Prior Increase in Metallothionein Levels Is Required to Prevent Doxorubicin Cardiotoxicity[1]

XIUHUA SUN* AND Y. JAMES KANG[2,*,#,†]

Departments of Medicine and #Pharmacology and Toxicology, University of Louisville School of Medicine, and †Jewish Hospital Heart and Lung Institute, Louisville, Kentucky 40202

Many studies have shown that metallothionein (MT) can be increased significantly by different oxidative insults in multiple organ systems. However, the increase in MT production often fails to protect against oxidative tissue injury. On the other hand, recent studies using a cardiac-specific, MT-overexpressing, transgenic mouse model have shown that MT protects against oxidative heart injury. Thus, the present study was undertaken to test the hypothesis that prior increase in MT levels is required to prevent oxidative injury. Oxidative heart injury was induced by doxorubicin (DOX), an important anticancer drug that causes severe cardiotoxicity through oxidative stress. Cardiac-specific, MT-overexpressing, transgenic mice and wild-type (WT) FVB mice were treated with DOX at 20 mg/kg. Four days after the treatment, MT concentrations were markedly elevated in the WT mouse heart. The elevated MT concentrations were comparable with those found in the transgenic mouse heart, which did not show further MT elevation in response to DOX challenge. Severe oxidative injury occurred in the heart of WT mice, including myocardial lipid peroxidation, morphological changes as examined by electron microscopy, high levels of serum creatine kinase activity, and decreased total glutathione concentrations in the heart. However, all of these pathological changes were significantly inhibited in the MT-transgenic mice. Therefore, this study demonstrates that there is a correlation between MT induction and oxidative stress in the DOX-treated mouse heart. However, MT can protect the heart from oxidative injury only if it is present prior to induction of oxidative stress.

[Exp Biol Med Vol. 227(8):652–657, 2002]

Key Words: cardiomyopathy; creatine phosphokinase; doxorubicin; ultrastructural alterations; transgenic mice

Metallothionein (MT) is a transition metal-binding protein that has been studied extensively since its discovery in 1957 (1). Many studies have focused on a comprehensive understanding of the physical, chemical, and biochemical properties of MT (2). The biological function of this protein has been a long debate among MT researchers, and the only consensus thus far is that it has a role in the detoxification of heavy metals (3, 4). However, the biological function of MT is likely related to the physiologically relevant metals that it binds. In mammals, MT is found to bind zinc and copper under normal physiological conditions. Both zinc and copper are trace metals that are essential for life. Recent studies have produced strong evidence to support the idea that MT functions as a metal chaperone for the regulation of gene expression and for the synthesis and functional activity of proteins such as metalloproteins and metal-dependent transcription factors (5–9).

An important hypothesis that has drawn extensive attention in the MT research field is that MT plays an antioxidant role in the cytoprotection from injury by reactive oxygen and nitrogen species. The first study to suggest that MT functions as an antioxidant was performed to examine the effect of MT on the radiosensitivity of the cultured human epithelial cell line and mouse fibroblast (C1-1D) cells (10). After this study, a detailed examination of the kinetics and mechanisms of the reaction of MT with superoxide and hydroxyl radicals was done (11). MT containing zinc or cadmium, or both, was shown to scavenge for hydroxyl and superoxide radicals produced by the xanthine/xanthine oxidase reaction system (11). The data suggest that all 20 of the cysteine sulfur atoms are involved in the radical quenching process and that MT appears to be an extraordinarily efficient scavenger (11). The rate constant for the reaction of the hydroxyl radical with MT ($K_{HO\bullet/MT}$ = 2700) is about 340-fold higher than that with glutathione (GSH; $K_{HO\bullet/GSH}$ = 8) (11). Additional studies have shown that MT is about 800-fold more potent than GSH (on a molar basis) in preventing hydroxyl radical-generated DNA degradation *in vitro* (12). A study using HL-60 cells has demonstrated a direct reaction of hydrogen peroxide with the sulfhydryl groups of MT (13). Moreover, the thiolate groups in MT were the preferred targets attacked by hydrogen peroxide compared with the sulfhydryls from GSH and other proteins (13).

However, these *in vitro* observations have not been demonstrated in intact animal studies, and most *in vivo* ex-

[1] This work was supported in part by the National Institutes of Health (grants HL59225 and HL63760) and by a research grant from the Jewish Hospital Foundation (to Y.J.K.).

[2] To whom requests for reprints should be addressed at Department of Medicine, University of Louisville School of Medicine, 511 South Floyd Street, MDR 530, Louisville, KY 40202. E-mail: yjkang01@athena.louisville.edu

Received January 15, 2002
Accepted April 10, 2002

1535-3702/02/2278-0652$15.00

perimental data provide only indirect evidence for the free radical scavenging action of MT. More troublesome is that many studies have failed to demonstrate a correlation between MT induction and antioxidant activity. In a study to examine the role of MT in myocardial protection against doxorubicin (DOX) toxicity, it was shown that there is no correlation between MT induction and protection from toxicity induced by DOX in the mouse heart (14). Studies in other tissues also show the lack of a correlation between MT induction and protection against oxidative injury (15–17).

Cardiac protection from DOX toxicity, on the other hand, has been consistently observed in cardiac-specific, MT-overexpressing, transgenic mice (MT-TG) (18–25). In particular, when the levels of MT in the hearts of transgenic mice were compared with those induced by oxidative stress, there were no significant differences. These results suggest that the induction of endogenous MT under oxidative stress conditions may occur as a consequence of oxidative injury when it may be too late for MT to protect against the injury. Oxidative stress-elevated MT may prevent further oxidative damage, but it may not repair the injury that occurred before its induction. Therefore, it is important to critically examine experimental details in the context of preexisting conditions to interpret results, and conclusions should not be derived only from a simple correlation analysis. Thus, the present study was undertaken to test the hypothesis that the preexisting high levels of MT are critical for cardiac protection from oxidative injury.

Methods

Chemicals. DOX hydrochloride (Adriamycin), GSH, NADH, NADPH, glutathione reductase, and ATP were purchased from Sigma Chemical Co. (St. Louis, MO). Sulfosalicylic acid and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Aldrich Chemical Co. (Milwaukee, WI). All reagents were of the highest purity available.

Animals and Drug Treatment. FVB mice were obtained originally from Harlan Bioproducts for Science, Inc. (Indianapolis, IN) and were bred and maintained at the University of Louisville animal facilities. Cardiac-specific, MT-TG mice were produced from the FVB stain as described previously (18). Animals were housed in plastic cages at 22°C on a 12:12-hr light:dark cycle and were given laboratory chow and tap water *ad libitum*. Adult male mice (6–8 weeks old, weighing 20–30 g) were randomly assigned to either the drug treatment group or the control group. The drug treatment group received 20 mg/kg DOX dissolved in physiological saline by a single intraperitoneal injection at a volume of 5 ml/kg, and the control group received an equal volume of saline without DOX. Animals were sacrificed on the 4th day of DOX injection and the hearts were removed and further processed for studies described below. All animal procedures were approved by the American Association of Accreditation of Laboratory Animal Care's certified Institutional Animal Care and Use Committee.

Cardiac MT Measurement. MT concentrations in the heart were determined by cadmium-hemoglobin affinity assay (26). Briefly, heart tissues were homogenized in 4 volumes of 10 m*M* Tris-HCl buffer, pH 7.4, at 4°C. After the centrifugation of the homogenate at 10,000*g* for 15 min, 200 μl of supernatant were transferred to microtubes for MT analysis as described previously (26). The MT concentrations in the heart tissue from nontransgenic and transgenic mice treated with or without DOX are expressed as micrograms per gram of heart tissue.

Creatine Phosphokinase (CPK) Activity. Serum CPK activity was assayed as described by Oliver (29). A CPK test kit (CK-20; Sigma Chemical Co.) based on this method was used. Blood was collected from the inferior vena cava of the anesthetized animals, and the serum was obtained by a serum separator apparatus (Becton Dickinson, Rutherford, NJ).

Morphological Examination by Electron Microscopy. After being anesthetized with sodium pentobarbital (60 mg/kg body wt), the hearts of all experimental animals were fixed *in situ* by vascular perfusion with saline for 10 min followed by a Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer, pH 7.4) for 15 min. The *in situ* fixative perfusion procedure was described previously (18). The tissue samples taken from the left ventricles were cut into 1-mm^3 blocks and kept in the same fixative overnight at 4°C. After rinsing with the same buffer, the blocks were postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series with 100% propylene oxide as a transitional solvent, and embedded in LX-112 resin (LADD Research Industries Co., Burlington, VT). Ultrathin sections were obtained with an ultramicrotome (LKB Instruments, Gaithersburg, MD) and were stained with uranyl acetate and lead citrate and observed with a Philip transmission electron microscope (Eindhoven, the Netherlands). Semiquantitative analysis of the morphological changes was done as described in Table I.

Lipid Peroxidation Assay. Cardiac lipid peroxidation was quantified by measuring thiobarbituric acid-reactive substance (TBARS) as described previously (28).

Table I. Semiquantitative Analysis of Mitochondrial Damage in WT and MT-TG Mouse Hearts

Treatment	WT	MT-TG
Saline	<1%	<1%
DOX	83%[a]	37%[b]

Note. Heart tissues of WT mice or MT-TG mice treated with DOX were examined by electron microscopy at x4500. Five random cells from each experimental group ($n = 3$) were examined for mitochondrial morphology, and the total numbers of mitochondria were counted in each cell. Mitochondria were classified as abnormal if they showed mitochondrial swelling, intramitochondrial vacuoles, loss of cristae, or had abnormal size or shape. The results are expressed as the percentage of abnormal mitochondria.

[a] Mitochondria showed many alterations, including swelling, intramitochondrial vacuoles, loss of cristae, and abnormal shape and size. [b] Mitochondria mainly exhibited abnormal shape and size.

Heart tissue was homogenized in 9 volumes of 1.15% KCl. To 0.2 ml of the tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid, 1.5 ml of 0.9% thiobarbituric acid, and 0.6 ml of distilled water were added and vortexed. The reaction mixture was placed in a water bath at 90°C for 70 min. After cooling on ice, 1.0 ml of distilled water and 5.0 ml of butanol/pyridine mixture (15:1, v/v) was added and vortexed. After centrifugation at 3000*g* for 15 min, the organic phase was determined at 532 nm. The TBARS concentration was expressed as nanomoles TBARS per gram of heart tissue.

Total GSH Concentration Heart tissues were homogenized in 10 volumes of 5% (w/v) 5-sulfosalicylic acid at 4°C. The homogenate was centrifuged at 10,000*g* for 5 min, and the supernatant was assayed for GSH by the DTNB-glutathione reductase recycling assay (27). The 1.0-ml reaction mixture contained 190 μl of stock buffer (143 m*M* sodium phosphate and 6.3 m*M* Na_4-EDTA, pH 7.5), 700 μl of 0.248 mg of NADPH/ml in stock buffer, 100 μl of 6 m*M* DTNB, and 10 μl of sample. The assay was initiated by the addition of 10 μl of 266 U/ml glutathione reductase. GSH was used as a standard and was assayed in parallel under the same condition as the tissue samples.

Statistical Analysis Data are expressed as mean ± SD and were analyzed according to a 2 × 2 (mouse strain: WT versus MT-TG; and treatment: saline versus DOX) factorial design. After a significant interaction was detected by the two-way analysis of variance (ANOVA), the significance of the main effects was further determined. The level of significance was considered at $P < 0.05$.

Results

Transgenic mice and wild-type (WT) mice were treated with a single dose of DOX at 20 mg/kg. Four days after the treatment, MT concentrations, serum CPK activity, myocardial morphological changes, lipid peroxidation, and total GSH concentrations in the heart were examined. To make a comparison in myocardial MT production in response to DOX between transgenic and nontransgenic mice, MT concentrations in the heart were measured before and after DOX treatment. As shown in Table II, MT concentrations were about five times higher in the transgenic mouse heart than those in the WT mouse heart before DOX treatment. After the treatment, MT concentrations were significantly increased in the WT mouse heart, reaching a level comparable with that in the transgenic mouse heart in which MT concentrations remained as the same as before DOX treatment.

Serum CPK activity changes have been used as an important indication of myocardial injury induced by DOX. As shown in Figure 1, CPK activities were significantly elevated by DOX in both the transgenic and WT mice; however, a significant inhibition was observed in the transgenic mice. Corresponding to serum CPK activity changes, DOX induced dramatic morphological changes in the WT mouse heart as examined by electron microscope. These changes include moderate nuclear chromatin margination with many clumped pieces of coarse chromatin, severe mitochondrial swelling with membrane disruption, cristae disappearance, matrix clear-out, and dilated sarcoplasmic reticula (Fig. 2). In contrast to these findings, the chromatin was distributed homogeneously within the nucleus, mitochondrial membrane and cristae were intact, and much less dilation in sarcoplasmic reticula was observed in the transgenic mouse heart. Semiquantitative morphometric analysis by counting the number of damaged mitochondria further confirmed the inhibition of the DOX-induced ultrastructural changes in the transgenic mouse heart as shown in Table I.

Because MT functions as an antioxidant *in vivo* to prevent tissue injury caused by oxidative stress, the effects of MT on lipid peroxidation induced by DOX in the heart was examined. As shown in Figure 3, the extent of lipid peroxidation as measured by TBARS concentrations in the heart was significantly enhanced in the WT mice. This increase was significantly inhibited in the transgenic mice. To further examine the oxidative changes induced by DOX, total GSH concentrations in the heart were measured. As shown in Figure 4, DOX significantly decreased total GSH concentrations in the hearts of the WT mice, but not in those of the transgenic mice.

Discussion

The results obtained from this study demonstrate that there are different responses to DOX-induced oxidative stress in myocardial tissue between MT-TG and WT mice. These differences include MT concentrations that were elevated by DOX treatment in the heart of WT mice, but not in the transgenic mice. The elevation in MT concentrations in the WT mouse heart reached a comparable level with that presented in the transgenic mouse heart, however, severe myocardial injury occurred in WT mice, but was inhibited in transgenic mice. Therefore, the most important information generated from this study is that elevation of MT concentrations in the heart prior to exposure to DOX is required to prevent this drug's cardiotoxicity.

Table II. Comparison between DOX-Induced MT Elevation in WT and MT-TG Mouse Hearts

	WT		MT-TG	
	SLN	DOX	SLN	DOX
MT (μg/g heart)	5.42 ± 0.81	20.14 ± 3.09[a]	25.34 ± 4.03[a]	26.23 ± 3.32[a]

Note. Values are means ± SD for five to eight mice.
[a] Significantly different from the SLN-treated WT mice ($P < 0.01$).

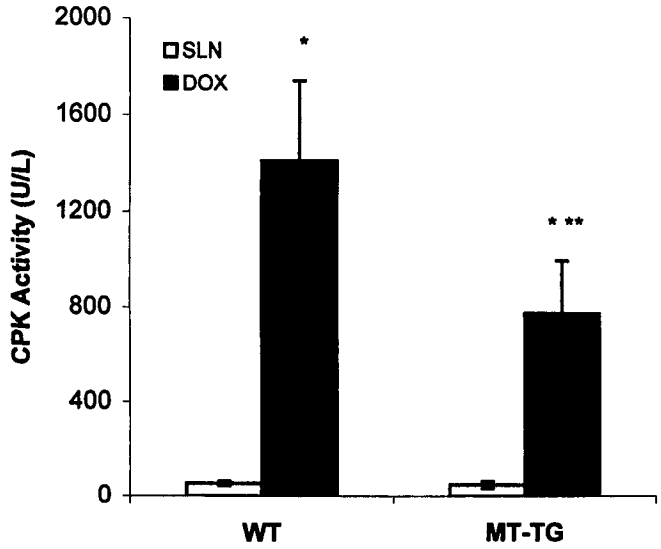

Figure 1. DOX-induced serum CPK release from the heart. Serum CPK activity was measured using a CK-20 kit from Sigma Chemical Co. as described previously (18). As shown, DOX significantly increased serum CPK level in the WT animal. This elevation was significantly inhibited in the MT-TG mice. $^{*}P < 0.01$ vs saline (SLN) controls; $^{**}P < 0.01$ vs DOX-treated WT mice.

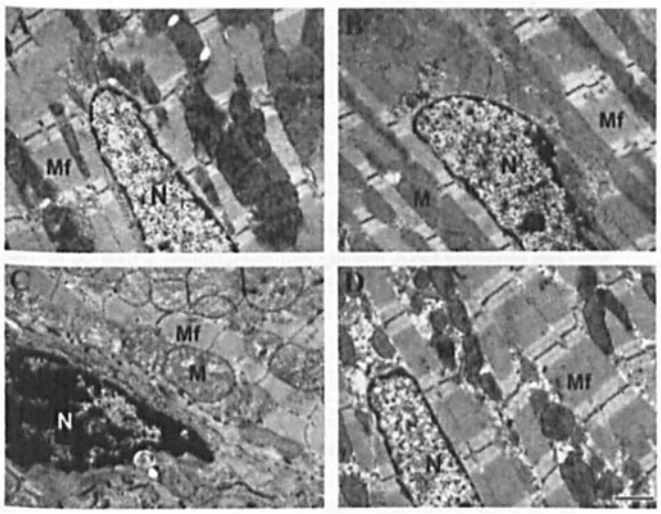

Figure 2. Electron micrograph showing different effects of DOX on myocardial ultrastructures in WT and MT-TG mice. (A) WT mice treated with saline (SLN) as control; (B) MT-TG mice treated with SLN; (C) WT mice treated with DOX; (D) MT-TG mice treated with DOX. M, mitochondria; Mf, myofiber; N, nucleolus. Scale bar = 1 μm.

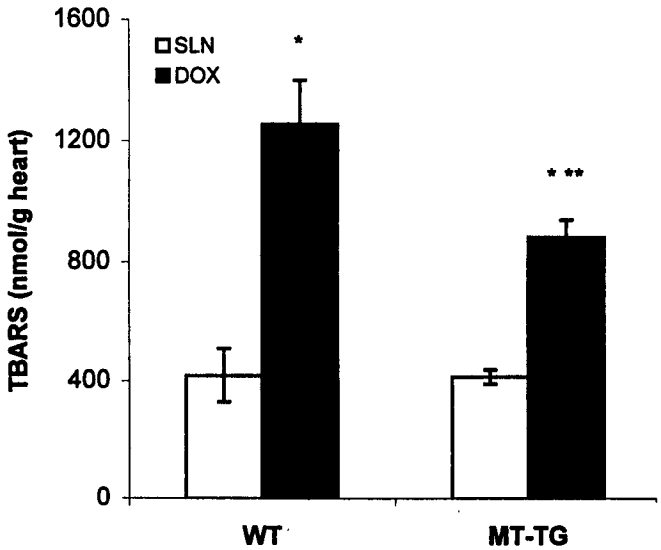

Figure 3. DOX-induced lipid peroxidation in the heart as determined by the TBARS assay. As shown, DOX significantly elevated the lipid peroxide concentrations in the WT animal. This elevation was significantly inhibited in the MT-TG mice. $^{*}P < 0.01$ vs saline (SLN) controls; $^{**}P < 0.05$ vs DOX-treated WT mice.

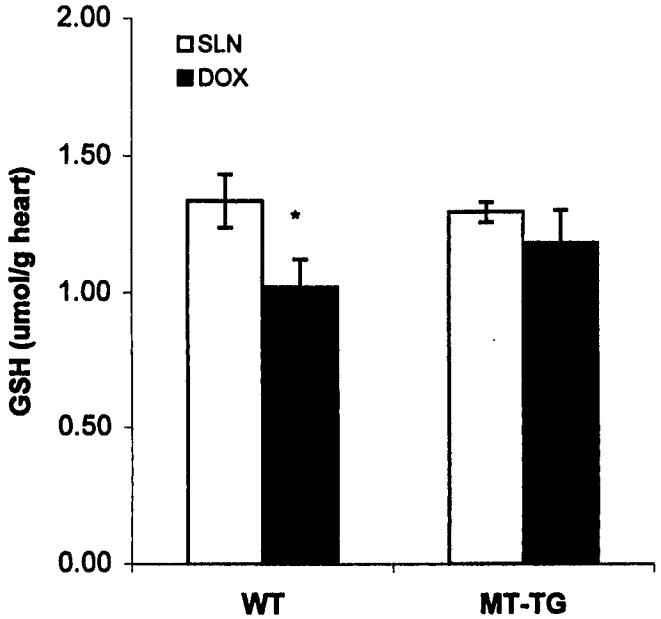

Figure 4. DOX-induced decrease in total GSH concentrations in the heart. As shown, DOX significantly decreased the total GSH concentrations in the WT animal ($^{*}P < 0.01$). This effect was significantly inhibited in the MT-TG mice.

The lack of a correlation between MT induction and cytoprotection against oxidative injury has been often reported (14–17). The same observation was obtained in the WT mice from the present study. In previous studies, we have also observed that treatment of mice with DOX at a single dose of 15 mg/kg for 4 days markedly increased MT mRNA abundance in the heart of the WT mice. However, even with a high level of myocardial MT concentration, DOX caused severe toxic effects in the heart, as shown in the present study. On the other hand, DOX-induced cardiotoxicity was significantly inhibited in the transgenic mice whose MT concentrations in the heart were comparable with that induced by DOX in the WT mice. Thus, it demonstrates that preexisting high levels of MT in the heart are critical for the protective function of this protein against oxidative stress. Under normal physiological conditions, MT concentrations in the heart are very low. Because the MT gene is regulated by free radicals (30), the induction of MT under oxidative stress is thus likely a stress or adaptive response to oxidative injury. This scenario is further suggested by the result obtained from transgenic mice. In these mice, the preexisting or constitutively high levels of MT

were expressed from the MT transgene driven by α-cardiac myosin heavy chain promoter (18). The regulation of this promoter is not responsive to oxidative stress. Due to the preexisting high levels of MT in the transgenic mice, myocardial oxidative injury was greatly inhibited so that the native MT gene in the heart was not upregulated, leading to no further production of MT in the heart.

Thus, the present study suggests that MT may not provide the first line of defense against oxidative stress under physiological conditions, that induction of endogenous MT under oxidative stress conditions may occur as a consequence of oxidative injury, when it may be too late for MT to protect from the occurrence of the injury, and that MT may prevent further oxidative injury, but may not repair the injury that has occurred prior to its induction. Therefore, it is important to critically examine experimental details in the context of preexisting conditions to interpret experimental results, and conclusions should not be drawn only from a simple correlation analysis.

The results obtained from the present study also demonstrate that the effective level of MT in myocardial protection against oxidative injury is pharmaceutically inducible. As shown in the transgenic mouse heart, the MT concentrations were about five times more than in the WT mouse heart. Under the treatment with a single dose of DOX at 20 mg/kg, MT concentrations in the WT mouse heart were elevated to about four times more than basic levels. It is obvious that DOX cannot be used as an inducer for MT production for the myocardial protection. There are several studies that have shown that several agents selectively elevate MT levels in the heart, such as bismuth subnitrate (31), isoproterenol (32), and tumor necrosis factor-α (33). The induction of MT in the heart also inhibited DOX cardiotoxicity (25). Exploring the potential of MT in protection against myocardial oxidative injury would likely result in novel approaches to cardiac medicine. However, for such a development of pharmaceutical inducers for MT production in the heart, several critical issues need to be addressed. First, can elevation of endogenous MT reach an effective level? This question was answered by the present study. Second, are selective agents that can be used to increase MT production in the heart? This question has been answered in several previous studies as indicated above. Third, what is the approach to sustain the induced high level of MT in the heart? This question needs to be addressed in future studies.

In conclusion, myocardial MT concentrations can be significantly elevated in response to oxidative stress. However, this elevation likely occurs postoxidative tissue injury as a myocardial adaptive response to damage. Although the elevated MT concentrations can reach a high level that is sufficient to provide myocardial protection against oxidative injury, the delayed production of this protein after damage makes the lack of a correlation between elevation of myocardial MT concentrations and myocardial protection against oxidative injury. Therefore, preexisting high levels of MT in the heart are required for this protein to be functional in protection against oxidative injury.

It should note that there are some limitations in this study. For example, metabolism of DOX in the myocardial tissue was not examined. Therefore, it cannot exclude the possibility that alterations in DOX metabolism in the MT-TG mouse heart might occur, such as a decrease in DOX accumulation in the heart. Another possibility is that although there are no other changes in the MT-TG mouse heart examined so far, some undetected alterations may occur that might affect the observation. These possibilities will be our future undertakings in the MT studies.

The authors thank Ms. Cathy Caple for technical assistance. Y.J.K. is a University Scholar at the University of Louisville.

1. Margoshes M, Vallee BL. A cadmium protein from equine kidney cortex. J Am Chem Soc **79**:1813–1814, 1957.
2. Kagi JH, Schaffer A. Biochemistry of metallothionein. Biochemistry **27**:8509–8515, 1988.
3. Klaassen CD, Liu J, Choudhuri S. Metallothionein: an intracellular protein to protect against cadmium toxicity. Annu Rev Pharmacol Toxicol **39**:267–294, 1999.
4. Templeton DM, Cherian MG. Toxicological significance of metallothionein. Methods Enzymol **205**:11–24, 1991.
5. Maret W. Metallothionein/disulfide interactions, oxidative stress, and the mobilization of cellular zinc. Neurochem Int **27**:111–117, 1995.
6. Zeng J, Vallee BL, Kagi JH. Zinc transfer from transcription factor IIIA fingers to thionein clusters. Proc Natl Acad Sci U S A **88**:9984–9988, 1991.
7. Zeng J, Heuchel R, Schaffner W, Kagi JH. Thionein (apometallothionein) can modulate DNA binding and transcription activation by zinc finger containing factor Sp1. FEBS Lett **279**:310–312, 1991.
8. Jacob C, Maret W, Vallee BL. Control of zinc transfer between thionein, metallothionein, and zinc proteins. Proc Natl Acad Sci U S A **95**:3489–3494, 1998.
9. Maret W, Larsen KS, Vallee BL. Coordination dynamics of biological zinc 'clusters'' in metallothioneins and in the DNA-binding domain of the transcription factor Gal4. Proc Natl Acad Sci U S A **94**:2233–2237, 1997.
10. Bakka A, Johnsen AS, Endresen L, Rugstad HE. Radioresistance in cells with high content of metallothionein. Experientia **38**:381–383, 1982.
11. Thornalley PJ, Vasak M. Possible role for metallothionein in protection against radiation-induced oxidative stress: kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim Biophys Acta **827**:36–44, 1985.
12. Abel J, de Ruiter N. Inhibition of hydroxyl-radical-generated DNA degradation by metallothionein. Toxicol Lett **47**:191–196, 1989.
13. Quesada AR, Byrnes RW, Krezoski SO, Petering DH. Direct reaction of H_2O_2 with sulfhydryl groups in HL-60 cells: zinc-metallothionein and other sites. Arch Biochem Biophys **334**:241–250, 1996.
14. Yin X, Wu HY, Chen Y, Kang YJ. Induction of antioxidants by Adriamycin in mouse heart. Biochem Pharmacol **56**:87–93, 1998.
15. Min KS, Terano Y, Onosaka S, Tanaka K. Induction of metallothionein synthesis by menadione or carbon tetrachloride is independent of free radical production. Toxicol Appl Pharmacol **113**:74–79, 1992.
16. Sato M. Dose-dependent increase in metallothionein synthesis in the lung and liver of paraquat-treated rats. Toxicol Appl Pharmacol **107**:98–105, 1991.
17. Conrad CC, Grabowski DT, Walter CA, Sabia M, Richardson A. Us-

ing $MT^{-/-}$ mice to study metallothionein and oxidative stress. Free Radical Biol Med **28:**447–462, 2000.
18. Kang YJ, Chen Y, Yu A, Voss-McCowan M, Epstein PN. Overexpression of metallothionein in the heart of transgenic mice suppresses doxorubicin cardiotoxicity. J Clin Invest **100:**1501–1506, 1997.
19. Kang YJ, Zhou Z, Wang GW, Buridi A, Klein JB. Suppression by metallothionein of doxorubicin-induced cardiomyocyte apoptosis through inhibition of p38 mitogen-activated protein kinases. J Biol Chem **275:**13690–13698, 2000.
20. Zhou Z, Kang YJ. Immunocytochemical localization of metallothionein and its relation to doxorubicin toxicity in transgenic mouse heart. Am J Pathol **156:**1653–1662, 2000.
21. Sun X, Zhou Z, Kang YJ. Attenuation of doxorubicin chronic toxicity in metallothionein-overexpressing transgenic mouse heart. Cancer Res **61:**3382–3387, 2001.
22. Wang GW, Kang YJ. Inhibition of doxorubicin toxicity in cultured neonatal mouse cardiomyocytes with elevated metallothionein levels. J Pharmacol Exp Ther **288:**938–944, 1999.
23. Wang GW, Klein JB, Kang YJ. Metallothionein inhibits doxorubicin-induced mitochondrial cytochrome c release and caspase-3 activation in cardiomyocytes. J Pharmacol Exp Ther **298:**461–468, 2001.
24. Wu HY, Kang YJ. Inhibition of buthionine sulfoximine-enhanced doxorubicin toxicity in metallothionein overexpressing transgenic mouse heart. J Pharmacol Exp Ther **287:**515–520, 1998.
25. Kang YJ. The antioxidant function of metallothionein in the heart. Proc Soc Exp Biol Med **222:**263–273, 1999.
26. Eaton DL, Cherian MG. Determination of metallothionein in tissue by cadmium-hemoglobin affinity assay. Methods Enzymol **205:**83–88, 1991.
27. Tietze F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem **27:**502–522, 1969.
28. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. Anal Biochem **44:**276–278, 1979.
29. Oliver IT. Improved method for determination of creatine kinase. J Lab Clin Med **62:**159–164, 1963.
30. Andrews GK. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem Pharmacol **59:**95–104, 2000.
31. Satoh M, Naganuma A, Imura N. Effect of preinduction of metallothionein on paraquat toxicity in mice. Arch Toxicol **66:**145–148, 1992.
32. Namikawa K, Okazaki Y, Nishida S, Kimoto S, Akai F, Tomura T, Hashimoto S. [Changes in myocardial metallothionein on isoproterenol-induced myocardial injury]. Yakugaku Zasshi **113:**591–595, 1993.
33. Sato M, Sasaki M, Hojo H. Tissue-specific induction of metallothionein synthesis by tumor necrosis factor-α. Res Commun Chem Pathol Pharmacol **75:**159–172, 1992.