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Bifunctional Anti-/Prooxidant Potential of Metallothionein: Redox Signaling of Copper Binding and Release

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ABSTRACT

Metallothioneins (MTs) are cysteine-rich metal-binding proteins that exert cytoprotection during metal exposure and oxidative stress. The roles of MT in copper (Cu) binding and release and modulation of redox cycling are unresolved. We hypothesized that Cu-binding to MT renders Cu redox inactive, but that oxidation of free thiols critical for metal binding can reduce MT/Cu interactions and potentiate Cu redox cycling. Overexpression of MT in cells by cadmium pretreatment or ectopic overexpression by gene transfer confers protection from Cu-dependent lipid oxidation and cytotoxicity. Using a chemically defined model system (Cu/ascorbate/H_2O_2) to study Cu/MT interactions, we observed that MT inhibited Cu-dependent oxidation of luminol. In the absence of H_2O_2, MT blocked Cu-dependent ascorbyl radical production with a stoichiometry corresponding to Cu/MT ratios ≤12. In the presence of H_2O_2, Cu-dependent hydroxyl radical formation was inhibited only up to Cu/MT ratios ≤6. Using low-temperature EPR of free Cu^{2+} to assess Cu/MT physical interactions, we observed that the maximal amount of Cu^{1+} bound to MT corresponded to 12 molar equivalents of Cu/MT with Cu and ascorbate alone and was reduced in the presence of H_2O_2. 2,2′-Dithiodipyridine titration of MT SH-groups revealed a 50% decrease after H_2O_2, which could be regenerated by dihydrolipoic acid (DHLA). DHLA regeneration of thiols in MT was accompanied by restoration of MT's ability to inhibit Cu-dependent oxidation of ascorbate. Thus, optimum ability of MT to inhibit Cu-redox cycling directly correlates with its ability to bind Cu. Some of this Cu, however, appears releasable following oxidation of the thiolate metal-binding clusters. We speculate that redox-dependent release of Cu from MT serves both as a mechanism for physiological delivery of Cu to specific target proteins, as well as potentiation of cellular damage during oxidative stress. Antiox. Redox Signal. 1, 349–364.

INTRODUCTION

SEVERAL ENDOGENOUS TRANSITION METALS, particularly copper (Cu), are potent inducers of oxidative stress if their redox activity is not limited due to sequestration by specialized proteins. Cu^{1+}, the form mostly likely encountered within the intracellular reducing environment, can react with oxygen and H_2O_2 to yield superoxide and hydroxyl radical species, respectively. Furthermore, the requirement of a number of enzymes for copper, such as cy-

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tochrome oxidase and superoxide dismutase among others, requires that Cu be efficiently shuttled from "storage depots" to apoenzymes with minimal redox consequences.

Metallothioneins (MTs) are small (60–62 amino acids) cysteine-rich (30%) metal-binding proteins (Kagi and Schaffer, 1988). Since its original discovery and isolation as a cadmium (Cd)-binding protein in horse kidney, it has become increasingly clear that MTs can serve to protect cells and animals from the toxic effects of various metals (Webb and Verschoyle, 1976; Lee *et al.*, 1989; Liu *et al.*, 1990; Waalkes and Goering, 1990). The ability of MT, however, to protect against Cu-dependent toxicity remains controversial. For example, Cu-containing MT has been shown to modify DNA oxidatively (Oikawa *et al.*, 1995), as well as to give rise to hydroxyl radical (Suzuki *et al.*, 1996). Cu/MT complexes accumulate in the liver and may contribute to the hepatitis observed in Long-Evans Cinnamon rats, an animal model for Wilson's disease (Suzuki, 1995).

The tertiary structure of MT is such that the 20 cysteines are arranged into two separate thiolate cluster domains termed α and β (Otovos and Armitage, 1985). The thiolate clusters serve to coordinate with various divalent metals (Zn, Cd, Pb) in a tetrahedral fashion resulting in the association of 7 metal ions/molecule of MT. In contrast, MT can bind up to 12 atoms of Cu^{1+} probably arranged in a trigonal and/or digonal configuration (Presta *et al.*, 1995). The stability constant for monovalent Cu is about 100-fold greater than for Cd, which in turn is about 1,000-fold greater than for Zn (Hamer, 1986). This suggests the possibility that significant amount of Cu can be associated with MT under *in vivo* conditions.

Free thiol groups on proteins, however, are exquisitely sensitive to oxidation. The large number of free thiol groups in MT suggests that the metal-binding capacity of this protein can be compromised under conditions of oxidative stress. Indeed, several studies have shown that oxidation of MT by H_2O_2 and other free radical species can mediate Zn release from MT (Kroncke *et al.*, 1994; Maret, 1994; Quesada *et al.*, 1996). In fact, it has been proposed that redox mechanisms can physiologically regulate Zn transfer from MT to specific Zn-containing target proteins (Cano-Gauci and Sarkar, 1996; Jacob *et al.*, 1998; Maret and Vallee, 1998). There is little information available, however, regarding the ability of sulfhydryl oxidation to mediate Cu release or transfer from MT.

Here we examined the effects of MT on modulation of Cu-dependent oxidative stress in intact cells, as well as, in a simple cell-free model system. We also compared the ability of native and partially oxidized MT to bind Cu and modulate its redox cycling. Furthermore, we assessed the ability of dihydrolipoic acid, an endogenous thiol antioxidant, to regenerate cysteines in thiolate clusters from oxidized MT and, hence, restore Cu-binding capacity.

METHODS

Reagents

MT_{II} from rabbit liver (approximately 5.5% Cd, 0.7% Zn), sodium ascorbate, ascorbate oxidase, catalase, H_2O_2, desferoxamine mesylate, 8-hydroxyquinoline, $CuSO_4$, and $CdCl_2$ were obtained from Sigma Chemical Co (St. Louis, MO). We purchased 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) from Aldrich (Milwaukee, WI) and ascorbic acid from Fisher Scientific (Pittsburgh, PA). *cis*-Parinaric acid (*cis*-PnA) was from Molecular Probes (Eugene, OR). Alamar Blue was from Alamar Biosciences (Sacramento, CA). To remove trace metal ions, we prepared and treated sodium phosphate buffer solutions (pH 7.4) and all other stock solutions with Chelex-100 resin (BIO-RAD, Hercules, CA).

Cell culture and estimation of viability

HL-60 cells were routinely split at 3- to 4-day intervals and cultured in RPMI 1640 containing 15% fetal bovine serum (FBS) under CO_2 at 37°C. For MT induction, cells were cultured in medium containing $CdCl_2$ (10 μM) for 24 hr. Cells were then centrifuged and resuspended in Cd-free medium and cultured for another 24 hr. To assess Cu toxicity, we resuspended Cd-exposed and unexposed cells in 115 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 5 m*M* NaH_2PO_4, 10 m*M* glucose, and 25 m*M* HEPES, pH 7.4, at 1×10^6 cells/ml containing various concentrations

of $CuSO_4$ and incubated them at 37°C for 2 hr. Viability was determined by exclusion of Trypan Blue.

Sheep pulmonary artery endothelial cells (SPAEC) were cultured in low-glucose Dulbecco's modified essential medium (DMEM) (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS under CO_2 at 37°C. SPAEC cells were transiently transfected with a plasmid containing a hMTIIA cDNA plasmid under the control of the β-actin promoter using lipofectamine (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's instructions. MT levels in transfected and wild-type cells were determined using the ^{109}Cd binding assay described by Eaton and Toal (1982) and normalized to total cellular proteins determined by a modified Bradford assay (BIO-RAD, Hercules, CA). Cu-dependent cytotoxicity of SPAEC cells was determined by plating cells into 24-well plates (75,000 cells/well). Cells were then exposed to indicated concentrations of $CuSO_4$ in serum-free medium containing 5 μM 8-hydroxyquinoline to aid the intracellular uptake of Cu (Crutchley and Que, 1995) for 2 or 24 hr. Medium also included 5 μM desferroxamine to chelate any adventitious iron. Viability was determined by the Alamar Blue assay essentially as previously described (Pitt *et al.*, 1997).

Electron paramagnetic resonance of ascorbyl radical and DMPO-OH adduct

Electron paramagnetic resonance (EPR) measurements were performed on a JEOL-RE1X spectrometer at 25°C using gas-permeable Teflon tubing (0.8-mm internal diameter, 0.013-mm thickness) obtained from Alpha Wire Corporation, (Elizabeth, NJ). The tube (approximately 8 cm in length) was filled with 60 μl of a mixed sample, folded into quarters, and placed in an open 3-mm internal-diameter EPR quartz tube so that all of sample was within the effective microwave irradiation area. Spectra of ascorbate radicals and DMPO-OH adducts were recorded at 335.5 mT–center field, 20 mW–power, 0.04 mT–field modulation, 10 mT–sweep width, 500–receiver gain, 0.03 sec–time constant.

Low-temperature EPR of Cu^{2+}

Cu/MT interaction was indirectly measured using the determination of free paramagnetic Cu^{2+} by low-temperature EPR. Various concentrations of MT, $CuSO_4$ (60 μM) and sodium ascorbate (4 mM) were incubated in 50 mM phosphate buffer (0.6 ml) for 2 min to allow Cu-MT binding. Remaining free Cu was then converted to Cu^{2+} by addition of 1 unit of ascorbate oxidase and incubation at room temperature for 10 min. Samples were frozen in liquid nitrogen (−196°C) until analysis. Low-temperature (77 K) EPR spectra were then obtained using a Jeol JES-RE1X spectrometer (9.078 GHz–frequency, 294.5 mT–center field, 100 mT–field range, 0.5 mT–amplitude modulation, 10 W–microwave power, 0.3 sec–time constant, 8 min–time scan). Double integration of the Cu^{2+} spectra and comparison to spectra obtained from Cu-EDTA standards of known concentration were used to estimate the amount of paramagnetic Cu^{2+} content. The amount of EPR-silent Cu resistant to oxidation and, presumably bound to MT, was determined as the difference between total paramagnetic Cu^{2+} measured in absence of MT and Cu^{2+} measured in the presence of MT.

Determination of reduced thiols in MT

To measure Cu/H_2O_2-dependent oxidation of MT sulfhydryls, we incubated MT (20 μM) alone or with $CuSO_4$ (20 μM)/ascorbate (200 μM)/H_2O_2 (400 μM) for 5 min at 25°C in 100 μl of 50 mM sodium phosphate buffer, pH 7.4. The reaction was stopped by addition of 2.5 U ascorbate oxidase (Sigma) and 3 U catalase (Sigma). Samples (100 μl) were then acidified in 0.2 M acetate buffer, pH 4.0, and free sulfhydryl groups were quantified by reaction with 2,2′-dithiodipyridine (DTDP) as described by Pedersen and Jacobsen (1980) and measuring the absorbance at 343 nm with a Shimadzu 160U UV/Visible recording spectrophotometer. The number of reduced thiols per molecule of MT was calculated based on the extinction coefficient, $\epsilon_{343} = 7600\ M^{-1}cm^{-1}$.

To measure sulfhydryl content of MT after oxidation and regeneration with dihydrolipoic acid, we developed a technique that would allow the elimination of DHLA thiols prior to as-

say of MT. For these experiments, MT (30 μM) was dissolved in 10 mM Tris, pH 7.4, and incubated with or without H_2O_2 (8 mM) for 20 min at 28°C. Reactions were stopped by the addition of 2.5 U/ml of catalase and centrifuged once through Microcon YM-3 (3,000 MW cutoff, Millipore, Bedford, MA) filters at 10,000 × g for 45 min at 4°C. The retentate containing MT was diluted to the initial volume and then exposed to various concentrations of dihydrolipoic acid (DHLA) in buffer containing 100 μM desferroxamine and 100 μM diethylenetriaminepentaacetic acid (Sigma) to chelate any adventitious iron or copper, respectively, for 30 min. MT was separated from DHLA by centrifugation four times through Microcon YM-3 filters. The retentate from each spin was reconstituted in buffer to its original volume before the next sequential centrifugation. The final retentate then analyzed for thiols based on DTDP titration or added to Cu/ascorbate system for EPR determination of asorbate radical as described above. Experiments with unoxidized MT demonstrated that recovery of MT under these conditions is greater than 95% (data not shown).

Lipid peroxidation

The oxidant-sensitive, fluorescent fatty acid, *cis*-PnA was employed as a sensitive probe to assess lipid peroxidation in live cells (Ritov *et al.*, 1996; Fabisiak *et al.*, 1997). SPAEC were metabolically labeled with *cis*-PnA/human serum albumin complex (5 μM *cis*-PnA/0.5 mg human serum albumin/1 × 10^6 cells per ml) for 2 hr at 37°C. After washing to remove any excess unincorporated *cis*-PnA, labeled cells were incubated in phenol red-free DMEM containing 5 μM 8-hydroxyquinoline, 0.5 μM desferoxamine in the presence or absence of 0.5 μM $CuSO_4$ for 1 hr at 37°C. After incubation, cells were scraped, lipids extracted by the Folch procedure, and phospholipids resolved by high-performance liquid chromatography (HPLC) as previously described (Ritov *et al.*, 1996; Fabisiak *et al.*, 1997). Oxidation of specific *cis*-PnA-containing phospholipid classes was assessed by the loss of fluorescence in each phospholipid peak.

Luminol-enhanced chemiluminescence

Incubations were carried out in 50 mM phosphate buffer, pH 7.4, containing luminol (400 μM), ascorbic acid (500 μM), and H_2O_2 (400 μM) in the absence and in the presence of MT (0.1–2.5 μM) and were initiated by addition of $CuSO_4$ (5 μM). The time-dependent chemiluminescence responses were recorded using a Luminescent Analyzer 633 (Coral Biomedical, San Diego, CA) over 3 min using a chart recorder (WR 3101, Graphtec, Inc., Japan).

RESULTS

MT protects cells from Cu-mediated toxicity

To determine the potential cytoprotective effects of MT against Cu toxicity, we used systems where MT levels could be easily manipulated in intact cells. It is well known that MT is a highly inducible protein whose expression can be rapidly upregulated by exposure of cells to a number of stimuli, including exposure to heavy metals (Durnam and Palmiter, 1981; Hager and Palmiter, 1981; Hamer, 1986). For our initial experiments, we exposed HL-60 cells to $CdCl_2$ for 24 hr and then compared the ability of various concentrations of $CuSO_4$ to induce cytotoxicity between induced and naïve untreated cells. Figure 1 shows the concentration-response relationships for Cu-mediated cell death between control and Cd-induced cells. It is clear that control cells were more sensitive to Cu-induced cell death with a 50% loss of viability being observed at the lowest concentration (2 μM) of Cu used. In contrast, Cd-treated cells showed little change in cell viability up to 4 μM at which point cell viability significantly fell. Cu-mediated cell death was greater in control cells compared to Cd-treated cells at all Cu concentrations tested.

The resistance of cadmium-treated cells to copper most likely reflects the enhanced expression of MT by induced cells. MT expression, however, may not be the only parameter to be altered by cadmium exposure. To identify MT specifically as a mediator of resistance to Cu toxicity, we employed the forced genetic overexpression of MT. SPAEC were transfected with the pβACThMTIIA plasmid, containing

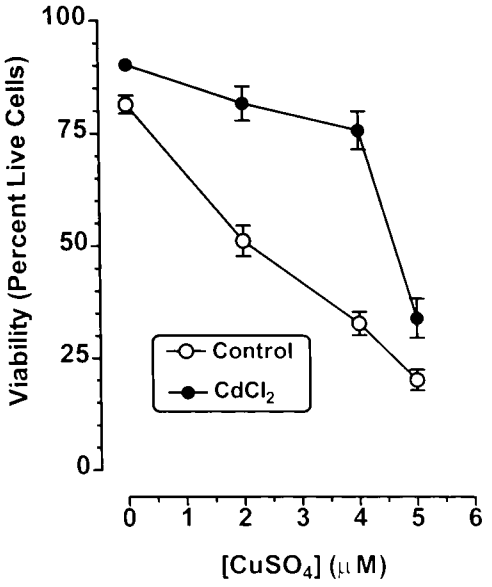

FIG. 1. Cadmium-induction of MT protects HL-60 cells from Cu-induced cytotoxicity. HL-60 cells were cultured in the presence of $CdCl_2$ (10 μM) in RPMI 1640 medium containing 15% FBS for 24 hr. Cells were collected by centrifugation, media was removed, and cells resuspended in fresh media without $CdCl_2$ and cultured for another 24 hr. Cells were then washed, resuspended in L1210 buffer, pH 7.4, and exposed to $CuSO_4$ at the indicated concentrations. Cell viability was assessed by Trypan Blue exclusion 1 hr after exposure. Data represent mean ± SEM, $n = 3$.

the hMTIIA cDNA under transcriptional control of the β-actin promoter, to create a cell line that overexpressed MT. Using the cadmium binding assay of Eaton and Toal (1982) we observed that MT levels were increased over 10-fold following transfection (0.38 ± 0.1 μg MT/mg protein for pβ-ACThMTIIA transfected cells vs. 0.02 ± 0.01 μg MT/mg protein for controls). Figure 2 compares the concentration-response for Cu-induced cytotoxocity between cells transfected with pβACThMTIIA and control cells transfected with vector alone. Overexpression of MT produced significant resistance to Cu. Figure 2A shows the amount of cell death 2 hr after the exposure to Cu in the presence of 8-hydroxyquinoline, which was included to enhance the cellular uptake of Cu (Crutchley and Que, 1995). Viability of control cells fell sharply as Cu concentration was raised. For example, viability was 69% at 0.5 μM and reached approximately 42% at 2 μM. In contrast, MT-overexpressing cells possessed greater viability than control cells at all Cu concentrations tested, although the difference was small at the highest (2 μM) Cu concentration.

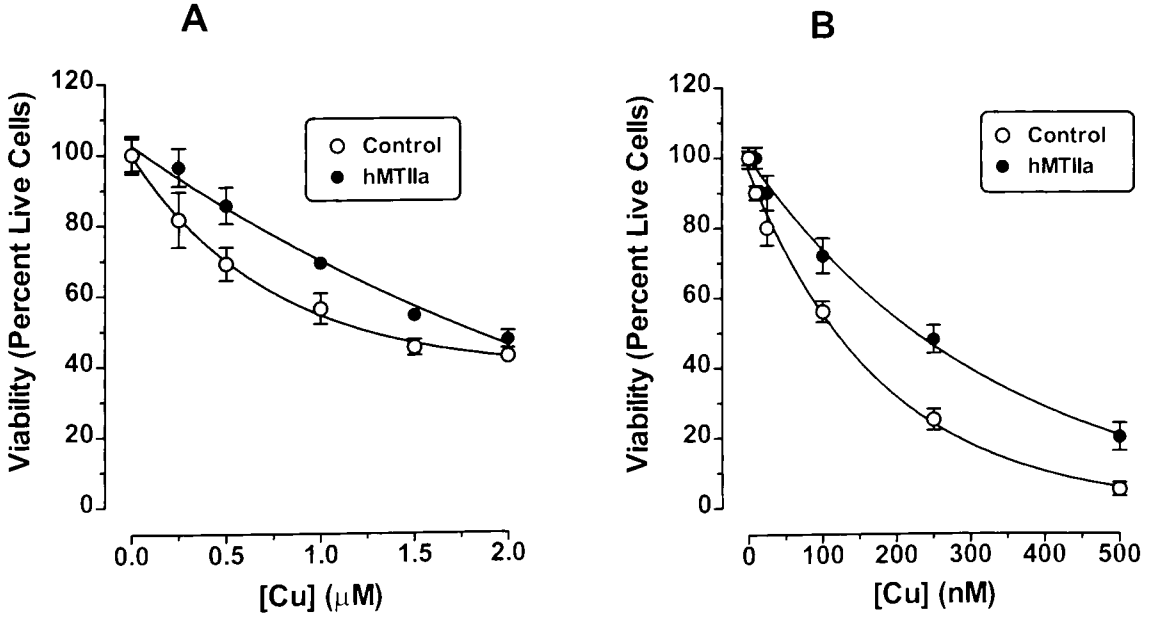

FIG. 2. Overexpression of hMTIIA protects SPAEC from Cu toxicity. SPAEC were transfected with pβACThMTIIA as described in Methods, plated into 24-well plates, and cultured overnight. Cells were then exposed to varying concentrations of $CuSO_4$ (as indicated), ascorbate (0.5 μM), 8-hydroxyquinoline (5 μM), and desferroxamine (0.5 μM) for 1 hr as described in Methods. Cu-dependent oxidations were stopped by addition of butylated hydroxytoluene and fresh medium was added to cells. Cell viability was determined by Alamar Blue fluorescence. **A.** Toxicity 2 hr after Cu exposure. **B.** Toxicity after 24 hr exposure. Note the difference in Cu concentrations used at these two times. Data represent mean ± SD for five and four observations per time point in **A** and **B**, respectively.

Similar experiments were also performed at lower Cu concentrations that necessitated longer exposure times (24 hr) to observe toxicity. Figure 2B shows that the protective effect of MT appears even more pronounced with exposure to lower Cu concentrations. Once again SPAEC/hMTIIA cells exhibited greater viability at all concentrations of Cu tested. The approximate LC_{50} (concentration for 50% cell death) for control cells was approximately 125 n*M* and about two-fold higher in MT-overexpressing cells (250 n*M*). Thus, it appears that MT has the ability to protect cells from Cu-induced cytotoxicity, presumably via its ability to bind Cu with high-affinity, and to prevent its redox-cycling.

MT could have induced cytoprotection through either direct inhibition of Cu-dependent oxidative stress or indirectly through modulation of cell death pathways activated in response to Cu-induced damage. To test this we examined the role of MT to modulate Cu-dependent lipid peroxidation in these cells. Lipid peroxidation was measured in cells after metabolic incorporation of the oxidant sensitive fatty acid, *cis*-PnA into control and MT-transfected SPAEC. Figure 3 shows representative content of *cis*-PnA in various phospholipid classes after 1 hr incubation in the presence or absence of $CuSO_4$ (0.5 μM). A comparison of Fig. 3, A and B, illustrates that exposure to Cu induced a pronounced decrement in content of fluorescent fatty acid residue in several phospholipid species including phosphatidylcholine (PC), phosphatidylethanoloamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). In contrast, the profiles of fluorescent phospholipids are essentially identical between SPAEC cells transfected with hMTIIA in the absence (Fig. 3C)

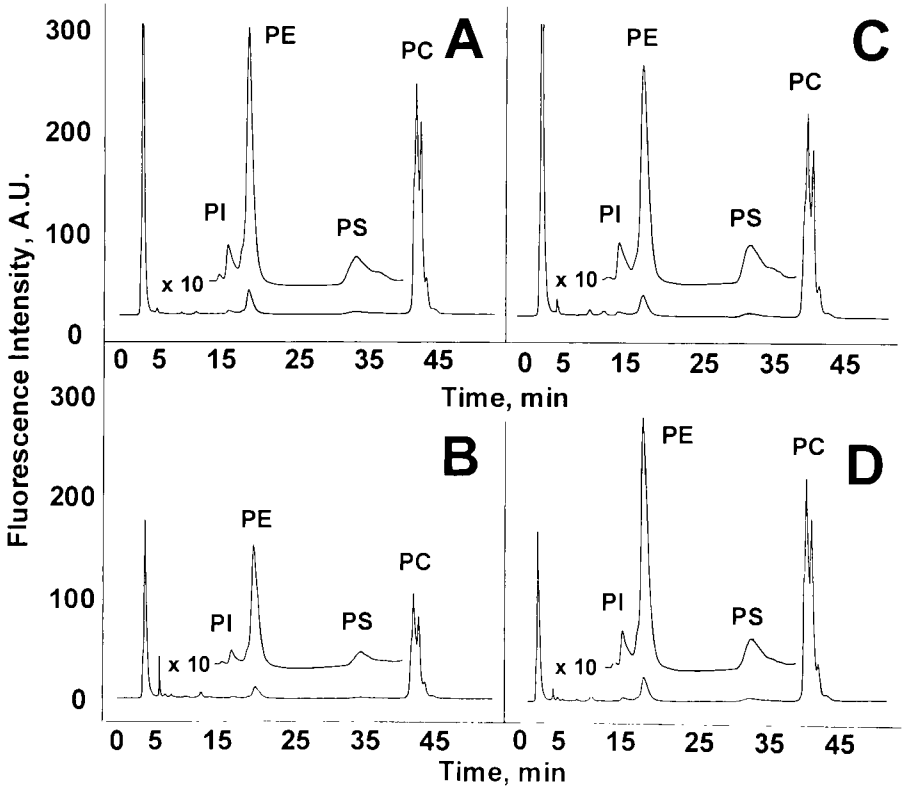

FIG. 3. MT-overexpression inhibits Cu-induced lipid peroxidation in SPAEC. Shown are representative HPLC chromatograms of total lipids extracted from 1×10^6 control SPAEC **(A, B)** and SPAEC/hMTIIA **(C, D)**. Cellular phospholipids were metabolically labeled by incubation of cells with *cis*-PnA as described in Methods. Cells were then treated with **(B, D)** or without **(A, C)** 0.5 μM $CuSO_4$ in DMEM containing 8-hydroxyquinoline (5 μM) and desferroxamine (0.5 μM) for 1 hr at 37°C. Lipids were then extracted, resolved by HPLC, and monitored for unoxidized *cis*-PnA content by fluorescence detection. PI, phosphatidylinositol; PE, phosphatidylethanoloamine; PS, phosphatidylserine; PC, phosphatidylcholine.

and following exposure to 0.5 μM $CuSO_4$ (Fig. 3D).

MT inhibits Cu-dependent redox cycling: difference between native and oxidized MT

To assess the molecular mechanisms responsible for MT protection against Cu toxicity, we used a cell-free model system where Cu-dependent redox cycling and Cu-binding to MT could be examined specifically. In addition, we examined the potential of MT sulfhydryls to be oxidized and compared the ability of oxidized MT to bind Cu and inhibit Cu-dependent redox cycling with native MT.

Our first goal was to demonstrate that MT could inhibit Cu-dependent redox cycling in a manner similar to its ability to protect cells from Cu-mediated oxidative stress. Our cell-free system contained 5 μM $CuSO_4$, as well as, 500 μM ascorbate and 400 μM H_2O_2, which were added to maximize the redox cycling of Cu. Ascorbate reduced Cu^{2+} to Cu^{1+}, thus providing the necessary catalyst for Cu-dependent production of hydroxyl radical from the substrate, H_2O_2. MT was then added at various concentrations to assess its ability to inhibit this process. We first monitored the potential for Cu-redox-cycling by oxidation of the chemiluminescent substrate luminol. Figure 4 (trace b) shows the typical chemiluminescence response observed in this system with Cu in the absence of MT. Note that in the absence of added $CuSO_4$ (trace a) no signal was observed, verifying that these responses were dependent on the presence of Cu. When MT was added to the system, a concentration-dependent quenching of luminol chemiluminescence was observed (traces c–i). Inhibition was first noticeable when Cu:MT ratio was between 20 and 15. Increasing MT concentrations continued to inhibit luminol oxidation further until it was completely abolished at Cu/MT ratio of 2. Therefore, MT could inhibit Cu-dependent oxidations in this model system.

Our model system with luminol, however, contained H_2O_2, which could have itself (or via hydroxyl radical production) directly oxidized sulfhydryl groups on MT. Because free thiols are required for metal binding, it was possible that the ability of MT to bind Cu and provide protection under these "severe" oxidizing conditions could be impaired. Therefore, we felt it important to compare the ability of MT to mitigate Cu-dependent redox cycling in the presence and absence of H_2O_2. For this, we chose to measure directly by EPR the ascorbyl radical and hydroxyl radical species produced by Cu-dependent redox cycling in this system. In

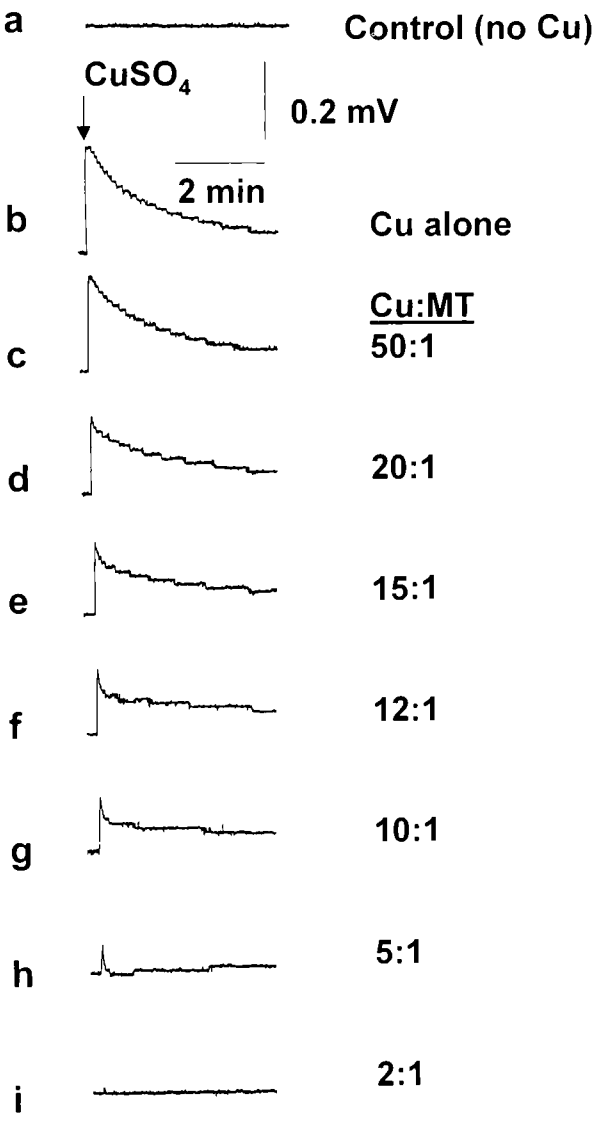

FIG. 4. MT inhibits Cu-dependent luminol oxidation. Reaction conditions were 5 μM $CuSO_4$, 500 μM ascorbic acid, 400 μM H_2O_2, 0.5 μM desferroxamine, and 400 μM luminol in 50 mM phosphate buffer. Each trace shows the typical luminol chemiluminescence response in the presence of various MT concentrations to create the Cu:MT ratios indicated. Trace a shows the response in the absence of any added $CuSO_4$ and trace b depicts maximal luminol oxidation in the absence of MT. Traces c–i contain progressively greater amounts MT to form Cu:MT ratios from 50:1 to 2:1.

the absence of H_2O_2, we could directly quantify the production of ascorbyl radical due to the relatively slow autooxidation of Cu^{1+} to Cu^{2+} under aerobic conditions and its recycling back to Cu^{1+} at the expense of ascorbate. In the presence of H_2O_2, however, it was impractical to measure the steady-state concentration of the short-lived ascorbyl radical because it was rapidly consumed under these conditions that permit extensive redox-cycling of Cu. In this case, we instead quantified the production of the hydroxyl radical using the spin trap DMPO.

Figure 5 compares the stoichiometric relationships between Cu and MT for inhibition of ascorbyl and hydroxyl radicals produced in the absence and presence of H_2O_2, respectively. Note that in the absence of H_2O_2, MT completely prevented the Cu-dependent formation of ascorbyl radical up to Cu:MT ratios of approximately 12. As MT concentration was lowered to achieve Cu:MT ratios greater than 12, ascorbyl radical could be detected and its level increased in parallel with increasing Cu:MT ratios. In contrast, complete prevention of hydroxyl radical formation in the presence of H_2O_2 was only achieved up to Cu:MT ratios ≤ 6 after which DMPO-OH adduct could be detected and rapidly increased with increasing Cu:MT ratio. Thus, the stoichiometry of MT prevention of redox cycling appears different in the presence and absence of oxidizing conditions.

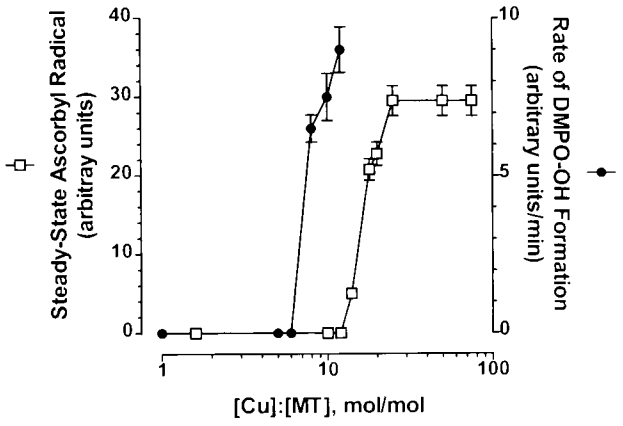

FIG. 5. Stoichiometry of MT inhibition of Cu-dependent redox-cycling in the presence and absence of H_2O_2. EPR spectra of ascorbyl radical were obtained in 50 m*M* phosphate buffer containing $CuSO_4$ (5 μM), ascorbate (500 μM), desferroxamine (0.5 μM) in the absence and presence of MT as described in Methods. Reactions were initiated by the addition of Cu, and steady-state concentration of the ascorbyl radical was determined from EPR spectra obtained 1 min later. Parallel incubations contained the same reactants with the addition of H_2O_2 (500 μM) and DMPO (75 m*M*). The amount of DMPO-OH adduct was assessed by EPR spectra obtained 15 min after the addition of H_2O_2. The magnitude of the signal was quantified and used to determine the rate of DPMO-OH adduct produced in the presence of various Cu/MT ratios. Data represent mean ± SEM of three to five observations per point.

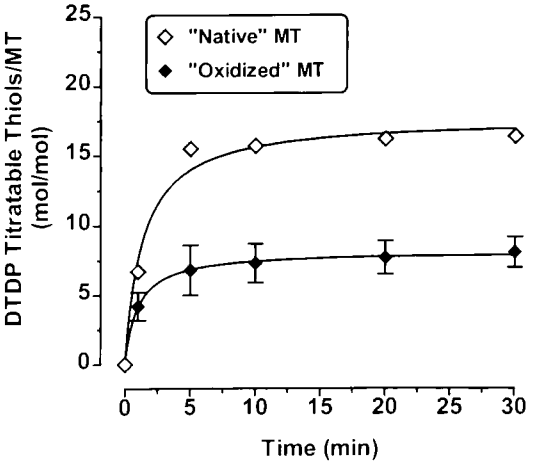

FIG. 6. Oxidation of free thiols in MT by H_2O_2. MT (20 μM) was preincubated alone or with $CuSO_4$ (20 μM), ascorbate (200 μM), and H_2O_2 (400 μM) for 5 min at 25°C in 50 m*M* phosphate buffer. Reaction was stopped by addition ascorbate oxidase and thiols were determined using DTDP as described in Methods. Data represent mean ± SD for three observations.

We next sought to determine whether H_2O_2 could oxidize free thiol groups on MT and thereby reduce the capacity of the protein to bind Cu as a mechanism to explain its reduced ability to inhibit Cu-dependent redox cycling under oxidizing conditions. We first quantified the number of free sulfhydryl groups on native and oxidized MT using the sulfhydryl reagent DTDP Figure 6 shows the time-dependent titration of free thiols in MT before and after treatment of MT under oxidizing conditions (20 μM $CuSO_4$, 200 μM ascorbate, 400 μM H_2O_2) for 5 min. We were able to titrate approximately 16.6 free thiols/MT molecule using the unoxidized native MT obtained from commercial sources. This number compares well with the 20 thiol groups theoretically available based on the amino acid sequence of MT. In contrast, after MT was exposed to oxidizing conditions, less than 8 thiols reacted with DTDP, suggest-

ing that exposure to Cu/ascorbate/H_2O_2 oxidized approximately 50% of the free sulfhydryl groups in MT within the metal-binding thiolate clusters.

We next determined if the potential of oxidized MT to directly bind Cu and, thereby, prevent its redox cycling was altered compared to native MT. For this, we measured the interaction of Cu/MT using low-temperature EPR of paramagnetic Cu^{2+} content of MT/Cu mixtures. Binding of Cu to native or oxidized MT was first allowed to proceed in the presence of excess ascorbate to provide primarily the cuprous ion (Cu^{1+}) species known to bind to MT. EPR-silent Cu^{1+} unbound to MT was then converted to the cupric state by addition of ascorbate oxidase to remove excess reductant. Preliminary studies using solutions of Cu^{2+} and ascorbate alone indicated that essentially all available free Cu was converted to Cu^{2+} within 10 min after addition of ascorbate oxidase (data not shown). Cu^{2+} signal was then measured by using low-temperature EPR, the magnitude of which corresponded to the amount of free Cu^{2+} unbound to MT after incubation. Figure 7 shows the EPR spectra obtained after incubation of native (Fig. 7A) and oxidized MT (Fig. 7B) at a Cu:MT ratio of 10. The top traces of each panel show the control spectra in the absence of $CuSO_4$. A small Cu^{2+} signal was obtained when $CuSO_4$ was preincubated with native MT. Most notable, however, was the significant increase in the free Cu^{2+} signal obtained after binding of Cu to oxidized MT. This result indicates that less Cu^{1+} could be bound to MT and, therefore, more was converted to paramagnetic Cu^{2+} in the presence of H_2O_2.

We quantified the amount of Cu bound to MT by estimating the EPR-silent Cu (Cu^{1+}) as the difference between the EPR-detectable Cu^{2+} in the absence (total Cu added) and pres-

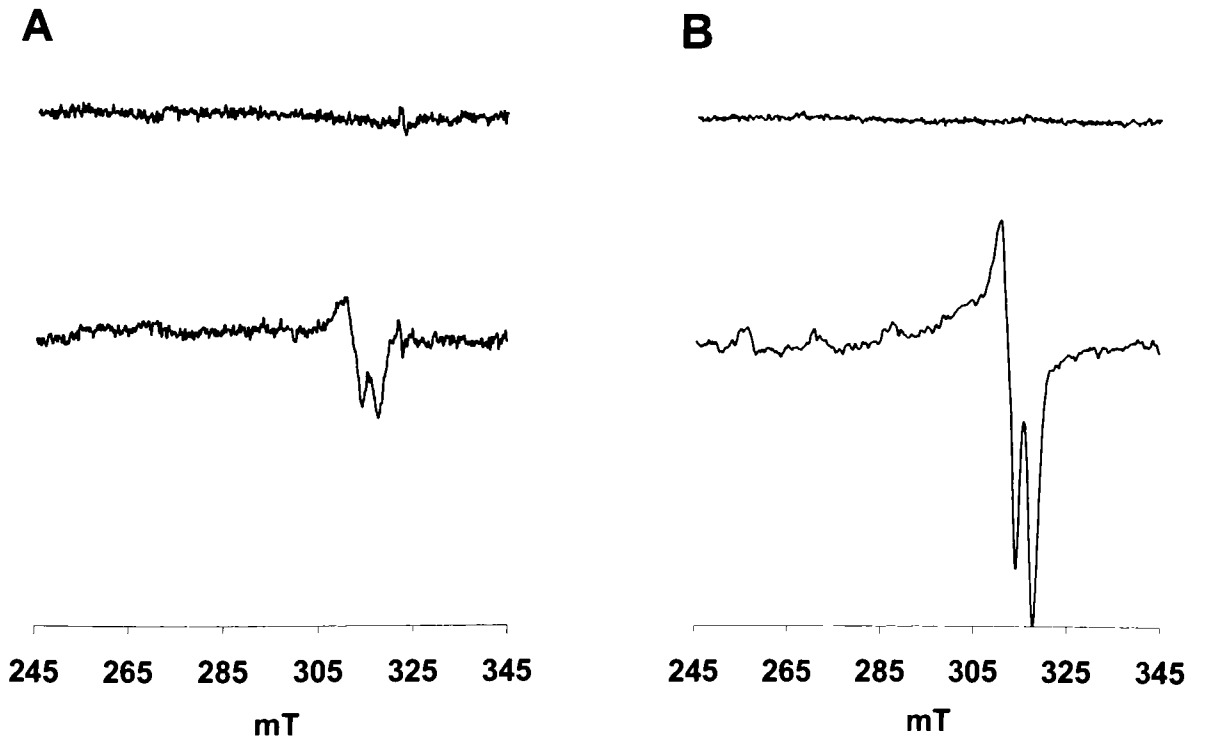

FIG. 7. EPR spectra of Cu^{2+} in the presence of MT treated with and without H_2O_2. MT (6 μM) was incubated with (bottom traces) and without (top traces) $CuSO_4$ (60 μM) in the presence 400 μM ascorbate in 50 mM phosphate buffer. **A.** Control conditions in the absence of H_2O_2. **B.** Incubation in the presence of 400 μM H_2O_2. Reactants were incubated for 2 min following which 1 U of ascorbate oxidase was added and incubated for 10 min to convert all free Cu^{1+} to Cu^{2+}. Low-temperature (77K) EPR spectra were obtained with settings of center field, 294.5 mT; field range, 100 mT; amplitude modulation, 0.5 mT; microwave power, 10 W. Time constants are 0.1 sec and 0.3 sec for **A** and **B**, respectively.

ence (unbound Cu^{2+}) of various concentrations of MT. EPR signals were double integrated and the absolute amount of Cu^{2+} estimated from a standard curve prepared using various Cu/EDTA concentrations. Table I shows the effect of H_2O_2 on the overall Cu-binding capacity of MT at various Cu:MT ratios. At lower Cu:MT ratios (6–10), essentially no change in the Cu-binding was detected; however, it became clear at higher Cu:MT ratios that the maximal amount of Cu that could be bound to MT was reduced in the presence of H_2O_2. Native MT appeared fully saturated at a total of 12 moles of Cu bound/mole of MT. In contrast, in the presence of H_2O_2, overall binding of Cu was reduced approximately 20% such that MT could only accommodate between 9 and 10 moles of Cu/mole of MT. Thus, oxidation of MT in the presence of H_2O_2 was accompanied by a loss in overall binding capacity for Cu.

Regeneration of oxidized MT by DHLA

Because the optimum functioning of MT in the binding of Cu (and presumably other metals) appears to depend on the maintenance of thiols in their reduced state, it seems logical to assume that cellular mechanisms exist to regenerate reduced MT following exposure to oxidative stress. One possible candidate for this role is the small-molecular-weight intracellular reducing agent, DHLA. To test the potential of this compound to regenerate cysteines on oxidized MT, we used our cell-free model system to determine if the number of sulfhydryls in oxidized MT could be increased after incubation with DHLA. For these experiments, MT was first oxidized by treatment with H_2O_2 for 20 min. Exposure of MT to H_2O_2 alone under these conditions decreased the sulfhydryl content to approximately 30% of that observed in native MT (Fig. 8). MT was then collected in the retentate after filtration through Microcon YM-3 filters and incubated with DHLA. Figure 8 shows the concentration-dependent increase in DTDP-titratable thiols after incubation of oxidized MT with DHLA. The number of reduced sulfhydryls progressively increased following incubation of MT with between 30 and 600 μM DHLA until they reached essentially the same level as that measured in native MT.

To determine if regeneration MT following treatment of DHLA could restore the ability of oxidized MT to inhibit Cu-dependent redox activity, we measured the ability of native, oxidized, and regenerated MT to inhibit the Cu-dependent generation of ascorbyl radical in mixtures of MT/Cu/ascorbate. Figure 9 shows the ascorbyl radical EPR signals detected in the cell-free system containing 10 μM $CuSO_4$, 500 μM ascorbate, and 1 μM of MT (native, oxidized, or regenerated). Spectrum 9a represents

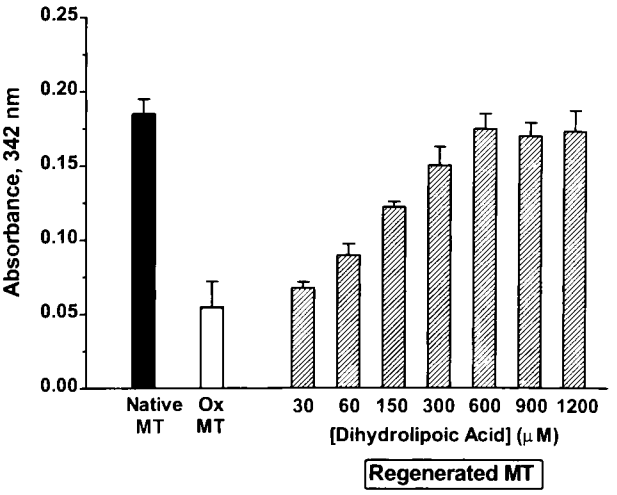

FIG. 8. DHLA regenerates reduced sulfhydryls in oxidized MT. Native MT (30 μM) was oxidized by exposure to H_2O_2 (8 mM) in 10 mM Tris, pH 7.4, for 20 min at 28° C. Reaction was stopped by addition of catalase (2.5 U/ml) for 10 min followed by recovery of oxidized MT in the retentate after centrifugation four times through Microcon YM-3 filters. MT thiols in the retentate were quantified by optical absorbance at 343 nm following reaction with DTDP. Data represent mean ± SEM obtained from at least three observations.

TABLE 1. QUANTIFICATION OF CU BINDING TO MT BEFORE AND AFTER OXIDATION

	Cu bound to MT (mol/mol)	
Cu:MT ratio (mol/mol)	*Before H_2O_2*	*After H_2O_2*
6:1	5.9 ± 0.1	5.5 ± 0.4
10:1	8.6 ± 1.1	8.4 ± 0.9
14:1	11.5 ± 0.4	9.1 ± 0.8
16:1	11.8 ± 0.1	9.7 ± 0.5
19:1	12.0 ± 0.1	9.4 ± 0.6

The amount of Cu bound to MT was considered as the EPR-silent Cu remaining after addition of ascorbate oxidase and was calculated as the difference between EPR detectable Cu before and after the addition of MT and ascorbate. Data represent mean ± SD obtained from at least three observations.

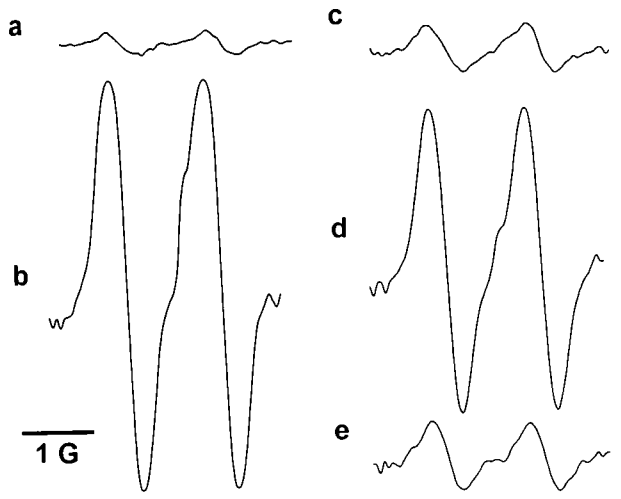

FIG. 9. DHLA restores ability of oxidized MT to inhibit Cu-dependent redox cycling. Native, oxidized, and DHLA-regenerated MT were incubated with $CuSO_4$ (10 μM) and sodium ascorbate (500 μM). The EPR signal for ascorbate radical was obtained by repeated scanning using 335.4 mT, center field; 0.3 mT, sweep width; 0.05 mT field modulation; 10 mW, microwave power; 0.3 sec, time constant; 20 sec, time scan. Data represent typical spectra obtained 8 min after incubation for ascorbate + native MT alone **(a)**, ascorbate + Cu alone **(b)**, ascorbate + Cu + native MT **(c)**, ascorbate + Cu + oxidized MT **(d)**, and ascorbate + Cu + regenerated MT **(e)**. In **c–e**, MT was present at a Cu:MT ratio of 10.

the control signal generated in the absence of Cu and, hence, measures Cu-independent background formation of ascorbyl radical and spectrum 9b shows the robust ascorbyl radical spectrum detected in the absence of any MT. Spectrum 9c shows that inclusion of native MT at a Cu:MT ratio of 10 significantly quenched the ascorbyl radical signal to almost background levels. In contrast, MT that had been oxidized in the presence of H_2O_2 provided little protection against Cu-dependent generation of ascorbyl radical (9d), whereas, treatment of oxidized MT with DHLA fully-restored the ability of MT to inhibit Cu-dependent formation of ascorbyl radical similar to that seen with native MT (9e).

DISCUSSION

Although the trace metal, copper, is required for life, it is clear that excess exposure to this transition metal leads to cytotoxocity (Halliwell and Gutteridge, 1990; Arora and Gores, 1996; Luza and Speisky, 1996). The primary mechanism of cell damage and death following Cu exposure is thought to be the generation of severe oxidative stress by Cu-dependent redox cycling. Cu^{1+} catalyzes the formation of superoxide and hydroxyl radical from oxygen and H_2O_2, respectively. These oxyradicals then initiate damage to cellular constituents such as lipids, proteins, and nucleic acids. Our studies indicate that indeed Cu-dependent oxidative stress occurs in cells and causes cell death. Moreover, the metal-binding protein MT confers significant protection to Cu toxicity and inhibits Cu-dependent redox cycling in model systems. This protective ability of MT, however, appears sensitive to oxidation of the metal-binding thiolate clusters whose integrity can be restored by dihydrolipoic acid.

Cellular protection by MT

Using two independent cell systems, we have demonstrated that expression of MT by cells confers resistance to Cu-dependent cytotoxicity. Although other reports have demonstrated a prooxidant effect of Cu-MT (Oikawa *et al.*, 1995; Suzuki *et al.*, 1996), it is clear that induction of MT by Cd exposure or forced genetic overexpression of MT protected cells from Cu-dependent oxidative stress and cell death. At the same time, MT inhibited Cu-induced lipid peroxidation observed in live cells prior to cell death, a fact that supports the hypothesis that MT action is to inhibit Cu-dependent redox cycling directly. It was likely, however, that pre-exposure to cadmium elicited other protective responses in addition to MT induction (*e.g.*, increased glutathione levels). This may be one reason why the difference in resistance to Cu is greater between the Cd-treated and control cells (Fig. 1) than in the experiments utilizing forced genetic overexpression (Fig. 2) where MT was more selectively upregulated. Other potential explanations include the use of disparate cell types and the use of 8-hydroxyquinoline.

Previously, we have identified the selective oxidation of PS as a component of apoptotic cell death (Fabisiak *et al.*, 1997; Fabisiak *et al.*, 1998a,b). The mechanism responsible for this is unknown, but suggests the existence of phos-

pholipid-specific oxidation-dependent signaling pathways in apoptosis. Since, however, Cu induced global oxidation of *cis*-PnA in all phospholipid classes within cellular membranes, lipid peroxidation as measured in these studies most likely represents the random oxidation of membrane lipids by Cu-generated oxyradical species. In addition, we have not observed other markers of apoptosis, such as characteristic changes in nuclear morphology, in these Cu-treated cells (data not shown). Thus, the mode of Cu-induced cell death, at least under these conditions, appears to be chiefly necrotic.

Oxidation of MT reduces Cu binding and increases Cu-dependent redox cycling

In addition to its ability to bind metals, we and others have suggested a direct antioxidant role for MT by scavenging reactive oxygen species (Thornalley and Vasak, 1985; Thomas *et al.*, 1986; Schwarz *et al.*, 1994). We feel that this plays little role in the ability of MT to mitigate Cu-dependent oxidative stress for several reasons. First, the observed stoichiometry between native MT and Cu for inhibition of ascorbyl radical formation corresponds with the ultimate binding capacity of 12 molecules of Cu/molecule of MT. Second, if MT was primarily acting as a savenger of hydroxyl and/or superoxide radical, then we might expect that the actual rates of Cu^{1+}/Cu^{2+} cycling would be unaltered in the presence of MT. This is not the case, however, because MT prevented the appearance of ascorbyl radical whose formation directly reflects the degree of Cu^{1+}/Cu^{2+} cycling. Third, we observed good correlation between the degree of Cu-binding to MT measured by EPR and the degree of inhibition of Cu-dependent oxidations.

It is clear, however, that MT thiols are indeed sites of oxidation. Treatment of MT with H_2O_2 reduces the number of free sulfhydryls and compromises the capacity of MT to bind Cu and render it redox inactive. Thus, its usefulness as a direct scavenger of radicals is limited because thiol oxidation would effectively increase the amount of free Cu available for redox cycling and, hence actually enhance Cu-dependent oxidative stress. Nitrosylation of MT sulfhydryls has been shown to enhance Cd release and toxicity in CHO-K1 cells (Misra *et al.*, 1996). Similarly, nitric oxide destroys thiolate clusters and releases Zn from MT (Kroncke *et al.*, 1994). It will be of interest to determine if MT/Cu complexes can indeed serve as releasable pools of redox-active transition metal when exposed to other non-metal-dependent oxidative/nitrosylative stresses.

In addition to its cytoprotective role, it has also been postulated that MT plays a pivotal role in control of the physiological delivery and release of endogenous metals ions to appropriate targets within the cells. A recent series of papers have very elegantly described the redox control of Zn transfer between MT and zinc proteins (Jacob *et al.*, 1998; Jiang *et al.*, 1998; Maret and Vallee, 1998). Therefore, selective oxidation of MT by endogenous NO or oxidants may serve to signal Cu transfer for MT to target proteins. It is possible that MT can function in the delivery of Cu to target proteins similar to other Cu chaperones such as Atx1 (Pufhal *et al.*, 1997) and Cox17 (Glerum *et al.*, 1997), and that control of Cu transfer is redox regulated.

Recently, it has been speculated that the α-thiolate domain of MT functions as a site for toxic metal sequestration, whereas the β-domain may be involved in transport of essential metal ions (Presta *et al.*, 1995). This would imply differences in the ability of each domain to release bound metals. Whether all thiols in MT are uniformily susceptible to H_2O_2 oxidation is not clear. Misra *et al.* observed that nitric oxide could only displace between two and three molecules of Cd out of the seven bound to saturated MT, but they did not measure sulfhydryl content (Misra *et al.*, 1996). Our group has observed selective oxidation of a single sulfhydryl on MT by the phenoxyl radical of etoposide formed by myeloperoxidase (Kagan *et al.*, 1994). It should be pointed out, however, that etoposide is a large polycyclic molecule whose access to the thiolate clusters of MT may be limited. Future concentration-response and time-response studies are planned to determine the degree of heterogeniety among various cysteines for sensitivity to oxidation.

Regeneration of oxidized MT by DHLA

For MT to retain its ability to bind Cu and other toxic metals in the face of oxidative stress, it is essential that oxidized MT be repaired or regenerated to preserve its cytoprotective function. Furthermore, if redox mechanisms serve to regulate physiological metal delivery by MT, it is likely that there exists an efficient regenerating system to reduce oxidized sulfhydryls after metals have been delivered to target proteins. Cells have evolved efficient protein-based systems to repair oxidized proteins as typified by thioredoxin and glutaredoxin (Fernando *et al.*, 1992; Gravina and Mieyal, 1993). In addition, small-molecular-weight thiol compounds can directly participate in redox-coupled exchange of electrons to regenerate reduced protein thiols at the expense of their own oxidation. The regeneration of MT, however, has not been studied so far.

DHLA represents one potential small-molecular-weight thiol with potential to regenerate reduced MT thiols after oxidation. α-Lipoic acid (as lipoamide) was first described as an essential co-factor in mitochondrial α-keto acid dehydrogenase complexes (Schmidt *et al.*, 1969). More recently, it has been described as a "metabolic antioxidant" (Packer *et al.*, 1997). The vicinal sulfhydryls can exist in either their reduced form (dihydrolipoic acid) or oxidized as an internal disulfide (lipoic acid). Dihydrolipoate can directly scavenge different reactive species including hydrogen peroxide, hydroxyl radical, phenoxyl radicals, and nitric oxide among others (Whiteman *et al.*, 1996; Packer *et al.*, 1997). α-Lipoate adminstration can preserve glutathione levels in a variety of cells (Busse *et al.*, 1992; Sen *et al.*, 1997) and has been shown to block the oxidant-induced activation of the transcription factor nuclear factor kappa B (NF-κB) (Sen and Packer, 1996).

We hypothesized that reduced dihydrolipoic acid would be cabable of regenerating MT by way of a disulfide exchange reaction as follows:

$$\text{MT-(S-S)} + \text{DHLA (SH)}_2 \rightarrow \text{MT-(SH)}_2 + \text{Lipoic acid (S-S)}$$

A similar reaction has been suggested for DHLA-mediated reduction of thioredoxin by mitochondrial α-ketoacid dehydrogenase complexes (Bunik and Follman, 1993), but not to our knowledge for any other oxidized cellular proteins. Our data clearly show the potential, at least *in vitro,* for DHLA to regenerate thiols in oxidized MT. Quesada *et al.* (1996) failed to regenerate free sulfhydryls on MT after H_2O_2 exposure by 2-mercaptoethanol or dithiothreitol and concluded that cysteines were irreversibly oxidized beyond the disulfide state. This is in contrast to H_2O_2 oxidation in our study where we observed nearly complete restoration of the number of reduced thiols and Cu binding capacity by DHLA. It is important to point out that these observations represent a direct effect of DHLA on the oxidized protein, and not protection by antioxidant scavenging, because the oxidation and regeneration steps were carried out separately. The most logical mechanism, therefore, is the formation of reduced thiols in MT at the expense of forming oxidized lipoic acid. Because reduced DHLA can be recycled from lipoate by lipoamide dehydrogenase (Haramaki *et al.*, 1997) or thioredoxin reductase (Arner *et al.*, 1996), DHLA serves to couple MT to an efficient protein repair system. Furthermore, because lipoic acid is readily taken up by cells, it is feasible to use as a pharmacologic agent to augment protein repair as it would be converted to the bioactive DHLA during intracellular metabolism. DHLA can also directly bind Cu; however, its effects in our experiments were to regenerate MT directly because the exposure of MT to DHLA occurred in the absence of Cu and after oxidation had already occurred.

Thus, we have shown here that MT is an important cytoprotective factor in Cu-dependent oxidative stress primarily by binding Cu and rendering it redox inactive. The multiple thiols on MT, however, are prone to oxidation, which reduces the ability of MT to bind Cu and inhibit Cu redox cycling. Optimal function of MT is likely maintained through an efficient system of protein repair that may utilize lipoate/dihydrolipoate as a proximal step in reduction of oxidized sulfhydryls.

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ABBREVIATIONS

DHLA, dihydrolipoic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DMEM, Dulbecco's minimum essential medium; DTDP, 2,2′-dithiodipyridine; EPR, electron paramagnetic resonance; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; MT, metallothionein; NF-κB, nuclear factor kappa B; PC, phosphatidylcholine; PEA, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; *cis*-PnA, *cis*-parinaric acid; SPAEC, sheep pulmonary artery endothelial cells.

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