecting cells from challenges by electrophiles and reactive oxygen species (ROS). The ability of redox cycling transition metals to activate MT transcription through the ARE has not been fully addressed.

One mechanism by which the transcription of detoxification genes are activated is via mitogen-activated protein kinase (MAPK) pathways, which include extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38. Many forms of cellular stress preferentially activate the JNK/SAPK and p38 pathways (16, 29). In human bronchial epithelial cell line BEAS 2B cells,
exposure to copper, vanadium, arsenic, zinc, or chromium results in the phosphorylation and subsequent activation of JNK, p38, and ERK (39). Once activated, ERK, JNK, and p38 can phosphorylate a number of proteins and transcription factors. This results in the enhancement of the transcriptional activity of a multitude of genes (28).

Copper has the potential to mediate several important biological processes through multiple signal transduction pathways. However, the cellular and molecular responses underlying copper-regulated gene expression and toxicity are poorly understood. In this study, we report that copper increases the transcription of MRE- and ARE-containing genes. Transcriptional activation by copper involves MAPK pathways and changes in cellular glutathione status. Results from this study suggest that copper is capable of activating transcription through both metal- and oxidative stress-mediated mechanisms.

**MATERIALS AND METHODS**

**Cell culture.** COS-7 (ATCC CRL-1651) and MTF-1 null cells (dko7) (37) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 5% nonessential amino acids, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C with 5% CO2.

**Reporter gene constructs.** The level of MT transcription was established by measuring the amount of chloramphenicol acetyltransferase (CAT) produced in cells transfected with −42CAT, which contains the minimal mouse MT-I promoter (−42 to +62); −153CAT, which contains the region of the mouse MT-I gene from −153 to +62; MREΔ CAT, which contains all of the tandem copies of MRE-d inserted upstream of the TATA box in −42CAT; and AREΔ,CAT, which contains all of the tandem copies of the upstream stimulatory factor (USF)/ARE from the mouse MT-I gene inserted into −42CAT. The −42CAT plasmid contains a TATA box but lacks metal- or stress-responsive regulatory elements (i.e., negative control). In contrast, −153CAT contains 153 bp of the mouse MT-I regulatory region and contains four functional MREs, as well as an ARE. Complete descriptions of the MT-CAT reporter genes can be found in Dalton et al. (7).

The level of oxidative stress-responsive transcription was measured by using a CAT reporter gene that contains the ARE from the rat NQO1 gene (pARE). pARE consists of a single copy of the ARE from NQO1 fused to a rat GST minimal promoter. As a negative control, a two-nucleotide mutant form of the ARE, which is unresponsive to oxidative stress, was used (pM1) (10, 11). The pM1 plasmid is identical to pARE with the exception of a mutation of the GC box in the consensus ARE sequence (GTGACNNNGC).

**Transient transfection and reporter gene assays.** For transient transfection studies, cells were grown to ~60% confluence in complete DMEM in 24-well dishes. The medium was removed, and cells were washed with Opti-MEM and then transfected using Lipofectin (COS-7) or LipofectAMINE (dko7), according to the manufacturer’s instructions (Life Technologies). Cells were transfected for 3–6 h with 650 ng/well of one of the CAT reporter plasmids and 160 ng/well of the control plasmid pSV-βgal (Promega). To inhibit JNK activity, cells were cotransfected with pcDNA3-Flag-MKK4 (Ala), which expresses a dominant negative form of the JNK-activating kinase MEK-4/SEK-1, thereby inhibiting JNK phosphorylation and activation (51). In experiments utilizing the dominant negative SEK plasmid, cells were transfected with 650 ng/well of CAT reporter plasmid, 160 ng/well of pSV-βgal, and 300 ng/well of pcDNA3-Flag-MKK4 (Ala).

After transfection, the medium was replaced with complete DMEM, and the cells were allowed to recover for ~12 h. Copper (as CuSO4), ROS modulators, or signal transduction inhibitors were then added as described above. CAT concentrations and β-galactosidase activities were determined by sandwich ELISA, using a CAT-ELISA kit (Roche Diagnostics) and the β-Galactosidase Enzyme Assay System (Promega), respectively. All assays were performed in triplicate, and CAT protein levels were normalized to β-galactosidase activity.

**Cytotoxicity.** Cytotoxicity assays were performed as described by Shokri et al. (45). Briefly, cells were seeded in a 48-well tissue culture dish in complete DMEM and incubated for 24 h. Copper sulfate was then added, and incubation continued for an additional 24 h. In experiments in which glutathione was depleted, cells were exposed to 1 mM buthionine sulfoximine (BSO) for 8 h before the addition of copper. After copper exposure, the medium was removed and the cells were then incubated with neutral red solution (40 μg/ml) in DMEM for 3 h at 37°C. The cells were subsequently washed and then fixed with calcium chloride (1%, wt/vol) in formaldehyde (0.5%, vol/vol). To extract the dye from viable cells, cells were lysed with acetic acid (1%, vol/vol) in 50% (vol/vol) ethanol. Optical density was then measured at 540 nm (Abs540). Positive controls, representing 100% cytotoxicity, consisted of cells exposed to distilled water. Cells incubated with complete DMEM in the absence of copper represented 100% viability and were included as a negative control. The level of cytotoxicity was calculated by using the following calculation:

\[
\% \text{Viability} = \frac{\text{mean Abs}_{540} \text{ treated cells}}{\text{mean Abs}_{540} \text{ negative control}} \times 100
\]

**Glutathione measurements.** Cells were grown for 72 h and then incubated in the presence of copper for an additional 24 h. Extracts were prepared from phosphate-buffered saline-washed cells by sonicing cell pellets suspended in 0.5 ml of 20 mM sulfosalicylic acid. The lysate was centrifuged at 10,000 g for 2 min, and the supernatants were collected. Total glutathione (GSH + GSSG) and GSSG concentrations were determined by using the enzymatic recycling assay for mouse [35S]GSH (Promega). The concentration of GSH was determined by using the method of Beaufay (1, 2).

**Statistical analysis.** All statistical analysis was performed using StatView software (SAS Institute). Results are presented as means ± SE. The significance of mean differences was detected by analysis of variance (ANOVA) 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.

**RESULTS**

**Effect of copper and glutathione depletion on cell viability.** The effects of copper exposure and the depletion of glutathione on COS-7 cell viability were determined. Significant decreases in cell viability were observed at copper concentrations of 400 μM and above (Fig. 1). The viability of cells exposed to 400 μM copper and depleted of GSH was significantly lower than cells treated with 400 μM copper alone (40 vs. 71% viable cells, Fig. 1). This finding suggests that glutathione protects the cell from copper-inducible stress. Depletion of GSH in the absence of copper did not significantly affect cell viability.

**Effect of copper on glutathione levels.** Exposure of COS-7 cells to 400 μM copper for 24 h caused a significant decrease (35%) in total glutathione (oxidized plus reduced) (Table 1). Treatment of cells with BSO resulted in a 93% decrease in total glutathione. In addition, treatment with BSO and 400 μM copper caused a 97% decrease in total glutathione. This level of depletion was significantly greater than that observed with BSO alone.

The ratio of reduced to oxidized glutathione is commonly used as an indicator of intracellular oxidative stress. In untreated cells, oxidized glutathione accounted for 1.5% of the total glutathione. This value significantly changed when cells
were treated with 400 μM copper for 24 h or when they were exposed to BSO for 8 h (Table 1). Likewise, cells exposed to 400 μM copper following GSH depletion showed a significant, synergistic increase of the level of oxidized glutathione (Table 1). These results suggest that exposure to copper alters the intracellular redox potential. It should be noted, however, that this observation is confounded by the high degree of toxicity observed in cells exposed to both copper and BSO (Fig. 1).

Effect of copper on metal- and oxidative stress-responsive transcription. The ability of copper to induce MT transcription (i.e., MT transcriptional activity) was investigated by using CAT reporter genes whose expression is regulated by portions of the mouse MT-I promoter, or concatenated copies of MT upstream regulatory elements (7). COS-7 cells, transfected with MT-I-based reporter plasmids, were exposed to 0–600 μM copper for 24 h. Copper at any concentration did not significantly induce transcription when assayed using the −42CAT reporter gene (i.e., negative control; Fig. 2A). In contrast, significant increases in transcriptional activity were observed at copper concentrations of 400 and 600 μM in cells that contained the −153CAT, MREd−CAT, and AREiCAT reporters. The maximum response (3.5-fold increase) was observed at 400 μM in cells transfected with AREiCAT. The decrease in reporter gene activity that was observed at higher concentrations may be attributed to the cytotoxicity observed at these metal concentrations (Fig. 1).

Table 1. Effect of copper and BSO treatment on glutathione status

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Glutathione, pmol/viable cell × 10⁶</th>
<th>Oxidized Glutathione, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>33.4 ± 3.3</td>
<td>1.5 ± 0.24</td>
</tr>
<tr>
<td>Copper (400 μM)</td>
<td>27.1 ± 2.4*</td>
<td>6.4 ± 0.73*</td>
</tr>
<tr>
<td>BSO (1 mM)</td>
<td>2.36 ± 0.08*</td>
<td>6.5 ± 0.93*</td>
</tr>
<tr>
<td>Copper (400 μM) + BSO</td>
<td>1.66 ± 0.04*</td>
<td>26.9 ± 1.9*</td>
</tr>
<tr>
<td>(1 mM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 3 observations. BSO, buthionine sulfoximine. *P < 0.05, significant difference from control cells (untreated).

The increase in AREiCAT transcriptional activity reported above suggests that copper may activate transcription by an oxidative stress-responsive mechanism. The ARE in the mouse MT-I promoter, however, overlaps with a USF sequence. The USF element may influence the responsiveness to copper exposure. To further define the role of oxidative stress in copper-inducible transcription, plasmids containing an ARE from the rat quinone reductase gene were used. A 24-h exposure to 400 μM copper caused a 5.5-fold increase in reporter gene expression in cells transfected with pARE (Fig. 2B). The addition of copper did not cause an increase in the level of reporter gene expression in cells transfected with the negative control plasmid pM1 (Fig. 2B).

MTF-I involvement in copper-induced MT transcription. To further investigate the role of metal-responsive gene regulation in copper-induced transcription, the effect of copper exposure on reporter gene activity was examined in the MTF-1 knockout cell line dko7 (18). Cells were transfected with −42CAT, −153CAT, MREd−CAT, or AREiCAT plasmids and then exposed to 600 μM copper for 24 h. There was no significant

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**Fig. 1.** Effect of copper and glutathione depletion on cell viability. COS-7 cells were exposed to 0–1,000 μM CuSO₄ for 24 h. For cells in which GSH was depleted, cells were exposed to 1 mM buthionine sulfoximine (BSO) for 8 h before the addition of 400 μM copper (Cu). The cytotoxic effects of copper and BSO exposure on cell viability were then determined using the neutral red assay. Data are expressed as means ± SE, n = 6 observations. *P < 0.05, significantly different from cells not exposed to added copper (by ANOVA).

**Fig. 2.** Effect of copper exposure on gene expression. A: COS-7 cells were transfected with metallothionein (MT) reporter genes −42CAT, −153CAT, MREd−CAT (MREi−CAT), and AREiCAT and then exposed to 0–600 μM CuSO₄ for 24 h. B: cells were transfected with reporter genes containing a wild-type antioxidant response element (pARE) or a nonfunctional, mutant form of the ARE (pM1) and then exposed to 400 μM CuSO₄ for 24 h. The level of CAT protein expression was then measured by ELISA and normalized to the amount of β-galactosidase enzyme activity. Data are expressed as means ± SE, n = 3 observations. *P < 0.05, significantly different from cells not exposed to added copper (by ANOVA).
induction of −153CAT and MREd’sCAT expression in dko7 cells, which indicates an essential role for MTF-1 in copper-inducible transcription, via the MRE (Fig. 3). In contrast, there was a significant (5.3-fold) increase in the level of expression when ARE2CAT was used. This finding suggests that copper also activates transcription by an oxidative stress-response mechanism. To further investigate the roles of AREs and oxidative stress in copper-inducible transcription, dko7 cells were transfected with pARE and pM1. Consistent with the earlier results, copper exposure resulted in a significant (4.6-fold) increase in pARE reporter gene expression. These results indicate that both metal- and oxidative stress-responsive mechanisms contribute to copper-inducible transcription. Furthermore, these pathways can function independently.

Effects of GSH depletion on copper-inducible transcription. Treatment of COS-7 cells with BSO caused a >90% depletion of total glutathione (Table 1). A significant increase in the level of copper-inducible expression was observed when glutathione levels were reduced before copper addition, compared with copper-exposed cells that were not pretreated with BSO (Fig. 4A). The depletion of glutathione alone caused a significant increase in the level of reporter gene expression (Fig. 4A). Thus glutathione depletion did not significantly increase the level of-153CAT and MREd’sCAT reporter gene expression. Statistical analysis shows that there was not a synergistic interaction between copper and BSO for any of the reporter genes.

In cells transfected with ARE2CAT (5.2–6.8 fold), a significant increase in transcription was observed, which suggests that an ROS-mediated mechanism may be involved in copper-activated transcription. To confirm the involvement of ROS, cells were transfected with pARE and pM1 plasmids. Consistent with the above results, glutathione depletion increased copper-inducible pARE expression compared with cells exposed to copper alone (Fig. 4B). However, the degree of induction for the pARE plasmid (1.5-fold) was much less than was seen for the ARE2CAT plasmid. In addition, reporter gene expression in the absence of added copper was higher in BSO-treated cells for all four MT plasmids compared with cells not treated with BSO. These results suggest that the levels of ROS generated in the absence of glutathione are sufficient to activate MT transcription via the ARE-mediated pathway.

In cells transfected with the −42CAT reporter, gene expression was significantly higher than in cells exposed to BSO and copper compared with cells treated with copper alone (4.1- to 4.4-fold). The same effect, however, was not observed in cells transfected with the pM1 plasmid. This finding indicates that GSH depletion increases the basal level of transcription.

Effects of ROS scavengers and antioxidants on copper-induced transcription. Previous experiments demonstrated that pretreatment of cells with the ROS scavengers aspirin and vitamin E partially protects cells from the cytotoxic effects of transition metals (34). Aspirin and/or vitamin E had no effect on levels of expression observed using the −42CAT reporter (Table 2). Aspirin or vitamin E treatment had no significant effect on the level of −153CAT expression, relative to the
Table 2. Effects of aspirin and vitamin E on copper-induced transcription

<table>
<thead>
<tr>
<th>Vector</th>
<th>Treatment</th>
<th>Normalized CAT Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>−42CAT</td>
<td>None</td>
<td>0.131±0.011</td>
</tr>
<tr>
<td></td>
<td>Copper</td>
<td>0.105±0.006</td>
</tr>
<tr>
<td></td>
<td>Copper + aspirin</td>
<td>0.096±0.010</td>
</tr>
<tr>
<td></td>
<td>Copper + vitamin E</td>
<td>0.131±0.008</td>
</tr>
<tr>
<td></td>
<td>Copper + aspirin + vitamin E</td>
<td>0.097±0.005</td>
</tr>
<tr>
<td>−153CAT</td>
<td>None</td>
<td>0.154±0.022</td>
</tr>
<tr>
<td></td>
<td>Copper</td>
<td>1.934±0.071</td>
</tr>
<tr>
<td></td>
<td>Copper + aspirin</td>
<td>1.831±0.027</td>
</tr>
<tr>
<td></td>
<td>Copper + vitamin E</td>
<td>1.97±0.031</td>
</tr>
<tr>
<td></td>
<td>Copper + aspirin + vitamin E</td>
<td>2.139±0.085*</td>
</tr>
<tr>
<td>MRECAT</td>
<td>None</td>
<td>0.197±0.012</td>
</tr>
<tr>
<td></td>
<td>Copper</td>
<td>1.726±0.054</td>
</tr>
<tr>
<td></td>
<td>Copper + aspirin</td>
<td>1.511±0.038*</td>
</tr>
<tr>
<td></td>
<td>Copper + vitamin E</td>
<td>1.532±0.031*</td>
</tr>
<tr>
<td></td>
<td>Copper + aspirin + vitamin E</td>
<td>1.506±0.014*</td>
</tr>
<tr>
<td>ARECAT</td>
<td>None</td>
<td>0.395±0.049</td>
</tr>
<tr>
<td></td>
<td>Copper</td>
<td>0.826±0.051</td>
</tr>
<tr>
<td></td>
<td>Copper + aspirin</td>
<td>0.526±0.019*</td>
</tr>
<tr>
<td></td>
<td>Copper + vitamin E</td>
<td>0.607±0.039*</td>
</tr>
<tr>
<td></td>
<td>Copper + aspirin + vitamin E</td>
<td>0.607±0.054*</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 3 observations. Cells were transfected with reporter genes and exposed to 400 μM CuSO₄ for 24 h. Aspirin (30 μM) or vitamin E (50 μM) were added 2 h before the addition of copper. CAT, chloramphenicol acetyltransferase. *P < 0.05, significantly different from treatment with copper alone (by ANOVA).

level of expression observed with copper alone. In combination, however, aspirin and vitamin E caused a 10.6% increase that was significantly higher than treatment with copper alone.

Pretreatment of cells with aspirin, vitamin E, or the combination of the two before copper addition significantly decreased MREdCAT expression (~12.5% for each). Similar results were obtained in cells transfected with ARECAT. Aspirin caused a 36.3% decrease in copper-inducible transcription, vitamin E caused a 26.5% decrease, and in combination, aspirin and vitamin E caused a 26.5% decrease. The combination of aspirin and vitamin E was not additive.

Effects of signal transduction inhibitors on copper-induced transcription. COS-7 cells were treated with kinase inhibitors to identify potential signal transduction pathways that may regulate copper-inducible transcription. The protein kinase C (PKC) inhibitor H-7 was used to determine the role of PKC in the transcriptional response to copper. Treatment with 100 μM H-7 completely blocked copper-induced −153CAT, MREdCAT, and pARE expression (Fig. 5). Copper (400 μM) did not significantly change the level of transcription of ARECAT during the 4-h incubation used in this and subsequent inhibitor studies. Time-course studies (results not shown) have shown that a 4-h exposure to 400 μM copper is insufficient to induce ARECAT reporter gene expression, whereas a 24-h exposure as used above induces significant levels of expression.

MAPK inhibitors were used to investigate the role of ERK1/2, p38, and JNK signaling pathways on copper-inducible gene expression. ERK1/2 and p38 were inhibited by the chemicals PD-98059 and SB-203580, respectively. JNK inhibition was achieved by transfecting the cells with a plasmid expressing a dominant negative form of SEK-1 that prevents the phosphorylation and activation of JNK (51). Kinase inhibitors were added individually (Fig. 6) or in combinations (Fig. 7). Inhibition of p38 kinase caused a significant increase in copper-inducible expression of −153CAT and MREdCAT. Similarly, inhibition of JNK activity caused an increase in −153CAT. The inhibition of ERK1/2 activity did not significantly affect copper-inducible transcription of any of the reporter genes.

Exposure to the combination of ERK and p38 inhibitors caused a significant decrease in the level of copper-inducible expression of −153CAT and MREdCAT. Similarly, inhibition of ERK in combination with either p38 or JNK inhibition caused a significant decrease in copper-inducible expression of −153CAT and MREdCAT. In contrast, the combination of JNK and p38 inhibitors caused a small but significant increase in −153CAT and MREdCAT. Simultaneous inhibition of the three MAPKs caused a significant decrease in copper-inducible expression of −153CAT and MREdCAT. In all of the inhibitor studies, treatment with inhibitors alone did not significantly affect reporter gene expression (data not shown).

DISCUSSION

The ability of several transition metals and oxidative stress to induce MT transcription has been well established (4, 7, 8). Studies of MT transcription, however, have primarily focused on the ability of zinc and cadmium to active gene expression through interactions between MTF-1 and MREs. In addition, the mechanism by which MT expression is induced by ROS has also been examined. Oxidative stress, produced by exposing cells to hydrogen peroxide or bHQ, activates transcription through MREs. The USF/ARE sequence in mouse MT-I mediates induction by hydrogen peroxide and cadmium but not by zinc and bHQ (7, 8). Unlike zinc and cadmium, copper can directly catalyze the formation of ROS. Thus copper has the potential to affect transcription by two separate mechanisms: a metal-responsive pathway and an oxidative stress-responsive pathway. The results presented in the current study provide...
evidence that copper is able to induce transcription via both pathways.

Copper activation of transcription via a metal-responsive pathway was demonstrated using reporter genes in wild-type and MTF-1 null cells. Copper activates transcription via the MRE in a concentration-dependent manner (Fig. 2). In addition, copper did not induce MRE-mediated transcription in the absence of MTF-1 (Fig. 3.). These results are consistent with those previously observed in BHK cells containing a MRE-βGeo reporter gene (36). Copper has also been shown to increase transcription factor binding to the MRE in the human MT-2A gene (20).

Several experiments indicate that copper activates transcription by inducing oxidative stress and that the oxidative stress response is independent of the metal response. First, exposure to copper induces transcription of reporter genes containing AREs from MT-I and NQO1 (Fig. 2). The magnitude of the response is attenuated when cells are exposed to chemicals that reduce ROS (Table 2) and increases following glutathione depletion (Fig. 4). The observation that ROS-mediated gene expression occurs in the absence of MTF-1 suggests that the
metal-responsive transcription machinery is not required for ROS-activated transcription.

Copper can induce oxidative stress by two mechanisms. First, it can directly catalyze the formation of ROS via a Fenton-like reaction (19, 44, 50). Second, exposure to elevated levels of copper significantly decreases glutathione levels (Table 1). An underlying mechanism of copper- and cadmium-induced oxidative damage is the depletion of glutathione. Depletion of intracellular GSH has also been shown to increase the cytotoxic effects of other classes of xenobiotics (5, 15, 46). A decrease in glutathione would allow the endogenous levels of ROS to become cytotoxic. The large increase in copper toxicity due to GSH depletion (Fig. 1) demonstrates that GSH contributes to the cellular defense against copper toxicity.

Depletion of glutathione by BSO treatment causes an increase in oxidative stress responsive MT transcription (Fig. 4). Glutathione has multiple functions in intracellular copper metabolism and detoxification. It has been proposed that glutathione quickly binds the metal following uptake. Once copper is bound, the metal can then be transferred to other proteins (13, 14). Glutathione also has several roles in copper detoxification. It can ameliorate copper toxicity by directly chelating the metal. Binding to the thiol group can protect cells by maintaining copper in a reduced state, making it unavailable for redox cycling. Glutathione is also a substrate for several enzymes that remove ROS as well as a hydroxyl radical scavenger. The depletion of glutathione may allow the metal to remain in a state that is 1) a stronger inducer of metal-responsive transcription and 2) more catalytically active, thus producing higher levels of ROS, which activates oxidative stress-responsive transcription.

Additional evidence that copper activates transcription via ROS was demonstrated in studies in which cells were treated with aspirin or vitamin E. Treatment with aspirin or vitamin E led to decreases in copper-induced transcription, using MRE\textsuperscript{d}CAT or ARE\textsuperscript{d}CAT reporters (Table 2). The decrease was greater for ARE\textsuperscript{d}CAT, which is consistent with reduction in oxidative stress. In addition, the level of reporter gene expression did not change when aspirin or vitamin E was added simultaneously with the metal, suggesting that the effects of copper were primarily intracellular (results not shown). The mechanisms by which aspirin and vitamin E treatment reduce copper-inducible transcription are currently unclear. Vitamin E is a lipid soluble vitamin that acts as an antioxidant in cells to interrupt the propagation of lipid peroxidation in the plasma membrane (6). Treatment with vitamin E may reduce the formation of secondary products of lipid peroxidation that can potentially trigger signal transduction pathways. The effects of aspirin are likely due to a different mechanism. Salicylic acid is commonly used as a trap for hydroxyl radicals, which suggests that aspirin may act as a radical scavenger. Aspirin also possesses some degree of metal-binding ability and may thus withdraw copper ions from the site of ROS formation.

To identify potential signal transduction pathways that may mediate copper-activated transcription, the effects of kinase inhibitors on metal-activated transcription were examined. Inhibition of PKC activity prevents both ARE- and MRE-mediated transcription (Fig. 5). This observation suggests that copper activates both metal- and oxidative stress-responsive cascades through signaling pathways that are regulated by PKC. This is similar to previous results that demonstrated the involvement of PKC in metal-inducible transcription, which is activated by zinc and cadmium (30, 41). In addition, it has been shown that inhibition of PKC in Chinese hamster ovary cells blocks MT mRNA transcription, suggesting that PKC is essential for MT expression (53). The inhibition of ARE-mediated, copper-inducible transcription is consistent with PKC regulation of the oxidative stress response (17).

MAPK inhibitors were used individually and in combinations to identify the MAPK pathways that may be involved in regulating copper-inducible transcription. Copper is capable of activating the three MAPK pathways (ERK, p38, and JNK), often in a cell type-specific manner (25, 39). Inhibition of ERK1/2 did not have a significant effect on copper activation of −153CAT and MRE\textsuperscript{d}CAT reporter genes (Fig. 6). Inhibition of JNK/SAPK or p38 activity led to an increase in copper-inducible transcription, which suggests that JNK/SAPK and p38 may be negative regulators of the response to copper. Whereas ERK1/2 inhibition alone had little effect, in combination with p38 inhibition there was a significant decrease in reporter gene activity (Fig. 7). A similar response was observed when ERK1/2 and JNK/SAPK were both inhibited. Inhibition of both JNK/SAPK and p38 led to a significant increase in copper activation, consistent with inhibition of either pathway alone. Inhibition of all three kinases led to a strong reduction in copper-inducible transcription, which suggests that ERK1/2 has a dominant effect over both p38 and JNK (Fig. 7). Comparable results were obtained when the three MAPK pathways and zinc- and cadmium-inducible transcription were examined (unpublished data). It has been suggested that ERK is capable of attenuating both p38 and JNK activity through its activation of MAP kinase phosphatase-1 (MKP-1). ERK phosphorylation of MKP-1 thereby activates it to dephosphorylate p38 and JNK, resulting in an attenuation of their activity (12). These findings may explain the decreases in copper-induced MT transcription in response to the inhibitor combinations (Fig. 7).

The kinase inhibitor studies are consistent with a model in which copper activates a MAPK signal transduction cascade to ultimately affect the level of MTF-1 phosphorylation to induce transcription. This model is similar to that previously reported for cadmium and zinc (41). It is unlikely that copper is directly binding to MTF-1 to activate transcription; however, an alternative model suggests that copper may displace zinc from MT (54). The “free” zinc would then bind to a zinc finger in MTF-1 that would allow the transcription factor to bind to the MRE and activate transcription.

The results presented in this report support a model in which copper activates transcription via both metal- and oxidative stress-responsive mechanisms. Copper is able to activate transcription through MREs and AREs. In addition, glutathione modulates the level of metal- and oxidative stress-inducible transcription. In COS-7 cells, the ERK1/2 pathway exerts a dominant effect relative to p38 and JNK in regulating metal-inducible MT transcription. Both metals and ROS can independently affect the activity of members of the three MAPK signaling pathways. Thus the roles of specific kinases in regulating transcription in response to these activators may be overlapping. Furthermore, there are additional signaling pathways (casein kinase II, calcium-activated kinase) that participate in the regulation of metal-responsive MT activation (41). Additional studies will be required to more adequately assess copper activation of signal transduction and transcription.
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