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Free Radical Scavenging Actions of Metallothionein Isoforms I and II

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By employing electron spin resonance spectroscopy, we examined the free radicals scavenging effects of hepatic metallothionein (MT) isoforms I and II (MTs-I and II) on four types of free radicals. Solutions of 0.15 mM of MT-I and 0.3 mM of MT-II were found to scavenge the 1,1-diphenyl-2-picrylhydrazyl radicals (1.30×10^{15} spins/ml) completely. In addition, both isoforms exhibited total scavenging action against the hydroxyl radicals (1.75×10^{15} spins/ml) generated in a Fenton reaction. Similarly, 0.3 mM of MT-I scavenged almost 90% of the superoxide (2.22×10^{15} spins/ml) generated by the hypoxanthine and xanthine oxidase system, while a 0.3 mM MT-II solution could only scavenge 40% of it. By using 2,2,6,6-tetramethyl-4-piperidone as a "spin-trap" for the reactive oxygen species (containing singlet oxygen, superoxide and hydroxyl radicals) generated by photosensitized oxidation of riboflavin and measuring the relative signal intensities of the resulting stable nitroxide adduct, 2,2,6,6-tetramethyl-4-piperidine-1-oxyl, we observed that MT-II (0.3 mM) could scavenge 92%, while MT-I at 0.15 mM µl/ml concentrations could completely scavenge all the reactive species (2.15×10^{15} spins/ml) generated.

The results of these studies suggest that although both isoforms of MT are able to scavenge free radicals, the MT-I appears to be a superior scavenger of superoxide and 1,1 diphenyl-2-picrylhydrazyl radicals.

Keywords: Metallothioneins, superoxides, hydroxyl radicals, oxidative stress

INTRODUCTION

Metallothionein (MT) was discovered in 1957, when Margoshes and Vallee[1] identified, in equine kidney, a protein responsible for accumulation of cadmium. Since then, it has been learned that all living organisms have developed efficient mechanisms to utilize essential trace elements such as zinc and copper in expressing their biological functions, and to minimize the cytotoxicity of nonessential post-transition metal ions such as cadmium and barium. The ability to bind excess metals and to limit concentration of "free" ions is achieved mostly by cysteine-rich polypeptides such as γ-glutamyl peptide (phytochelatin) found in plants, and MTs found in animals.[2–7]

Since MTs have no enzymatic functions, they have been traditionally classified according to their structural features, including possessing the

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following characteristics: high content of heavy metals (typically 4–12 atoms/molecule) bound exclusively by clusters of thiolate bonds; high content of cysteine (typically 23–33 mol%) and paucity of aromatic and hydrophobic amino acid residues; low molecular weight (typically less than 10,000 Da); and demonstrated structural or functional homology to mammalian metallothionein.[5–8] A similar nucleotide and amino acid sequence has made it difficult to obtain cDNA probes and antibodies capable of distinguishing indisputably among MT isoforms. MT-I and MT-II isoforms are found mostly in the liver and kidneys and other peripheral tissues; MT-III isoform, possessing an additional seven amino acids, is expressed mostly in the brain and to a very minute extent in the intestine and pancreas,[9–11] whereas MT-IV isoform is found in tissues containing stratified squamous epithelial cells.[12] The various MT isoforms have been postulated to participate in cell proliferation and differentiation; in the activation and synthesis of metalloenzymes; in regulation of metal homeostasis; and in protection against metal toxicity and oxygen radicals (for reviews and references see Refs. [13–19]).

Active oxygen species are continually produced in tissues by the action of the mitochondrial electron transport system[20] and of reduced nicotinamide adenine dinucleotide phosphate oxidase,[21] but several antioxidant defense systems prevent the damage to the tissues by oxygen radicals. These systems include a range of specific antioxidants such as catalase for hydrogen peroxide, superoxide dismutase for superoxide, and glutathione peroxidase for hydrogen peroxide and lipid peroxide, and nonspecific antioxidants such as reduced glutathione, ceruloplasmin, and transferrin. However, there is no specific defense mechanism against hydroxyl radicals, which are the most potent of the active oxygen species.[22] Recently, growing attention has been focused on a role for MT as a radical scavenger, because the rate constant for its reaction with hydroxyl radicals *in vitro* is very high.

By using electron spin resonance (ESR) spectrometry which provides direct evidence for formation of oxygen radicals,[23–29] we investigated the efficacy of MT-I and II in scavenging four types of free radicals. The results of these studies suggest that although both isoforms of MT are able to scavenge free radicals, MT-I appears to be a superior scavenger of superoxide and 1,1-diphenyl-picrylhydrazyl radicals.

MATERIALS AND METHODS

Experimental Procedures

Animals Adult male Sprague-Dawley rats, weighing between 200 and 250 g, were purchased from Sasco, Inc. (Wilmington, MA) and were maintained three per cage using corn cob bedding, in air-conditioned rooms, of windowless quarters, with a temperature ranging between 68°F and 72°F, and a humidity ranging between 40% and 50%. The rats were fed Purina rat chow, and provided with tap water *ad libitum*. The animals were subjected to a 12 h light–dark cycle, with an automatic switch turning off the light at 6:00 pm and turning it back on at 6:00 am.

Chemicals for induction of hepatic metallothionein isoforms $ZnSO_4 \times 7H_2O$ was purchased from Fisher Scientific Company (Fair Lawn, NJ). Sephadex G-75 was purchased from Sigma Chemical Company (St. Louis, MO). DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals (Piscataway, NJ).

Chemicals for electron spin resonance spectrometry studies Riboflavin was purchased from Merck Chemical Company (Giessen, Germany), while 2,2,6,6-tetramethyl-4-piperidone (TMPD) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Hypoxanthine and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Tokyo Kasei (Tokyo, Japan). Diethylene triamine penta acetic acid (DETAPAC), xanthine oxidase, and 5,5-dimethyl-1-pyrroline-N-oxide

(DMPO) were purchased from Sigma Chemical Company.

Induction of hepatic metallothionein isoforms The experimental rats were injected with 7.5 mg/kg of zinc sulfate intraperitoneally, whereas the control rats received an identical volume of physiological saline. The rats were decapitated 18 h post-zinc treatment, and the livers were removed and perfused with ice-cold nitrogen-saturated 0.01 M Tris-HCl buffer, pH 7.5, containing 1 mM dithiothreitol.

Determination of hepatic metallothionein isoforms One-hundred grams of zinc-treated livers or control livers were homogenized in 5 vol of nitrogen-saturated Tris-HCl buffer (0.01 M, pH 7.5) containing 1 mM dithiothreitol. The homogenates were centrifuged at 750*g* for 15 min. Then, the supernatants were recentrifuged at 43,000*g* for 30 min. The pellets were stored, and the supernatants were recentrifuged at 105,000*g* for 60 min in a Beckman Model L3-50 ultracentrifuge (Palo Alto, CA). Soluble fractions were concentrated in an Amicon ultrafiltration cell using ultrafiltration membranes (YM2, 43 mm Lot AS 02034A) purchased from Amicon Corporation (Danvers, MA). In all cases, 5–10 ml of concentrated supernatant containing 315–330 mg proteins was applied to 4.8 × 115 cm Sephadex G-75 columns, and the low molecular weight MT isoforms were eluted at 4°C with 0.01 M Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol.[30] The concentrations of protein and zinc were determined in the column eluents. The MT was purified further by applying anion exchange chromatography using DEAE-Sephadex A25, and the MT isoforms I and II were eluted with a linear gradient using a Tris-acetate buffer (0–200 mM, pH 7.5), according to a technique described by Karin and Herschman.[31]

The proteins pooled from DEAE-Sephadex A25 fractions were desalted on Sephadex G-25 columns (1.6 × 35 cm) and eluted with triple distilled water. The protein fractions were pooled, lyophilized and analyzed for hepatic MT isoforms using a reverse phase high performance liquid chromatographic technique[32] and by using the buffer system suggested by Suzuki *et al.*[33]

Atomic absorption spectrophotometer For determination of zinc, a Perkin Elmer Atomic Absorption Spectrophotometer Model 1100 equipped with a graphite furnace, Model HGA-300, was used utilizing the established procedure.[30] Control of metal contamination from numerous sources including water, acids, bases, buffer salts, plastics, rubber, dialysis tubing, and glassware was strictly observed.[34]

Determination of protein The concentrations of proteins were determined by the method of Bradford[35] and by using bovine serum albumin as a standard.

Electron Spin Resonance Spectrometry

Instrumentation and assay procedure Free radicals were analyzed by an ESR spectrometer (JEOL JES-FEIXG, Tokyo, Japan). Manganese oxide (left $g = 2.0340$, right $g = 1.9810$) was used as an internal standard and spin numbers were calculated using the ratio of signal height intensities of 2,2,6,6-tetramethyl-4-hydroxyl-piperidine-oxyl with known spin quantities as described by Hiramatsu *et al.*[28] and Kumari *et al.*[29]

1,1-Diphenyl-2-picrylhydrazyl radicals One-hundred µl of 30 µM DPPH in ethanol solution and 100 µl of various concentrations of MT I and II (0–0.3 mM) were placed in a test tube and were mixed for 10 s. The mixture was transferred to a special flat cells (JEOL) for the analysis of the amount of DPPH radicals and measurement was performed 60 s after addition of each sample. The signal intensities were evaluated by the peak height of the 3rd signal of five line spectra of DPPH radicals. The conditions of ESR spectrometeric analysis were as follows: room temperature of 21°C; magnetic field, 335 ± 5 mT; field modulation width, 0.1 mT; receiver gain, 2.5 × 100; time constant, 0.1 s; sweep time, 2 min.

Superoxide Fifty µl of 2 mM hypoxanthine, 35 µl of 10.98 mM DETAPAC, 50 µl of various

concentrations of MT-I and II (0–0.3 mM), 15 µl of DMPO and 50 µl of xanthine oxidase were added to a test tube and mixed for 10 s. The mixture was transferred to a special flat cell for the analysis of the amount of DMPO spin adducts of superoxide (DMPO-O_2^-). The signal intensities were evaluated by the peak height of the 1st signal of twelve line spectra of superoxide radicals. The conditions of ESR spectrometeric analysis were as follows: room temperature of 21°C; magnetic field, 335 ± 5 mT; field modulation width, 0.1 mT; receiver gain, 1.6 × 100; time constant, 0.1 s; sweep time, 2 min.

Hydroxyl radicals Seventy-five µl of 1 mM $FeSO_4$ and 1 mM DETAPAC solution, 50 µl of various concentrations of MT-I or II (0–0.3 mM), 20 µl of 0.92 M DMPO and 50 µl of 1 mM hydrogen peroxide were mixed in a test tube and were transferred to a special flat cell for the analysis of the amount of DMPO spin adducts of hydroxyl radicals (DMPO-OH). The conditions of ESR spectrometeric analysis were as follows: room temperature of 21°C; magnetic field, 335 ± 5 mT; field modulation width, 0.1 mT; receiver gain, 2 × 100; time constant, 0.1 s; sweep time, 2 min.

Reactive oxygen species (singlet oxygen, superoxide and hydroxyl radicals generated by the photosensitization of riboflavin) One-hundred µl of TMPD (0.2 M), 100 µl of riboflavin (50 µM), 700 µl of phosphate buffer (0.1 M, pH 7.4) and 100 µl of MT-I or II (0–0.3 mM), were mixed and transferred into a special flat cell. This reaction mixture was then photosensitized using a mercury lamp (USH-500 D, 500 W) designed by Ushio Electric Company (Japan), in conjunction with UV filter (UV-D36C, Toshiba Glass Chemical Co. Ltd., Japan) that has a transmission range of 300–400 nm for exactly 2 min and the ESR spectrum was taken precisely 60 s later. The signal intensities of 2,2,6,6-tetramethyl-4-piperiodine-1-oxyl were evaluated by the peak height of the first signal of a three line spectrum. The conditions of ESR spectrometeric analysis were as follows: room temperature of 21°C; magnetic field, 335 ± 5 mT; field modulation width, 0.1 mT; receiver gain, 2.5 × 100; time constant, 0.1 s; sweep time, 2 min.

The test solutions of MT isoforms were made in sodium phosphate buffer (pH 7.4).

Statistical evaluation of data Data among the various groups were expressed as mean ± SEM values. The significance of the difference between various groups was tested by independent *t*-test. For multiple comparison, ANOVA followed by *post hoc* test was performed, and p values of $p < 0.05$ were taken as showing statistical significance.

RESULTS

As it has been established during the last two decades, the administration of 7.5 mg/kg of zinc sulfate induced the synthesis of hepatic MT isoforms I and II, and the chromatographic properties (Figure 1) and retention times (Figure 2) of these proteins are identical to those reported by Münger *et al.*,[36] Karin and Richards,[37] Kägi and Kojima,[7] and Kägi.[38]

By employing ESR spectroscopy, we examined the free radicals scavenging effects of hepatic MT-I and II on four types of free radicals. Solutions of 0.15 mM of MT-I and 0.3 mM of MT-II were found to scavenge the DPPH radicals

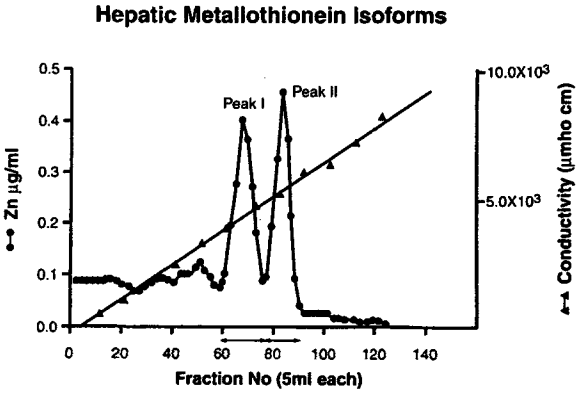

FIGURE 1 Zinc sulfate induces the synthesis of hepatic metallothionein isoforms I and II which bind 0.40 and 0.45 µg zinc/ml elution.

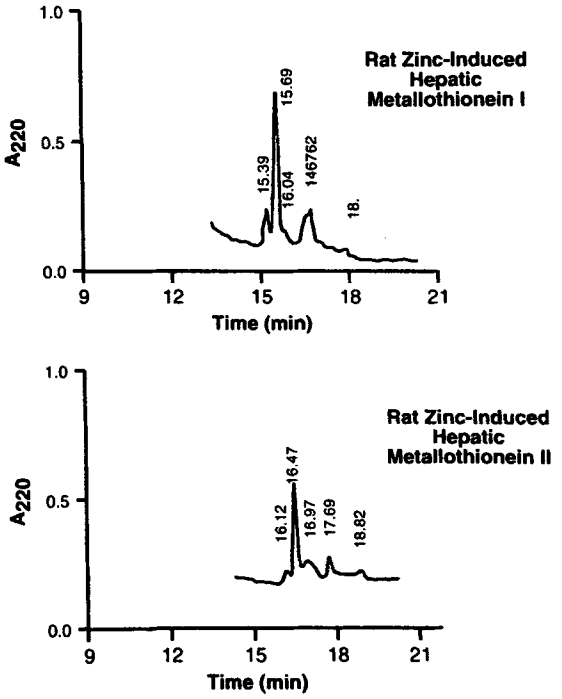

FIGURE 2 A typical profile of the zinc-induced hepatic metallothionein I and II exhibiting two peaks on HPLC with retention times of 15.69 min (MT-I, top panel) and 16.47 min (MT-II, bottom panel), respectively.

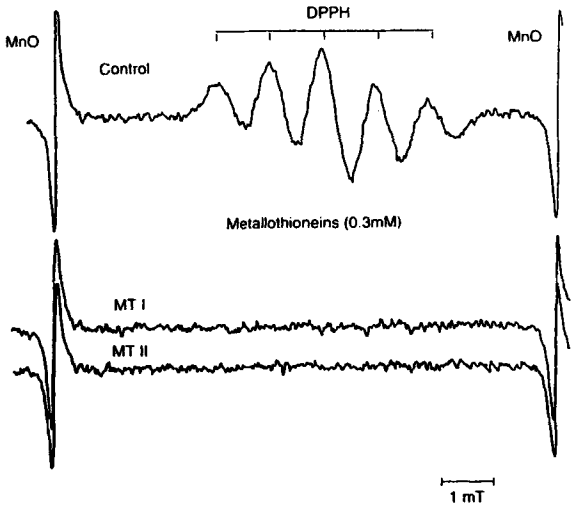

FIGURE 3 Electron spin resonance (ESR) spectra showing that the hepatic MT-I (0.15 mM) and MT-II (0.3 mM) scavenged completely 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (1.3×10^{15} spins/ml).

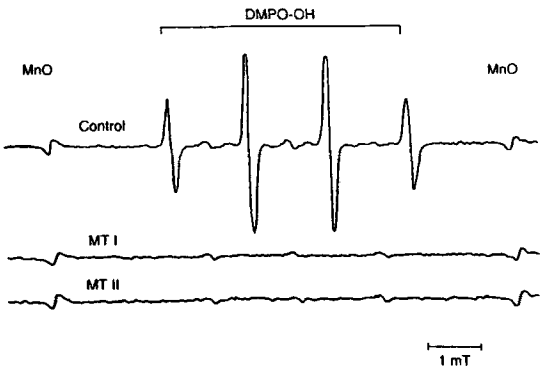

FIGURE 4 Electron spin resonance (ESR) spectra showing that the hepatic MT-I and II (0.3 mM) scavenged hydroxyl radicals (1.75×10^{15} spins/ml) generated in a Fenton reaction.

(1.30×10^{15} spins/ml) completely (Figure 3). Moreover, we contend that in the experiment where the scavenging effect of MTs on DPPH radicals was being studied, since the DPPH solution itself was made in ethanol, any possible influence of ethanol on the free radical scavenging action would have been uniform in the control as well as the control plus MT samples. The values are therefore represented as percentage of the control free radicals scavenged. In addition, both isoforms exhibited total scavenging action against the hydroxyl radicals (1.75×10^{15} spins/ml) generated in a Fenton reaction (Figure 4). Similarly, 0.3 mM of MT-I scavenged almost 90% of the superoxide (2.2×10^{15} spins/ml) generated by the hypoxanthine and xanthine oxidase system while a 0.3 mM MT-II solution could only scavenge 40% of it (Figure 5). By using TMPD as a "spin-trap" for the reactive oxygen species (containing singlet oxygen, superoxide and hydroxyl radicals) generated by photosensitized oxidation of riboflavin and measuring the relative signal intensities of the resulting stable nitroxide adduct, 2,2,6,6-tetramethyl-4-piperiodine-1-oxyl, we observed that MT-II (0.3 mM) could scavenge 92%, while MT-I at 0.15 mM μl/ml concentrations could completely scavenge all the reactive species (2.15×10^{15} spins/ml) generated simultaneously (Figure 6).

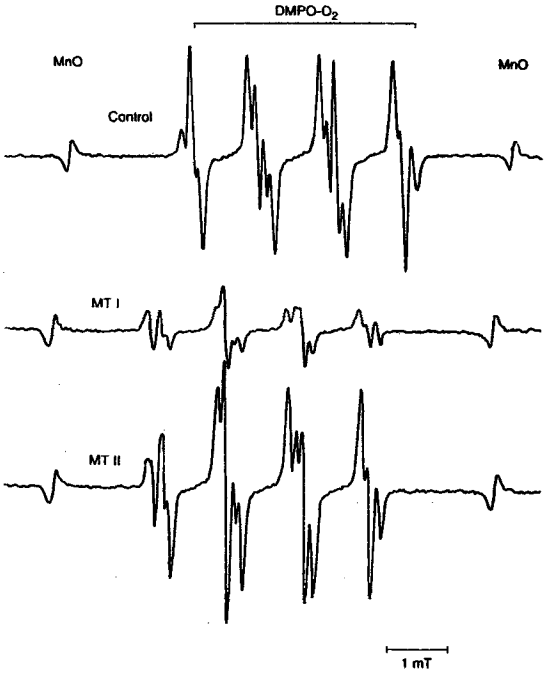

FIGURE 5 Hepatic MT-I (0.15 mM) and MT-II (0.30 mM) scavenged 40% and 90% of the superoxide (2.22×10^{15} spins/ml) generated by the hypoxanthine and xanthine oxidase system.

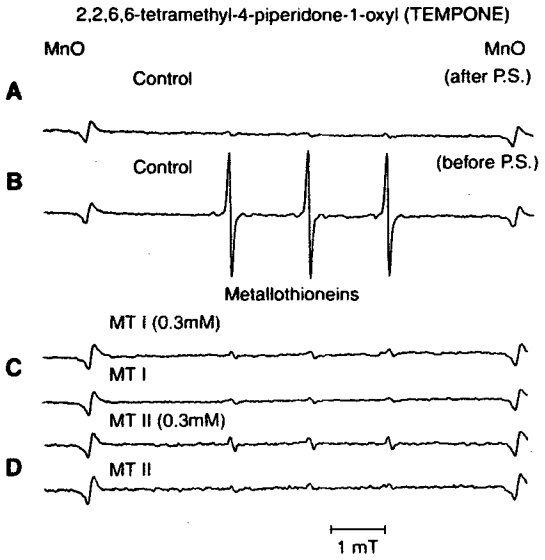

FIGURE 6 Electron spin resonance (ESR) spectra of 2,2,6,6-tetramethyl-4-piperidone (TMPD) riboflavin solutions (Control), before and after photosensitization are shown in A and B, respectively. C and D represent the spectra depicting the effect of metallothioneins on the control signal intensity (B) of the nitroxide adduct, 2,2,6,6-tetramethyl-4-piperidone-1-oxyl (TEMPONE), that corresponds to the overall TMPD-reactive oxygen species interactions, generated during the photosensitization of riboflavin. We observed that MT-II (0.3 mM) could scavenge 92%, while MT-I at 0.15 mM could completely scavenge all the reactive species (2.15×10^{15} spins/ml) generated.

To the best of our experimental and theoretical knowledge, no damage to the thiol group of MT isoforms nor any alteration in the confirmation of MT isoforms occur in the 300–400 nm range as determined by circular dichroism and magnetic circular dichroism spectroscopy of MT isoforms.[39]

The results of these studies suggest that although both isoforms of MT are able to scavenge multiple free radicals, the MT-I appears to be a superior scavenger of superoxide and DPPH radicals.

DISCUSSION

The physiological and structural characteristics of MT suggest that it plays an important role in cell functions during stressful states induced by a broad range of mediators such as catecholamines, oxygen free radicals, cytokines, and steroids.[18,19] MT is characterized by a high thiol content and absence of aromatic amino acids, including tyrosine, tryptophan, and phenylalanine.[2]

The synthesis of MT is increased by various stressful conditions causing oxidative stress including high oxygen tension,[40] X-ray irradiation,[41–43] UV irradiation,[44] food restriction,[45] fasting,[6,46] restrain,[47] heavy exercise,[48] and exposure to cold temperature.[48] Many of the aforementioned conditions cause lipid peroxidation and induce the synthesis of MT isoforms.[22]

The results outlined in this report provide the direct evidence using ESR that MT-I and II are able to protect against diphenyl picrylhydrazyl radicals (Figure 3), hydroxyl radicals (Figure 4), superoxides (Figure 5), and other reactive oxygen species (Figure 6); and support the result of previous studies on the induction of hepatic MT following administration of paraquet.[49]

Although both MT-I and II were efficacious in scavenging free radicals, the reason for a greater potency of MT-I is not clear at this time. Twenty of the 61 amino acid residues in the molecule are cysteinyl residues, all of which are involved in metal binding. MTs contain many cys-x-cys and cys-cys sequences, and the position of the cysteinyl residues along the polypeptide chain is highly conserved in evolutionary terms. Two major isoforms of MT are found in most vertebrate tissues and are designated as MT-I and MT-II. The isoforms have very similar amino acid composition. For example, the two isoforms of rat metallothionein differ in only 12 of the 61 amino acids.[7] MT-II has a higher affinity for zinc than MT-I. MT-II is often predominant, but the relative proportions of the isoproteins depend on the particular stimulus, although the physiological significance of this variation is still unknown. The mechanism by which MT scavenges oxygen free radicals is still unclear, and may be due to cysteine residues[50] or due to zinc released from MT,[51] which in turn protects against lipid peroxidation and stabilizes membrane. Zinc suppresses lipid peroxidation, by decreasing the uptake of iron, by inhibiting the activity of NADPH-cytochrome C reductase, or by increasing the activity of glutathione peroxidase.[52] Indeed, the formation of an iron–MT complex has been shown to occur in an *in vitro* system.[53] Moreover, zinc deficiency state, which reduces the level of MT[54] enhances lipid peroxidation.[55] In addition, the antioxidant property of MT is 50 times greater than glutathione.[56] A study by Maret[57] has shown that oxidative stress, or any other event shifting the balance of the glutathione GSH/GSSG cycle to a more oxidized state, leads to release of zinc from MT.

Although MT isoforms have been characterized well biochemically, the comprehension and elucidation of their principal physiological functions are still at rudimentary stages of development. Due to a high thiol content, MT is a strong nucleophil that can bind not only metal cations but also reactive oxygen intermediaries and organic radicals. Preinduction of MT expression correlates with cell survival subsequent to exposure to otherwise lethal doses of metal cations, alkylating agents and free radicals and overexpression of MT has been demonstrated to protect cells from the toxic side effects of several antineoplastic agents.[58] In view of MT's nuclear and cytoplasmic locations, MT can confer protection to both DNA and cytoplasmic components.[59–61] Damage to MT itself can be repaired (*in vitro*) by glutathione,[50,57] and overexpression of MT protects against hydroperoxide toxicity.[62]

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