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# Metallothionein blocks oxidative DNA damage in vitro

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## Abstract

The role of metallothionein (MT) in mitigation of oxidative DNA damage (ODD) induced either by cadmium (Cd) or the direct oxidant hydrogen peroxide ($H_2O_2$) was systematically examined by using MT-I/II double knockout (MT-null) or MT-competent wild-type (WT) cells. Both toxicants were much more lethal to MT-null cells (Cd $LC_{50}$ = 6.6 μM; $H_2O_2$ $LC_{50}$ = 550 μM) than WT cells (Cd $LC_{50}$ = 16.5 μM; $H_2O_2$ $LC_{50}$ = 930 μM). Cd induced concentration-related MT increases in WT cells, while the basal levels were undetectable and not increased by Cd in MT-null cells. ODD, measured by the immuno-spin trapping method, was minimally induced by sub-toxic Cd levels (1 or 5 μM; 24 h) in WT cells, but markedly increased in MT-null cells (> 430%). Similarly, ODD was induced to higher levels by lower concentrations of $H_2O_2$ in MT-null cells than WT cells. Transfection of MT-I into MT-null cells reduced both Cd- and $H_2O_2$-induced cytolethality and ODD. Cd increased expression of the oxidant defense genes, *HO-1* and *GSTa2* to a much greater extent in MT-null cells than WT. Cd or $H_2O_2$ exposure increased expression of key transport genes, *Mrp1* and *Mrp2,* in WT cells but not in MT-null cells. MT protects against Cd- and $H_2O_2$-induced ODD in MT competent cells possibly by multiple mechanisms, potentially including direct metal ion sequestration and sequestration of oxidant radicals by MT. MT-deficient cells appear to adapt to Cd primarily by turning on oxidant response systems, while MT-competent cells activate MT and transport systems.

### Keywords

DNA; cadmium; oxidative damage; metallothionein; cancer

## Introduction

Cadmium (Cd) is a ubiquitous toxic environmental pollutant metal and known human carcinogen (IARC 2012; ROC 2011). However, the mechanism of Cd carcinogenesis is

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**CONFLICT of INTEREST STATEMENT**
None.

incompletely understood. Some studies link Cd exposure to production of reactive oxygen species (ROS; Filipic, et al. 2004; Chiaverini and Ley 2010). ROS could produce oxidative DNA damage (ODD), a hallmark of many metals, possibly leading to chronic diseases (Jomova and Valko 2011). In this regard, oxidative damage to DNA may initiate the carcinogenic process (Mates and Sanchez-Jimenez 2000), and it has been proposed that ROS plays a role in Cd carcinogenesis (Filipic, et al. 2004). Although Cd is not a redox active metal and it would not be expected to undergo typical Fenton-type reactions (O'Brien and Salacinski 1998; Jomova and Valko 2011), it could indirectly form ROS (Waisberg et al. 2003). For instance, Cd can displace iron and copper from various proteins (e.g. ferritin), increasing the amount of unbound metal ions that can directly cause oxidative stress via Fenton reactions (Jomova and Valko 2011). Cd can also deplete cellular glutathione and thereby disrupt "redox homeostasis" leading to DNA damage (Nemmiche et al. 2011). In this fashion Cd can act as an oxidation "facilitator" that enhances ROS formation, causing damage to critical macromolecules (Gioacchino et al. 2008), presumably including DNA.

Metallothionein (MT) is a small cysteine-rich, metal-binding family of proteins (Klaassen et al. 1999, 2009). The major isoforms of MT, MT-I and MT-II, are common in mammalian cells, and are induced by a variety of metals, including Cd (Klaassen et al. 1999, 2009; Chiaverini and Ley 2010). MT plays a key role in the homeostasis of essential metals like zinc (St Croix, 2002) but clearly functions with toxic metals like Cd (Klaassen et al. 1999, 2009). With bioaccumulation, Cd is often associated with MT, which generally detoxicates the metal by direct binding (Klaassen et al. 1999, 2009). The role of MT in carcinogenesis is not well defined. MT-transgenic mice, which greatly overexpress MT, are resistant to treatments that induce oxidative stress and hyperplastic lesions of the liver (Quaife et al., 1999). Conversely, MT deficiency causes hypersensitivity to chemically-induced skin cancer (Suzuki et al. 2003). Previous data indicate Cd causes a dose-related increase in liver tumors in MT-deficient mice (Waalkes and Liu 2009). Overall, however, the role of MT in Cd carcinogenesis has not been fully defined.

Although Cd exposure has been associated with production of cellular ROS and oxidant stress, this is likely an indirect effect (Nzengue et al. 2012; Qu et al. 2005). One important manifestation of oxidant stress is ODD. ROS and oxidative stress can also induce MT gene expression (Braithwaite et al. 2010). MT may act as an antioxidant by sequestering ROS and thus protecting against cellular injury (Chiaverini and Ley, 2010). The cysteines in MT appear able to react directly with ROS (Chiaverini and Ley 2010; Halliwell et al. 2000) and the protein may act as an antioxidant by mitigating ROS-induced cellular injury independent of a function in metal sequestration (Fridovich et al. 1986; Chiaverini and Ley 2010). In this regard, MT-I/II double knockout (MT-null) cells and their wild-type (WT) control cells are an excellent model for the study of MT-related mitigation of toxicity (Lazo et al. 1995; Leslie et al. 2006; Klaassen et al. 2009; Chiaverini and Ley 2010; Fujishiro et al. 2011).

Cd toxicity can be influenced by various biological defense systems beyond MT. Such influencing factors clearly include transport proteins like the multidrug resistance-related protein 1 (Mrp1) and 2 (Mrp2), heme oxygenase-1 (HO-1), multiple glutathione S-transferase (GST) isoforms such as glutathione S-transferase-pi 1(GST-π) and glutathione S-transferase-α2 (GSTα2) (Hoffmann and Kroemer 2004; Klaassen et al. 2009; Chiaverini and Ley 2010). These protective factors can act to help detoxicate Cd, either by direct actions (efflux), or by acting upon Cd-induced ROS (Chiaverini and Ley 2010). The precise mechanisms of such interactions are complex and remain to be fully elucidated.

Thus, the purpose of this study was to systematically determine if MT protects from Cd- or $H_2O_2$-induced ODD in vitro using the immuno-spin trapping (IST) method (Ramirez et al., 2006), a sensitive assay with low background which detects DNA radicals *in situ*, and

avoids many issues of isolation artifacts associated with other methods (Ramirez et al., 2006; Ramirez et al., 2007). To fully test the role of MT, we utilized MT-null cells and the otherwise isogenic WT cells as a test system (Lazo et al. 1995) and assessed ODD following exposure to several concentrations of Cd and the direct oxidant $H_2O_2$ at levels that were non-cytotoxic.

## Material and methods

### Chemicals and reagents

$CdCl_2$ was purchased from Sigma Chemical (St. Louis, MO). Monoclonal mouse anti-MT antibody was obtained from Dako Corporation (Carpinteria, CA) and reacts well to both MT-I and MT-II. The spin trapping reagent, 5,5-dimethyl-1-pyrroline N-oxide (DMPO) came from Alexis Biochemicals (San Diego, CA), and was purified twice by vacuum sublimation at 15-25°C and stored under argon atmosphere at −80°C until use. $H_2O_2$ was purchased from Fisher Scientific (Pittsburgh, PA). The cell Titer 96 Non-Radioactive Cell Proliferation Assay kit was purchased from Promega (Madison, WI).

### Cells and culture conditions

A cell line originating from the embryonic cells of mice with the targeted disruption of the MT-I/II genes, termed MT-null cells, and a WT control cell line from the parental strain of mice were obtained from Dr. John Lazo, University of Pittsburgh (Lazo et al. 1995). Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Lazo et al. 1995).

### Cytotoxicity

Acute cytolethality of Cd and $H_2O_2$ in WT and MT-null cells was defined by metabolic integrity using the cell proliferation assay kit as previously described (Qu et al. 2002). After media containing various levels of Cd or $H_2O_2$ were added, cells were then incubated for 24 h and viability was determined. Viability is expressed as lethal concentration 50% ($LC_{50}$) values. $LC_{50}$ values are the level of a toxicant that causes lethality to 50% of the cells and were determined from analysis of the linear portion of four separately derived metabolic integrity curves.

### MT transfection into MT-null cells

MT-null cells were made MT-competent by transfection of the MT-I gene. Complexes between pcDNA3-MT and the FuGen 6 Transfection Reagent (Roche, Mannheim, Germany) were made according to the manufacturer using MT-I gene. After construction, pcDNA3-MT-reagent complexes were incubated with MT-null cells for 16 h. FuGen 6 transfection system was used with 6-well plates and 3 μl FuGen 6 per well after a method established in prior work (Zuo et al. 2009).

### ODD measurement by IST method

ODD was measured in the various cell types by the IST method (Ramirez et al., 2006; Ramirez et al., 2007). The IST method measures the formation of DNA radicals in cells by reaction with DMPO causing *in situ* conversion of radicals to stable nitrone adducts, subsequent isolation of DNA and then immunochemical quantification of DNA adducts. Because this method fixes DNA radicals in cells before isolation and quantitation of DNA, it avoids artifacts introduced during DNA isolation and significantly reduces background signals (Ramirez et al., 2006; Ramirez et al., 2007).

### Gene expression at transcript level

Total RNA was isolated from cells using TRIzol (GIBCO/BRL Life Technologies) and then subjected to DNase digestion using RNase-Free DNase Set (Qiagen, Valencia, CA) followed by the cleanup using RNeasy Mini kit (Qiagen, Valencia, CA). The resultant DNA-free RNA was quantitated by UV spectroscopy at 260 nm and stored in RNase-free $H_2O$ at −80°C. Quantitative real-time reverse transcription polymerase chain reaction (real-time RT-PCR) was conducted as described previously (Zuo et al., 2009). The primers were designed using Primer Express software (Applied Biosystems) and included: *MT-I*: Forward 5′-AAT GTG CCC AGG GCT GTG T-3′; Reverse, 3′-GCT GGG TTG GTC CGA TAC TAT T-5′; *MT-II*: Forward 5′-TGT GCC TCC GAT GGA TCC T -3′; Reverse, 3′-GCA GCC CTG GGA GCA CTT-5′; *Mrp1*: Forward 5′-TGG TGA CAG ACA CCG TAG GAA A-3′; Reverse 3′-TGT GTT GCT GGC TGG TAT CC -5′; *Mrp2*: Forward 5′-TGC AGC TTC CTT GAC CAT GA-3′; Reverse 3′-CCT GCT GCC GGA CCT AGA G -5′; *HO-1*: Forward 5′-CCT CAC TGG CAG GAA ATC ATC-3′; Reverse 3′-CCT CGT GGA GAC GCT TTA CAT A-5′; *GST-π*: Forward 5′-TGG GCA TCT GAA GCC TTT TG-3′; Reverse 3′-GAT CTG GTC ACC CAC GAT GAA-5′; *GSTα2*: Forward 5′-CTT GTT GGG CCC CAC ATC T-3′; Reverse 3′-CTG GGA TGC CCT TCA AAG ACT-5′; -*actin*: Forward 5′-GGC CAA CCG TGA AAA GAT GA-3′; Reverse 3′-CAG CCT GGA TGG CTA CGT ACA-5′.

### Gene expression at translational level

Cells were lysed by adding 1X SDS sample buffer with 1% Protease Inhibitor Cocktail and 1% Phosphatase Inhibitor Cocktail 1 (Sigma/Aldrich, St. Louis, MO). The cells were immediately scraped off the plate and transferred to a microcentrifuge tube on ice. Samples were sonicated for 10-15 seconds to shear DNA and reduce viscosity, and then centrifuged at 18,000 *g* for 10 min. The resulting supernatants (termed cytosol) were used for determining MT protein levels by Western blot (20 μg protein). Relative densities of the bands were digitally quantified by using Bio-Rad Quantity One-4.4.0 analysis software (Qu et al. 2005).

### Statistical analysis

Data are expressed as the mean SEM. A Student's t-test was used when comparing responses of different cell lines at the same toxicant concentration. An ANOVA with subsequent Dunnett's multiple comparison test was used when comparing multiple concentration responses to a given toxicant within a single cell line to the appropriate control. Values are derived from 3 or more replicates. Differences were considered significant at a level of $p < 0.05$.

## Results

### MT protects against Cd- or $H_2O_2$-induced cytotoxicity and ODD

MT-null and WT cells were treated with Cd for 24 h and cytolethality was measured. Cd was much less cytolethal in MT-competent WT cells than in MT-deficient MT-null cells. The $LC_{50}$ was 2.5-fold greater in WT cells (16.5 ± 2.1 μM [mean ± SE]) than that in MT-null cells (6.6 ± 1.6 μM; $p < 0.05$ from WT). To test if MT also confers a generalized resistance to oxidant stress, MT-null and WT cells were exposed to $H_2O_2$, a direct oxidant. Similar to cells treated with Cd, the $LC_{50}$ after $H_2O_2$ treatment was 1.7-fold greater in WT cells (930 ± 20 μM) than in MT-null cells (550 ± 10 μM; $p < 0.05$ from WT).

To determine if MT mitigates ODD, MT-null and WT cells were then exposed to Cd or $H_2O_2$ at sub-toxic levels and ODD was assessed (Fig. 1). Cd exposure induced more ODD

in MT-null cells than WT cells (Fig. 1a). Likewise, treatment with $H_2O_2$ caused levels of ODD that were markedly higher in MT-null cells than WT cells (Fig. 1b).

### MT expression after Cd or $H_2O_2$ exposure

*MT-I* and *MT-II* transcripts and MT protein levels were measured after exposure to non-toxic levels of Cd- or $H_2O_2$. Cd caused concentration-related increases in expression of *MT-I* and *MT-II* transcripts (Fig. 2a, b) and MT protein (Fig. 2c) in WT cells. Cd had no effect on *MT-I* and *MT-II* transcript in MT-null cells (not shown) and did not impact MT protein, which was not detectable in control or treated cells (see Fig. 2c inset). In Wt cells $H_2O_2$ did not increase expression of *MT-I* (Fig. 2d), but did increase expression of *MT-II* at the highest concentration tested (Fig. 2e). $H_2O_2$ also caused concentration-related increases in MT protein in WT cells (Fig. 2f). In contrast, the basal levels of MT protein were not detectable and were not increased by $H_2O_2$ in the MT-null cells (see Fig. 2f inset), and, like Cd, in MT-null cells $H_2O_2$ did not increase expression of *MT-I* or *MT-II* transcript (data not shown).

### Expression of efflux genes and oxidant stress response genes after Cd or $H_2O_2$ exposure

Enhanced metal transport may be a method of natural or acquired tolerance to inorganics. Thus, expression of two genes that can function in metal efflux, namely *Mrp1/Abcc1* and *Mrp2/Abcc2* were examined after Cd treatment. Cd treatment increased *Mrp1* and *Mrp2* expression in WT cells but not in MT-null cells (Fig. 3a and 3b). In fact, in most cases, *Mrp* transcripts were decreased by Cd exposure in MT-null cells and *Mrp1* and *Mrp2* mRNA levels were lower in MT-null cell compared to WT cells (Fig. 3a and 3b). In addition, Mrp1 expression can be modified by oxidative stress (Jungsuwadee et al., 2009) while Mrp2 is functionally modified by cellular oxidant burden (Sekine et al., 2006). In the present study, $H_2O_2$ treatment increased expression of *Mrp1* and *Mrp2* in WT cells in a concentration-dependent manner (Fig. 3c and 3d) and decreased expression of *Mrp2* transcript in MT-null cells compared to control (Fig. 3d). *Mrp1* and *Mrp2* expression was consistently lower in MT-null cells compared to WT cells after $H_2O_2$ treatment.

MT-null or WT cells were tested for oxidant stress response gene expression after exposure to Cd or $H_2O_2$. Cd caused concentration-related increases in the expression of *GST-π, HO-1*, and *GSTα2* in MT-null cells (Fig. 4). However, in WT cells, expression of these genes either did not increase, such as with *GST-π* (Fig. 4a) or increased only modestly at the highest concentration of Cd used, such as with *HO-1* and *GSTα2* (Fig. 4b, 4c). $H_2O_2$ increased expression of *GST-π, HO-1, GSTα2* both in MT-null and WT cells (Fig. 4). The increases were greater in WT cells for *HO-1* and *GSTα2* (Fig. 4e and 4f). None of the increases induced by $H_2O_2$ in WT or MT-null cells appeared concentration-related.

### Introduction of MT into MT-null cells and Cd- or $H_2O_2$-induced responses

To determine whether MT expression is the key factor in protection against Cd-induced ODD, MT-null cells were transfected with the MT-I gene. Basal MT-I protein levels subsequently became easily detectable in the MT-null cells transfected with MT-I (Fig. 5a), but remained essentially undetectable in MT-null cells (not shown). MT-null cells transfected with MT-I exposed to Cd or $H_2O_2$ showed markedly increased *MT-I* expression, particularly with Cd treatment (Fig. 5b). In MT-null cells transfected with MT-I gene the $LC_{50}$ was elevated for both Cd and $H_2O_2$ compared to MT-null cells, indicating these agents were less toxic in the transfected cells (Fig. 5c). Similarly, Cd-induced much less ODD in MT-null cells transfected with MT-I gene than that in MT-null cells (Fig. 5d). $H_2O_2$-induced ODD was also markedly reduced in MT-null cells transfected with the MT-I gene (Fig. 5d) compared to MT-null cells (Fig. 5d). Thus, transfection of the MT-I gene into MT-null cells provided protection against both Cd- and $H_2O_2$-induced ODD.

## Discussion

In many instances, the binding of Cd with MT is considered to reduce the toxic potential of the metal (Klaassen et al., 1999, 2009). Here, we used MT-null and the otherwise isogenic MT-competent WT cell lines (Lazo et al., 1995) to help define the role of MT in protection from Cd-induced ODD. When comparing general cellular toxicity induced by Cd it was evident that MT-competent WT cells were resistant to Cd cytolethality compared to MT-null cells, which is not unexpected. This is consistent with in vivo findings of Cd-resistance in MT-transgenic mice that over-express MT (Klaassen et al., 2009) compared to MT-null mice which are very sensitive to Cd toxicity (Park et al., 2001). Likewise, we find MT-null mice are sensitive to liver cancer induced by Cd (Waalkes and Liu, 2009) or cisplatin (Waalkes et al., 2006). Cisplatin is thought to be toxic in many cases via oxidant stress (Pabla and Dong, 2008) and like Cd, can be bound by MT (Knipp 2009). More importantly, the present study also showed MT clearly mitigated ODD induced by Cd. In early work, activation of MT production in cultured rat liver cells by zinc was shown to prevent Cd-induced single strand DNA damage, as assessed by alkaline elution assay, using very high concentrations of Cd (500 μM; Coogan et al., 1992). This high level of Cd used in the prior work (Coogan et al., 1992) serves to point out the lack of sensitivity of such assays. Colangelo et al. (2004) applied a qualitative (single measurement) ODD assessment using 8-oxoguanine FITC-conjugation technique with mouse fibroblasts derived from MT-I and MT-II double knockout mice (named GKA2 cells) compared to cells from normal mice of the same strain (named GKA1 cells) and found the MT deficient cells showed much more oxidative damage after six days of continuous Cd (0.4 μM) exposure. The MT deficient GKA2 cells were also shown to be qualitatively more sensitive to ODD caused by $H_2O_2$ (Colangelo et al. 2004). A microfiltration assay measuring DNA strand breaks and DNA-protein crosslinks showed an association with Cd exposure in four cell lines, and in at least one cell line found pre-induction of MT did mitigate DNA damage (Misra et al. 1998). Other work indicates Cd-induced single strand damage is more pronounced in cells that poorly express MT (Shiraishi et al. 1995). Taken together, these data support the concept that MT can protect against Cd-induced DNA damage. An ability to protect against ROS-induced ODD by MT was confirmed in the present work with a general cellular oxidant, $H_2O_2$, which was much less toxic and caused much less ODD in WT cells than MT-null cells. Thus, MT showed an ability to confer a generalized resistance to oxidant stress and attack on DNA.

Although the carcinogenic potential of Cd is clearly established, the mechanism of Cd carcinogenesis remains unclear (IARC, 2012) as do the mechanisms by which the metal causes DNA damage, which are likely multifactorial (O'Brien and Salacinski, 1998). Some evidence points towards Cd-generated ROS as a potentially important factor (Keshava et al., 2000; Joseph et al., 2001; Filipic and Hei, 2004). For instance, in an in vitro system, BALB/c-3T3 cells acutely exposed to cytotoxic levels of Cd produced transformation in surviving cells (Filipic and Hei, 2004). Cells transformed by this high-dose, short-term method show evidence of elevated cellular levels of ROS (Keshava et al., 2000; Joseph et al., 2001) although the relevance of such a model to in vivo events could be questioned. Thus, although Cd is not a redox active metal and would be unable to form ROS directly, the metal has been shown to have the potential to induce oxidative stress (Bertin and Averbeck 2006; Thévenod, 2009). Oxidative stress could be viewed as a disturbance of cellular redox balance causing increased formation of ROS, thereby exceeding antioxidant protective systems (Nemmiche et al. 2011). This can lead to ODD (Nemmiche et al. 2011) and this damage presumably precipitates eventual development of cancer (Cuypers et al., 2010; Jomova and Valko, 2011). For instance, Cd-induced oxidative stress can occur in target organs of Cd carcinogenesis in vivo such as liver and lung (Nawrot et al., 2008) although it has never been directly correlated to tumor formation. Likewise, although our study showed

Cd induces ODD formation in vitro based on MT status, this has not been translated to ODD formation in vivo. MT-null mice do appear sensitive to liver tumor formation (Waalkes and Liu, 2009) at dose much lower (single 5 μmol/kg s.c. injection) than needed for a liver tumor induction in non-genetically altered mice (accumulated dose 640 μmol/kg, multiple s.c. injections; Waalkes and Rehm 1994). Also, it is clear that different rodent organs show different capacities for MT synthesis in response to Cd exposure (Waalkes and Klaassen 1985), and that basal human MT expression varies widely (Liu et al., 2007), which might be seen as a basis for target tissue or individual sensitivity. Accordingly, studies in liver cells, which are known to be quite adept at MT expression, indicate essentially no role for oxidative stress during Cd-induced malignant transformation in vitro (Qu et al., 2005). Clearly more experimentation is needed to define the role of MT and ROS production in sensitivity to Cd-induced DNA damage and carcinogenesis.

The MT-Cd complex is an important intracellular form for Cd storage (Thévenod, 2003; Klaassen et al., 1999, 2009). It is thought that Cd associated with MT makes less free Cd available within the cell and prevents interaction with critical targets (Klaassen et al., 1999, 2009), and, in the present case would thereby block Cd-induced oxidative stress and subsequent ODD. MT can also act as a scavenger for superoxide and hydroxyl radical *in vitro* (Klaassen et al. 1999). MT is enriched in thiol groups with a strong antioxidant capacity (Cuypers et al., 2010), which could presumably protect against $H_2O_2$-induced toxicity and ODD. The fact that transfection of MT-I into MT-null cells in the present work protected against both Cd- and $H_2O_2$-induced cytotoxicity and ODD clearly points to a direct role for MT protein in prevention of ODD. Others have found that poor expression of MT apparently reduces various types of Cd-induced or $H_2O_2$-induced DNA damage depending on the cell and detection system used (Coogan et al., 1992; Misra et al., 1998; Shiraishi et al., 1995; Nemmiche et al. 2011; Colangelo et al. 2004). The results of the present study are consistent with these data and show a quantitative concentration response for ODD in MT-null cells exposed to Cd or $H_2O_2$.

*Mrp1* and *Mrp2* are multidrug resistance-associated genes that produce the efflux transporter MRP1 and MRP2 proteins. MRPs can be involved in Cd transport (Carriére et al., 2011). For instance, MRP2 can enhance the biliary excretion of Cd in rats while MRP1 induction is associated with cellular Cd-resistance (Lim et al., 2010). In the present study, Cd increased expression of both *Mrp1* and *Mrp2* in WT cells, suggesting a link to Cd tolerance. The expression of Mrp1 is also increased by oxidative stress (Jungsuwadee et al. 2009) and cellular oxidant levels impact Mrp2 function (Sekine et al. 2006). $H_2O_2$ in the present study increased *Mrp1* and *Mrp2* expression in WT cells, but not MT-null cells, again suggesting these genes are activated as part of an adaptive process in MT competent cells to oxidant stress. Antioxidant genes such as *HO-1, GST-π, GSTa2* (Hoffmann and Kroemer 2004; Klaassen et al. 2009; Chiaverini and Ley 2010) were induced in the present work in primarily in MT-null cells potentially to offset Cd-induced oxidative stress, indicating that these become important in protection against Cd in cells with poor MT expression. However, $H_2O_2$ stimulated higher expression of *HO-1, GSTa2* in WT cells, so this did not hold true for the direct oxidant. Exactly how these cellular defense mechanisms are triggered during Cd exposure remains to be defined.

In summary, the present work suggests that MT protects against Cd- or $H_2O_2$-induced cytolethality and ODD possibly by multiple mechanisms. This includes the direct metal sequestration by MT and the sequestration of oxidant species since the presence of MT reduced direct oxidant-induced ODD. WT cells also increase transport systems that aid in tolerance while MT-null cells may try to adapt to Cd by turning on the oxidant stress response genes other than MT.

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## Abbreviations

| | |
|---|---|
| **Cd** | Cadmium |
| **$CdCl_2$** | Cadmium chloride |
| **DMPO** | 5, 5-dimethyl-1-pyrroline N-oxide |
| **GST** | Glutathione S-transferase |
| **GST-π** | Glutathione S-transferase pi 1 |
| **GSTα2** | Glutathione S-transferase-α2 |
| **HO-1** | Heme oxygenase 1 |
| **$H_2O_2$** | Hydrogen peroxide |
| **IST** | Immuno-spin trapping |
| **$LC_{50}$** | Lethal concentration 50% |
| **Mrp1** | Multidrug resistance-related protein 1 |
| **Mrp2** | Multidrug resistance-related protein 2 |
| **MT** | Metallothionein |
| **MT-null** | MT-I/II knockout |
| **ODD** | Oxidative DNA damage |
| **RT-PCR** | Reverse transcription-polymerase chain reaction ROS Reactive oxygen species |
| **WT** | Wild type |

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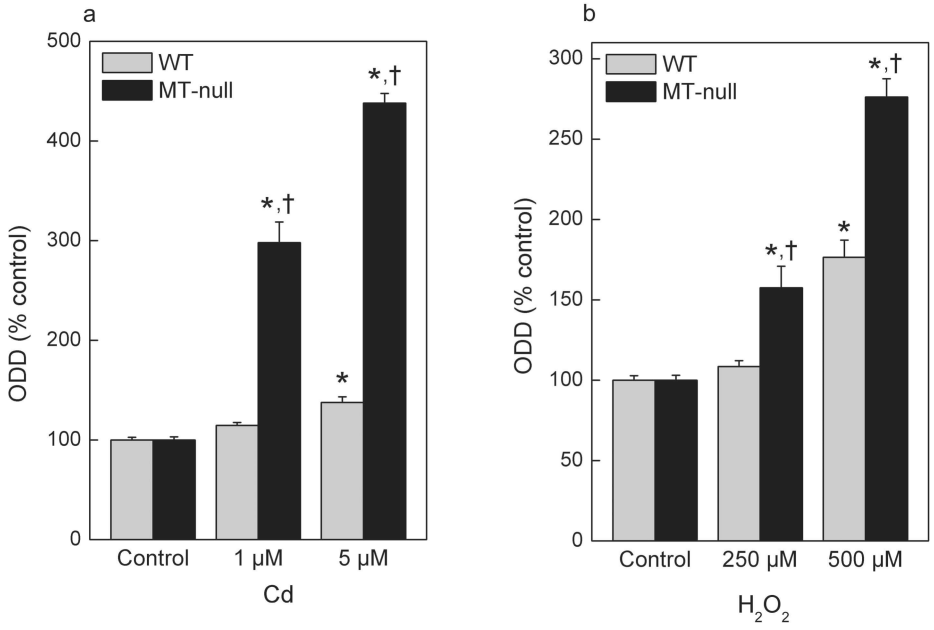


**Figure 1. MT and Cd- or $H_2O_2$-induced oxidative ODD**

MT-null or WT cells were exposed to various concentrations of Cd or $H_2O_2$ for 24 h, and ODD was measured. a, ODD induced by Cd exposure; b, ODD induced by $H_2O_2$ exposure. An asterisk (*) indicates a significant ($p < 0.05$) difference from control. A cross (†) indicates a significant ($p < 0.05$) difference from concentration-matched WT cells.

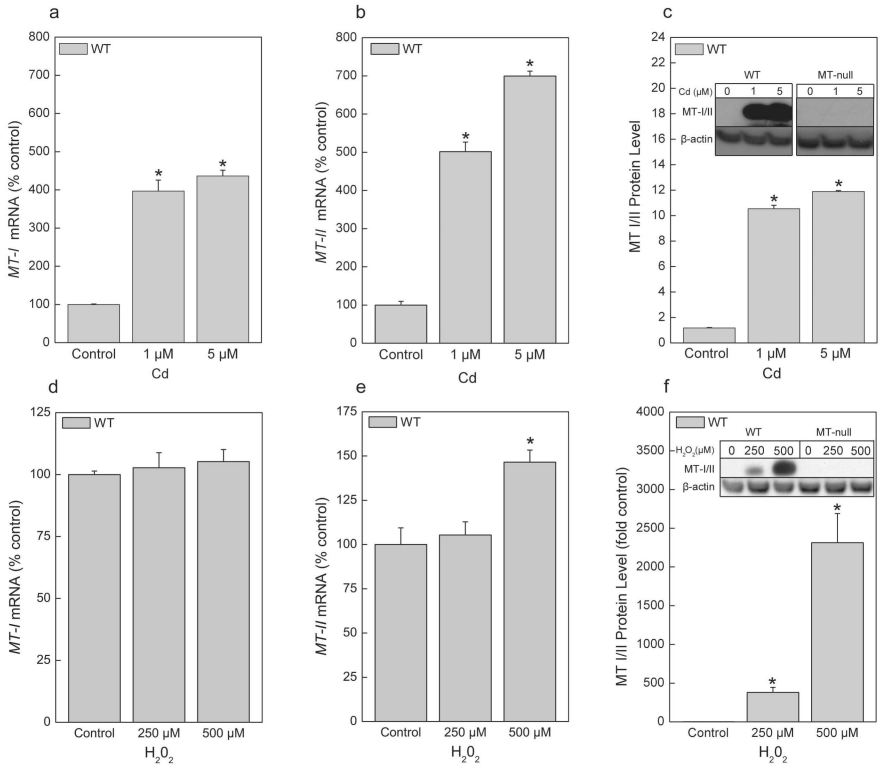


**Figure 2. MT transcript and protein expression after to Cd and $H_2O_2$ exposure**

MT-null or WT cells were exposed to various concentrations of Cd or $H_2O_2$ for 24 h. *MT-I* and *MT-II* transcript levels were measured. Results were normalized to β-actin and are expressed as % control. MT protein levels were assessed using an antibody that reacts with both MT-I and MT-II. Blots were analyzed by densitometry and normalized to β-actin. a, *MT–I* transcript in cells treated with Cd; b, *MT–II* transcript in cells treated with Cd; c, MT protein in cells treated with Cd. d, *MT–I* transcript in cells treated with $H_2O_2$; e, *MT–II* transcript in cells treated with $H_2O_2$; f, MT protein in cells treated with $H_2O_2$. An asterisk (*) indicates a significant ($p < 0.05$) difference from control.

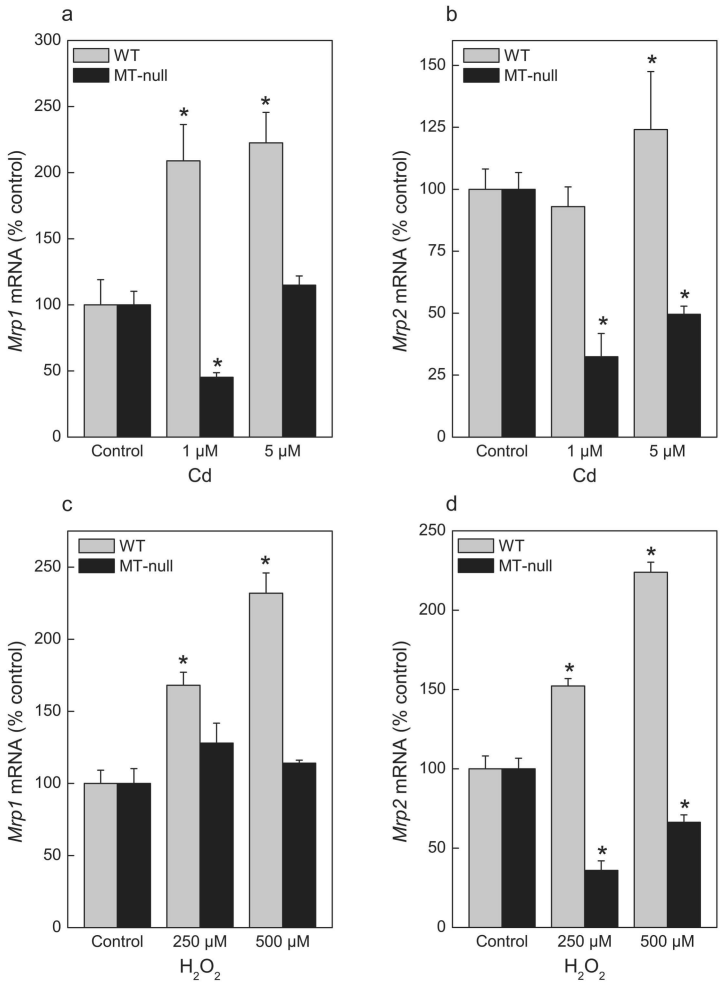


**Figure 3. Expression of *Mrp1* and *Mrp2* after Cd or $H_2O_2$ exposure**

MT-null or WT cells were exposed to various concentrations of Cd or $H_2O_2$ for 24 h and *Mrp1* and *Mrp2* transcript levels were measured. Results were normalized to β-actin and are expressed as % control. a, *Mrp1* transcript in cells treated with Cd; b, *Mrp2* transcript in cells treated with Cd; c, *Mrp1* transcript in cells treated with $H_2O_2$ ; d, *Mrp2* transcript treated with $H_2O_2$. An asterisk (*) indicates a significant ($p < 0.05$) difference from control. A cross (†) indicates a significant ($p < 0.05$) difference from concentration-matched WT cells.

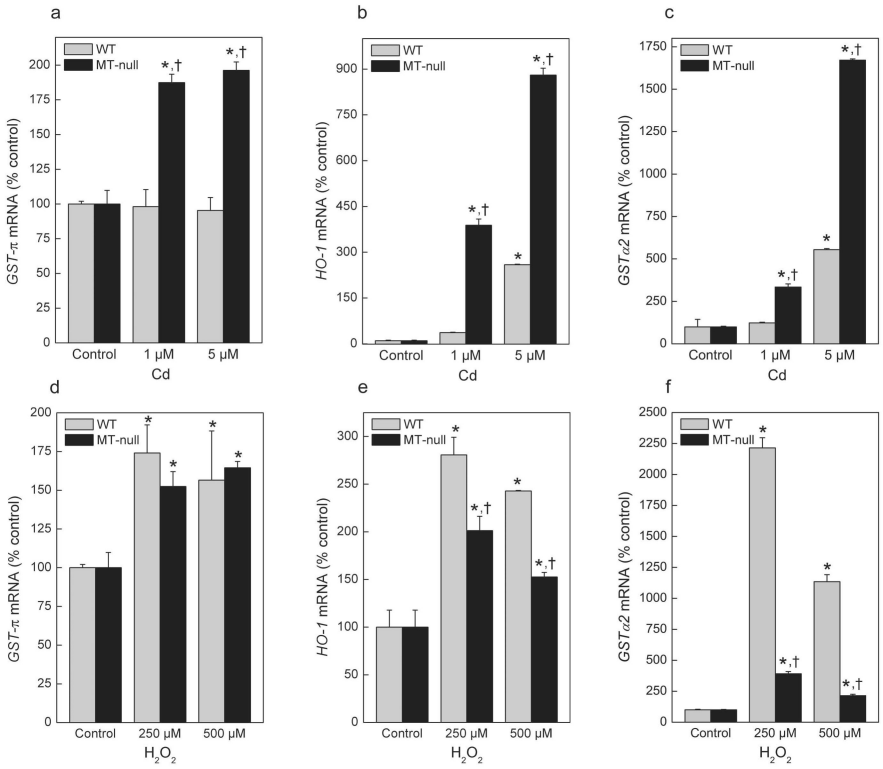


**Figure 4. Expression of oxidant stress defense genes after Cd or $H_2O_2$ exposure**

MT-null or WT cells were exposed to various concentrations of Cd or $H_2O_2$ for 24 h and transcript levels of *GST-π, HO-1*, and *GSTα2* were measured. Results were normalized to β-actin and are expressed as % control. a, *GST-π* transcript in cells treated with Cd; b, *HO-1* transcript in cells treated with Cd; c, *GSTα2* transcript in cells treated with Cd; d, *GST-π* transcript in cells treated with $H_2O_2$; e, *HO-1* transcript in cells treated with $H_2O_2$; f, *GSTα2* transcript in cells treated with $H_2O_2$. An asterisk (*) indicates a significant ($p < 0.05$) difference from control. A cross (†) indicates a significant ($p < 0.05$) difference from concentration-matched WT cells.

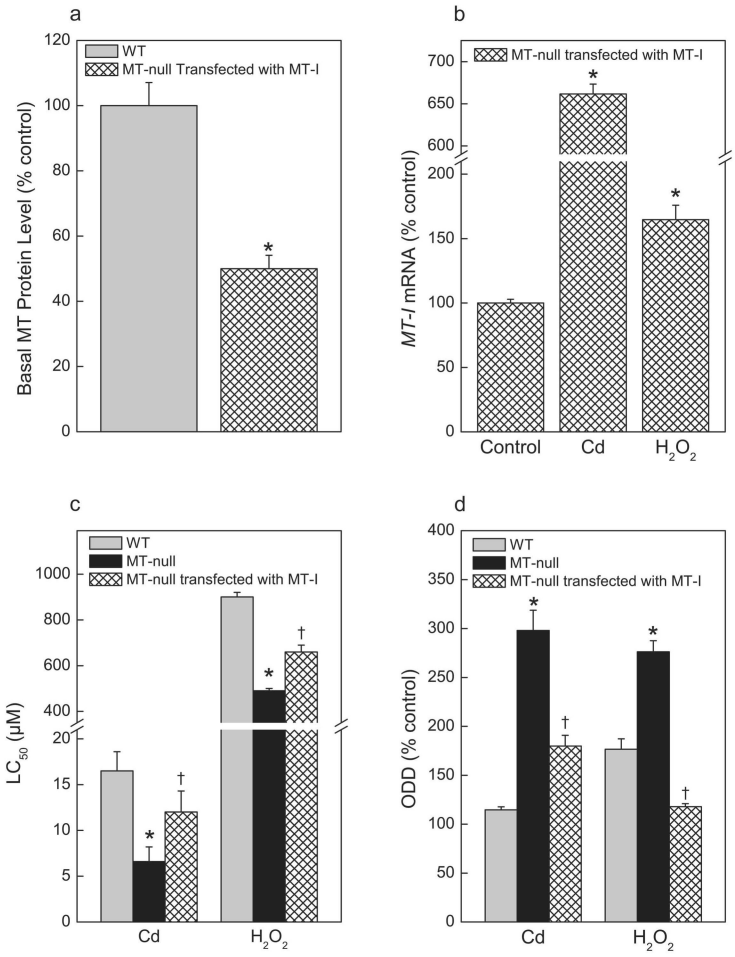


**Figure 5. MT transfection into MT-null cells protects against Cd or $H_2O_2$**

MT-null cells were transfected with the MT-I gene then underwent various treatments. a, Basal MT protein levels in MT-null transfected with MT-I cells or WT cells; b, *MT* transcript levels in MT-null transfected with MT-I cells after exposure to unaltered media (control), Cd (1 μM) or $H_2O_2$ (250 μM) for 24 h. Results were normalized to β-actin and are expressed as % control. c, $LC_{50}$ values for Cd or $H_2O_2$ exposure for 24 hours in MT-null, WT or MT-null transfected with MT-I cells. d, ODD was assessed in MT-null, WT or MT-null transfected with MT-I cells exposed to unaltered media (control), Cd (1 μM) or $H_2O_2$ (250 μM) for 24 hours. In all cases an asterisk (*) indicates a significant ($p < 0.05$) difference from control and a cross (†) indicates a significant ($p < 0.05$) difference from concentration-matched treated WT cells.