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The Metallothionein-Null Phenotype Is Associated with Heightened Sensitivity to Lead Toxicity and an Inability to Form Inclusion Bodies

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Susceptibility to lead toxicity in MT-null mice and cells, lacking the major forms of the metallothionein (MT) gene, was compared to wild-type (WT) mice or cells. Male MT-null and WT mice received lead in the drinking water (0 to 4000 ppm) for 10 to 20 weeks. Lead did not alter body weight in any group. Unlike WT mice, lead-treated MT-null mice showed dose-related nephromegaly. In addition, after lead exposure renal function was significantly diminished in MT-null mice in comparison to WT mice. MT-null mice accumulated less renal lead than WT mice and did not form lead inclusion bodies, which were present in the kidneys of WT mice. In gene array analysis, renal glutathione *S*-transferases were up-regulated after lead in MT-null mice only. *In vitro* studies on fibroblast cell lines derived from MT-null and WT mice showed that MT-null cells were much more sensitive to lead cytotoxicity. MT-null cells accumulated less lead and formed no inclusion bodies. The MT-null phenotype seems to preclude lead-induced inclusion body formation and increases lead toxicity at the organ and cellular level despite reducing lead accumulation. This study reveals important roles for MT in chronic lead toxicity, lead accumulation, and inclusion body formation. *(Am J Pathol 2002, 160:1047–1056)*

Lead is widely recognized as an important environmental toxicant that poses a substantial risk to the human population throughout the world.[1] Toxic effects of lead occur in multiple organ systems but particularly the developing nervous system of infants and children.[2,3] Renal effects are also common in adults with chronic lead exposure.[3] Lead produces renal tumors in rodents, and lead and inorganic lead compounds have been classified as possible human carcinogens.[4] However, the precise mechanisms of lead toxicity or carcinogenicity are incompletely defined.

A remarkable pathogenic feature of lead poisoning is the presence of inclusion bodies composed of lead-protein complex.[5–15] Blackman[6] first reported the formation of lead inclusion bodies in the 1930s in renal epithelial cells of lead-poisoned children. Since then, many investigators have reported inclusion body formation with lead exposure in humans and animals.[5,7,8] Lead-induced inclusion bodies are frequently nuclear, roughly spherical, and typically consist of an electron-dense core with a fibrillary network at the periphery.[2] These inclusion bodies, although common in the kidney, also form in cells of nervous tissue origin such as astrocytes,[9] neuroblastoma cells,[10] and in other cell types such as osteoclasts.[11] Metal analysis shows that lead is highly concentrated within the inclusion bodies.[12] Inclusion bodies may be protective in that, when lead accumulates in the inclusion bodies, it prevents injury to more sensitive cellular targets.[12,13] It is thought that inclusion bodies probably have an important role in the intracellular partitioning and, perhaps, transport and toxicity of lead.[14] Thus, the formation of lead-binding inclusion bodies may function to detoxify lead,[15] although this has yet to be definitively established.

Metallothionein (MT) is a low-molecular-weight metal-binding protein with one-third of its amino acids as cysteine.[16] These cysteinyl sulfhydryls coordinate a variety of metal atoms.[17] Various metals increased the concentration of MT in major organs of rats.[18] MT has been assigned pleiotropic roles from gene regulation to metal homeostasis, transport, and detoxification.[19] For instance, MT has been shown to play a protective role in cadmium-induced hepatotoxicity and nephrotoxicity.[20] Similarly, MT-I/II knock-out (MT-null) mice are more sensitive than wild-type (WT) mice to the nephrotoxicity produced by chronic exposure to cadmium and/or other

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inorganic metals.[21] MT is highly inducible by many metals, particularly zinc, cadmium, copper, and mercury, and clearly plays a role in mitigating the toxicity of these metals.[17] However, any mitigating role for MT in lead toxicity is still only poorly defined. In this regard, lead has been shown to induce the synthesis of MT in several instances,[19,22–24] which implicates, but does not definitively establish, a role in lead metabolism. On the other hand, this induction seems rather modest compared to many other metals and occurs only in the liver,[18] perhaps indicating stress-mediated induction. Others have found that lead is unable to stimulate the synthesis of MT in human blood lymphocytes.[25] Lead appears to bind to MT or MT-like proteins in human erythrocytes,[26] which suggests sequestration into a nonbioavailable, and thus nontoxic form. The presence of zinc-induced MT will modestly mitigate the toxicity of lead in cultured primary rat hepatocytes[27] and lead can avidly bind to MT *ex vivo* displacing zinc in the process.[28] Furthermore, the binding of lead to MT seems to reduce lead-induced inhibition of the enzyme δ-aminolevulinic acid dehydratase, at least *ex vivo*.[29] Although there are indications that MT mitigates lead toxicity, the data are far from convincing and additional work is warranted.

Therefore, the purpose of the present study was to investigate the role of MT in lead toxicity using genetically engineered systems. Initial studies used MT-null mice that are unable to produce the major forms of MT (MT-I and MT-II isoforms) and compared them to WT controls. Despite accumulating less renal lead, MT-null animals were significantly more sensitive than WT mice to the nephrotoxic effects of lead, as assessed by nephromegaly, renal function, and molecular evidence of a toxic response. Surprisingly, MT-null mice did not form inclusion bodies. Additional work *in vitro* showed MT-null cells similarly accumulated less lead but were still more sensitive to lead-induced cytotoxicity than WT cells. MT-null cells also did not form inclusion bodies after lead exposure, although they were common in WT cells. These data indicate that MT may play a role in lead toxicity and, possibly, in inclusion body formation. In addition, because the inability to produce MT seems to be related to enhanced susceptibility to lead toxicity, individuals that poorly express MT may have increased susceptibility to lead intoxication.

Materials and Methods

Chemicals and Materials

Lead nitrate, lead acetate, and glutamic acid were obtained from Sigma Chemical Company (St. Louis, MO). Nonradioactive cell proliferation assay kit was obtained from Promega (Madison, WI).

Animals and Treatments

Homozygous MT-I/II knock-out mice (129-Mt1$^{tm/Bri}$, Mt2$^{tm/Bri}$ 129/SvPCJ background)[30] were obtained from Jackson Laboratories (Bar Harbor, ME). The homozygous mutants were mated *inter se* to maintain the line. Male MT-null mice and the corresponding WT mice were housed in an American Association for Acreditation of Laboratory Animal Care (AAALAC) accredited facility under conditions that met or exceeded recommendations outlined in the Guide for Care and Use of Laboratory Animals (National Institutes of Health Publication no. 86-23, 1985). Mice were provided food (NIH-31 diet; Zeigler Brothers, Gardners, PA) and water *ad libitum*. At 10 weeks of age, MT-null and WT mice were randomly divided into three treatment groups of 10 mice each and one control group of 20 mice. They were given acidified drinking water containing lead acetate at concentrations of 1000, 2000, or 4000 ppm lead. Control groups of mice received acidified drinking water. Animals were weighed weekly. Mice were killed after 10 weeks of treatment. Their kidneys were removed and weighed individually. For one-half of the controls ($n = 10$), and the 1000 and 2000 ppm groups, one kidney was fixed in 10% buffered formalin for histopathological analysis and a portion of the contralateral kidney was frozen in liquid nitrogen and used for subsequent lead determination. For the 2000 ppm group and one-half of the controls ($n = 10$), half of one kidney was frozen in liquid nitrogen for later RNA isolation. Both kidneys in the 4000-ppm group were used for histopathological analysis including quantitation of inclusion bodies. Formalin-fixed kidneys were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) for histological examination.

In a separate experiment, urine and orbital blood samples were taken from individual male MT-null and WT mice that were part of an on-going chronic carcinogenesis bioassay and had been exposed to 4000 ppm lead for 20 weeks. Blood urea nitrogen, blood creatinine, and total urinary protein were assessed as biomarkers of renal function and determined through a commercial clinical chemistry laboratory (Ani Lytics, Inc., Gaithersburg, MD).

Renal Lead Accumulation

Kidneys removed from WT and MT-null mice were digested in nitric acid (J.T. Baker, Philipsburg, NJ) overnight at 65°C. These digests were used for determination of the renal lead levels by graphite furnace atomic-absorption spectrophotometry with a Perkin-Elmer Model 5000 spectrophotometer.

Quantitation of Inclusion Bodies

The number of inclusion bodies was counted in three randomly selected H&E-stained kidney sections from each group. In each case a total of 200 randomly selected cells from the inner cortex were scored.

Microarray Analysis

The Atlas Mouse 1.2 cDNA expression microarray (1178 genes) was performed according to the manufacturers' instructions. Briefly, 10 to 20 μg of total RNA isolated from MT-null control and lead-treated (2000 ppm) mouse kid-

neys were converted to [α-^{32}P]-dATP-labeled cDNA probe using MMLV reverse transcriptase and Atlas Mouse Stress CDS primer mix (Clontech, Palo Alto, CA). The ^{32}P-labeled cDNA probe was purified using chroma spin-200 columns, denatured in 0.1 mol/L NaOH, 10 mmol/L ethylenediaminetetraacetic acid at 68°C for 20 minutes, followed by neutralization with an equal volume of 1 mol/L NaH_2PO_4 for 10 minutes. The membrane was prehybridized with Ultrahyb (Ambion, Austin, TX) for 30 to 60 minutes at 42°C, followed by hybridization overnight at 42°C. Arrays were washed two times in 2× standard saline citrate/0.1% sodium dodecyl sulfate, 5 to 10 minutes each, and two times in 0.1× standard saline citrate/0.1% sodium dodecyl sulfate for 15 to 30 minutes. The arrays were then sealed in a plastic bag, and exposed to a phosphoimage screen or X-ray film. The images were analyzed densitometrically using AtlasImage software. The gene expression intensities were normalized with the sum of eight housekeeping genes on the array (40S ribosomal protein S29, 45-kd calcium-binding protein, β-actin, ornithine decarboxylase, myosin 1-α, G3PDH, hypoxanthine-guanine phosphoribosyltransferase, and phospholipase A2) except for ubiquitin (the hybrid intensity of ubiquitin was saturated). Means and SEM of four hybridizations were calculated for this analysis.

Cell Culture and Treatments

A cell line created from the embryonic cells of transgenic mice with a targeted disruption of MT-I/II genes (MT-null cells; also known as MT−/−), along with the corresponding WT control cells (WT; also known as MT+/+) from normal mice, were graciously supplied by Dr. John Lazo, University of Pittsburgh, Pittsburgh, PA. Cells were cultured in Dulbecco's modified Eagle's medium media containing 5% fetal bovine serum as described previously.[31] The precipitation of lead in the medium was controlled by complexing lead nitrate with glutamic acid in equimolar amounts, as detailed in a previous report.[32] Thus, cells were exposed to lead nitrate (200 μmol/L) with glutamic acid in equimolar amounts for the time specified throughout this study.

Metabolic Integrity Assay

Promega Cell Titer 96 Nonradioactive Cell Proliferation Assay kits were used to determine acute cytotoxicity of lead in MT-null and WT cells as defined by metabolic integrity. The assay measures the amount of formazan produced by metabolic conversion of Owen's reagent [(3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium, inner salt; MTS] by dehydrogenase enzymes found in the mitochondria of metabolically active cells. The quantity of formazan product, as measured by absorbance at 490 nm, is directly proportional to the number of living cells. A minimum of 4 replicates of 10,000 cells per well were plated in 96-well plates and allowed to adhere to the plate for 24 hours at which time the media was removed and replaced with media containing various concentrations of lead. Cells were then incubated for an additional 24 hours and cell viability was determined.[33] LC_{50} values were determined from analysis of the linear portion of the metabolic integrity curves and compared between WT and MT-null cells.

Electron Microscopy

WT and MT-null cells were treated with lead (200 μmol/L) for 48 hours. The cells were harvested by trypsinization and fixed overnight in 3% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.3. After primary fixation, the cells were rinsed in 0.1 mol/L of phosphate buffer for 15 minutes. Postfixation was done in 1% osmium tetroxide in 0.1 mol/L of phosphate buffer, pH 7.3, for 2 hours. The cells were rinsed again in the phosphate buffer for 15 minutes and were followed by treatment with an aqueous solution of 5% uranyl acetate for 2 hours. After dehydration in graded ethanol, the specimens were embedded in Poly-Bed resin. The resin blocks were cut at ~90 nm, collected on coated grids, and stained with uranyl acetate and lead citrate. The examination of the grids was done using a Philips 400 electron microscope.[32]

Determination of Cellular Lead Accumulation and Efflux

WT and MT-null cells were grown to ~50% confluence, then the medium was removed and replaced with either fresh control medium or medium containing lead (200 μmol/L). Cells were harvested 24 hours later, counted, and pelleted by centrifugation. The cell pellets were digested overnight in 50% perchloric:nitric acid (2:1). These digests were used for determination of the amount of total lead that had accumulated after 24 hours of exposure. To estimate lead efflux, replicate sets of cells were washed after 24 hours of exposure to lead and allowed to incubate an additional 24 hours in fresh media. These cells were then digested and analyzed for lead. Total cellular lead levels were determined by graphite furnace atomic-absorption spectrophotometry using a Perkin-Elmer Model 5000 spectrophotometer and adjusted to cell numbers. Triplicate determinations were used for each data point.

Determination of MT Levels

WT cells were treated with lead (200 μmol/L) for 48 hours. Cells were harvested by trypsinization and resuspended at a density of 2.5×10^6/ml in 10 mmol/L Tris buffer (pH 7.4) at 4°C. Cells were then lysed by sonication on ice. Complete lysis was confirmed microscopically and cellular debris was removed by centrifugation (15 minutes, 16,000 × *g*). MT levels were determined in the supernatant using the Cd-hemoglobin method of Onosaka and colleagues[34] as modified by Eaton and Toal.[35]

Statistical Analysis

Student's *t*-test or analysis of variance with subsequent Dunnett's test were used as appropriate. All values are

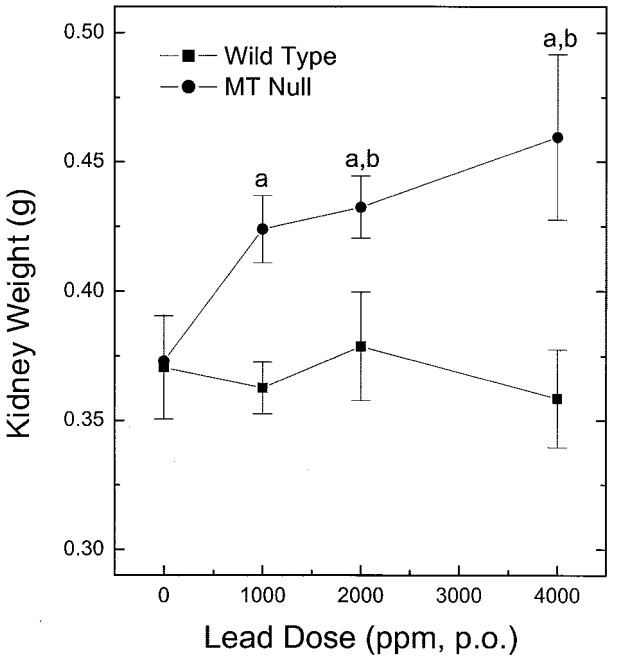

Figure 1. Kidney weight analysis. MT-null and WT mice were given drinking water containing lead acetate at concentrations of 1000, 2000, or 4000 ppm for 10 weeks. Their kidneys were removed and weighed individually. Results are presented as the mean ± SEM ($n = 10$); **a** indicates a significant ($P < 0.05$) difference from appropriate dosage-matched WT; **b** indicates a significant ($P < 0.05$) difference from appropriate untreated control.

expressed as mean ± SEM of three or more replications. Differences were considered significant at level of $P < 0.05$.

Results

Pathological and Functional Analysis of Kidneys from Lead-Exposed MT-Null and WT Mice

Male MT-null and WT mice received lead in drinking water (0 to 4000 ppm; 10 to 20 weeks) and renal pathology and function were assessed. Lead did not alter body weight in either MT-null or WT mice throughout the exposure period (data not shown). MT-null mice showed a dose-related nephromegaly indicative of renal toxicity, although the kidneys of WT mice were unaffected by lead (Figure 1).

Although, no microscopically obvious pathological lesions occurred in lead-exposed kidneys, after chronic exposure to 4000 ppm lead in the drinking water MT-null mice showed evidence of diminished renal function when compared to WT mice. Specifically, there were significant ($P \leq 0.05$) increases in blood creatinine (0.53 ± 0.03 mg/dl; mean ± SEM, $n = 3$ to 4) and total urinary protein (206 ± 14.1 mg/dl) in lead-treated MT-null mice when compared to similarly treated WT mice (blood creatinine = 0.33 ± 0.03 mg/dl; total urinary protein = 158 ± 1.76 mg/dl). Additionally, increases in blood urea nitrogen occurred in lead-exposed MT-null mice (30.3 ± 0.32 mg/dl) that approached significance ($P = 0.062$) when compared to WT mice (26.7 ± 1.86 mg/dl). This pattern of increases in blood urea nitrogen, blood creatinine, and total urinary protein is typically considered functional evidence of nephrotoxicity, and is consistent with reports on lead-induced nephrotoxicity.

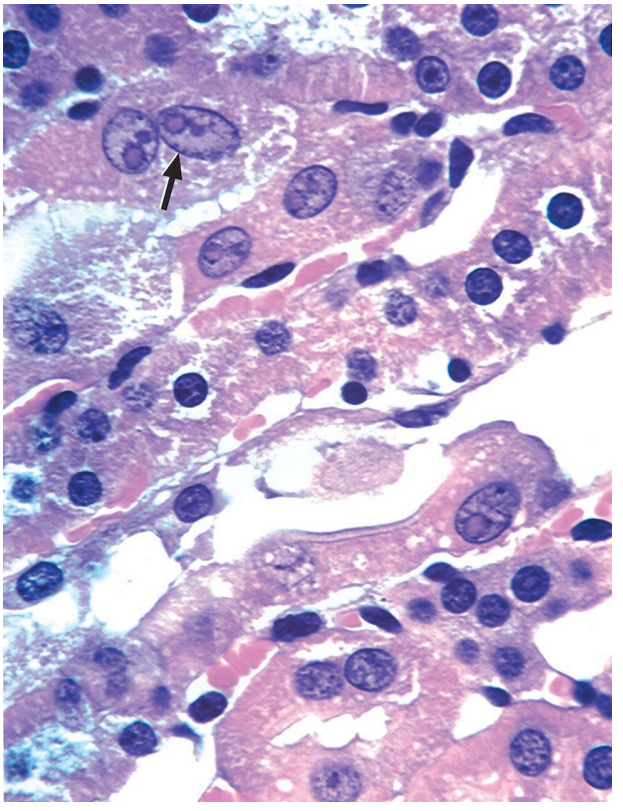

Figure 2. Lead-induced inclusion body formation in kidneys from WT mice. MT-null and WT mice were treated with lead as described in the legend to Figure 1. Portions of kidneys were fixed in 10% buffered formalin. Acid-fast stain was used to determine the presence of lead-induced inclusion body in renal tubular cells. **Arrows** indicate typical karyomegaly of P3 proximal tubular cell in WT mice given lead (lead, 1000 ppm; H&E: original magnification, ×300).

Surprisingly, MT-null mice did not form renal lead-containing inclusion bodies, whereas inclusion bodies were common at all doses in WT mice (Figure 2). These inclusion bodies were primarily nuclear. Quantitative analysis of cells from the inner cortex of lead-treated and control sections of kidneys showed that inclusion bodies were increased in a dose-dependent manner in WT mice, but, again were completely absent from MT-null animals (Table 1).

Table 1. Quantitation of Lead-Induced Inclusion Body Formation in Kidney From WT Mice

	Lead dose (ppm, p.o.)			
Mouse strain	0	1000	2000	4000
WT	N.D.	10 ± 1	16 ± 1	21 ± 1
MT-null	N.D.	N.D.	N.D.	N.D.

WT and MT-null mice were given lead p.o. at 0, 1000, 2000, or 4000 ppm for 10 weeks and renal inclusion body formation was assessed. In each case 200 nuclei selected from random fields of the inner cortex of lead-treated and control sections of kidneys were scored. Data are given as the mean ± SEM ($n = 3$).

N.D., not detected.

Table 2. Lead Accumulation in Kidney from WT and MT-Null Mice (μg/g Wet Weight)

	Lead dose (ppm, p.o.)		
Mouse strain	0	1000	2000
WT	N.D.	10.9 ± 0.3	14.4 ± 0.3
MT-null	N.D.	8.9 ± 0.2*	11.0 ± 0.7*

WT and MT-null mice were given lead p.o. at 0, 1000, or 2000 ppm for 10 weeks, renal lead levels were measured by AAS. Data given as the mean ± SEM (n = 10).

*Significant ($P < 0.05$) difference from appropriate dose-matched WT mice.

N.D., not detectable.

Lead-treated WT kidneys were analyzed immunohistochemically for MT localization to see if MT played a direct role in lead-induced inclusion body formation. MT in lead-treated kidneys from WT mice was primarily cytosolic with minimal nuclear staining and no apparent association with inclusion bodies (data not shown).

Renal Lead Accumulation in MT-Null and WT Mice

After 10 weeks of exposure to 0, 1000, or 2000 ppm lead in drinking water, renal lead levels were determined in MT-null and WT mice. Surprisingly, MT-null mice accumulated significantly less renal lead than WT mice at all doses tested (Table 2).

Microarray Analysis of Lead-Treated Kidneys from MT-Null and WT Mice

To help define more subtle differential toxicity after lead exposure, gene expression array studies were performed with RNA isolated from the kidneys of lead-treated (2000 ppm for 10 weeks) WT and MT-null mice. Lead exposure altered the expression of a variety of genes, and such alterations were generally much more common in MT-null mice. Details of gene expression changes are given in Table 3. Among the 1178 genes investigated, more than 60 genes (5.0%) were aberrantly expressed in MT-null mice after lead exposure whereas only 35 genes (2.9%) were aberrantly expressed in WT mice. Specifically, various oxidative stress and cellular defense-related genes were up-regulated in MT-null mice because of lead treatment, indicative of a molecular response to a toxic insult. Notably, the expression of the genes encoding for glutathione *S*-transferase-5 (GST-μ), glutathione *S*-transferase θ 1 (GST-theta), and glutathione *S*-transferase π (GST-π) were increased ~2.5-fold to threefold in MT-null mice treated with lead as compared with untreated MT-null mice. However, expression of GST-μ, GST-θ, and GST-π, was not altered in WT mice. Thus, although lead does not induce overt pathology at the microscopic level in MT-null mice, it did induce gross pathological changes (nephromegaly), as well as diminished renal function, and clearly caused more subtle lesions leading to altered gene expression much more commonly in MT-null mice.

Lead Toxicity in MT-Null and WT Cells in Vitro

To help further define sensitivity to lead and propensity to form inclusion bodies, an additional study was conducted *in vitro* using cell lines derived from MT-null and WT mice.[31] MT-null and WT cells were treated with lead for 24 hours and cytotoxicity was measured as metabolic integrity (Figure 3). MT-null cells were much more sensitive than WT cells to lead cytotoxicity. The LC_{50} value for lead in WT cells was 645 ± 26 μmol/L as compared to 230 ± 17 μmol/L in MT-null cells, which constitutes a 2.8-fold difference in sensitivity to the metal.

Formation of Inclusion Bodies in WT Cells But Not in MT-Null Cells

WT and MT-null cells were exposed to lead and the formation of lead inclusion bodies was examined by electron microscopy (Figure 4). Both WT and MT-null cells appeared to have normal ultrastructural features regardless of lead exposure. However, groups of irregularly shaped inclusion bodies were observed around the nuclear membrane in the cytoplasm only in lead-treated WT cells. No inclusion bodies were observed in MT-null cells.

Lead Accumulation in MT-Null and WT Cells

To determine whether cellular lead disposition could play a role in the lack of inclusion body formation in MT-null cells, WT and MT-null cells were exposed to lead and cellular lead levels were measured (Table 4). MT-null cells accumulated significantly less lead in comparison to the WT cells. As an indication of lead efflux, lead-loaded cells were placed in lead-free medium for an additional 24 hours and remaining cellular lead was measured. The amount of lead effluxed during this period was not significantly different between MT-null and WT cells (data not shown).

Effect of Lead on MT Levels

To detect the effects of lead on cellular MT levels, WT cells were exposed to lead for 48 hours and MT levels were measured (Table 5). Lead caused a significant dose-dependent increase in MT levels in WT cells.

Discussion

Lead is one of the most important environmental toxicants in the United States and throughout the world because of its ubiquitous nature and the spectrum of toxicological effects it induces, potentially including carcinogenicity.[36] Chronic nephropathy from lead exposure often shows interstitial fibrosis and cystic hyperplasia in humans and animals.[2,9,37,38] Lead is also a renal carcinogen in animals[14,39] and possibly in humans.[1] The profound nephrotoxicity induced by lead is characterized by cellular inclusion bodies and renal tubular dysfunction.[14] These inclusion bodies are a diagnostic feature of lead poison-

Table 3. Differentially Expressed Genes in MT-Null and WT Mice Treated with Lead*

Gene	MT-null control	MT-null lead 4000 ppm	MT-null lead/ control	WT control	WT lead 4000 ppm	WT lead/ control
Stress-response proteins (first group)	Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM	
Heat shock protein HSP27	1915 ± 2	4181 ± 891	2.18	3411 ± 364	3302 ± 934	0.97
Heat shock protein HSP84	10222 ± 4143	14301 ± 640	1.40	14991 ± 811	12755 ± 925	0.85
Microsomal GST (MGST1)	4784 ± 596	8721 ± 306	1.82	6681 ± 27	7029 ± 1134	1.05
Glutathione *S*-transferase-5 (GST mu)	14369 ± 1999	35330 ± 2752	2.46	33379 ± 6472	35428 ± 5084	1.06
Glutathione *S*-transferase theta 1	1409 ± 33	4213 ± 898	2.99	3216 ± 78	2909 ± 601	0.91
Glutathione *S*-transferase Pi	7920 ± 1609	20892 ± 4302	2.64	17357 ± 7238	17715 ± 4877	1.02
Oxidative stress protein A170	2477 ± 67	4526 ± 419	1.83	2534 ± 211	2571 ± 154	1.26
Cell signaling and transducers (second group)						
Insulin-like growth factor IGFB6	4675 ± 3115	10823 ± 2335	2.32	10567 ± 5353	11596 ± 3393	1.10
Insulin-like growth factor II IGF-II	1792 ± 215	4275 ± 2500	2.39	3519 ± 3216	4701 ± 3062	1.34
Wingless MMTV integration WNT 3	3218 ± 1088	6683 ± 1962	2.07	N.D.	N.D.	
WNT5B	2424 ± 260	4972 ± 655	2.05	N.D.	N.D.	
Tumor necrosis factor-beta	744 ± 214	2744 ± 1913	3.68	524 ± 139	298 ± 156	0.57
Cytokine inducible CISH7	6492 ± 2264	10727 ± 2423	1.65	12660 ± 1620	13995 ± 3714	1.11
Nuclear factor-kappa B (IkB-beta)	1626 ± 88	8941 ± 5194	5.45	1162 ± 9	1272 ± 418	1.09
DNA synthesis, repair (third group)						
DNA excision repair protein ERCC1	711 ± 266	1840 ± 779	2.59	N.D.	N.D.	
DNA repair protein XPBC	3170 ± 740	5819 ± 1410	1.84	2767 ± 201	5141 ± 1569	1.86
UV excision repair protein RAD23	4186 ± 1417	7080 ± 878	1.69	508 ± 389	1716 ± 1028	3.42
DNA polymerase delta POLD1	1324 ± 373	3903 ± 2341	2.95	1826 ± 763	1454 ± 173	0.80
DNAse 1	33264 ± 9000	21764 ± 4110	0.65	29969 ± 2862	28308 ± 3807	0.95
Apoptosis-associated proteins (fourth group)						
Tumor necrosis factor receptor 1	1121 ± 155	4601 ± 1409	4.11	2043 ± 2007	1619 ± 1450	0.79
Tumor necrosis factor receptor 2	1018 ± 81	13492 ± 5031	13.3	3062 ± 1100	2382 ± 1060	0.78
Caspase-7	2858 ± 2734	245 ± 138	0.09	200 ± 61	75 ± 40	0.37
MCL-1	5178 ± 1850	2221 ± 540	0.43	2296 ± 270	3236 ± 509	1.40
Sentrin; ubiquitin-like protein	7783 ± 1849	3532 ± 606	0.45	1484 ± 817	1654 ± 1454	1.11
Inhibitor of neuronal NO synthetase	12867 ± 2830	8963 ± 1697	0.71	12010 ± 753	10204 ± 408	0.85
Cell surface antigens (fifth group)						
Connexin 26	10096 ± 2018	4518 ± 1057	0.46	5944 ± 1841	7544 ± 2178	1.27
Connexin 40	2835 ± 210	4737 ± 712	1.67	2942 ± 531	7067 ± 2054	2.40
m-numb (m-NB)	3592 ± 453	1642 ± 193	0.46	1688 ± 285	1464 ± 768	0.87
Glutamate receptor subunit	2864 ± 1204	696 ± 493	0.24	1189 ± 519	1222 ± 513	1.03
Transcription factors (sixth group)						
Msx-interacting Zn finger protein	5069 ± 1976	1675 ± 842	0.33	2537 ± 944	3565 ± 1390	1.40
Brain-specific homebox (BRN-1)	7308 ± 1780	3992 ± 727	0.55	4347 ± 931	3855 ± 1216	0.91
TBX2 protein	4752 ± 1154	1980 ± 483	0.43	10279 ± 2927	8546 ± 4260	0.83
Homoboxcux2 (CUTL2)	2918 ± 235	8661 ± 3018	2.97	3233 ± 1825	2496 ± 404	0.78
Eyes absent homolog2	4352 ± 2058	10837 ± 4030	2.49	12694 ± 1847	11597 ± 5639	0.91
Transcriptional coactivator of AML-1	5533 ± 1372	2119 ± 1126	0.38	11081 ± 1791	7959 ± 3366	0.72
Transcription factor 3B	6794 ± 202	3160 ± 596	0.47	6085 ± 1097	4219 ± 1330	0.69
Ret finger protein	599 ± 15	1842 ± 638	3.10	906 ± 371	1565 ± 899	1.73
Trans-acting transcription factor 3	4190 ± 670	1545 ± 794	0.37	1724 ± 805	569 ± 184	0.33
A T motif-binding factor (ATBF1)	14324 ± 2979	25596 ± 6946	1.79	23293 ± 3372	21088 ± 76826	0.91
D-binding protein (DBP)	10812 ± 2609	169 ± 316	0.29	2604 ± 41	3512 ± 1418	1.34
Cell cycle Regulators (seventh group)						
Protein kinase-β cAMP dependent	6192 ± 1787	3016 ± 464	0.49	2421 ± 1045	2558 ± 1303	1.06
G2/M-specific cyclin G	12799 ± 3658	8715 ± 3360	0.68	9080 ± 1522	10282 ± 4090	1.13
Extracellular signal regulator ERK1	5473 ± 380	2126 ± 663	0.39	1981 ± 765	2312 ± 1011	1.16
T ob antiproliferative factor	11180 ± 938	7322 ± 1260	0.66	2039 ± 77	2793 ± 969	1.36
Cell adhesion receptors and proteins (eighth group)						
α-E-catenin	9458 ± 2098	5941 ± 1800	0.63	8413 ± 1211	8876 ± 852	1.05
Cell surface glycoprotein MAC-1	1840 ± 228	5140 ± 1516	2.79	3498 ± 444	6519 ± 2306	1.86
Integrin alpha 6	3516 ± 844	2268 ± 601	0.65	3188 ± 390	2943 ± 570	0.92
Oncogenes and tumor suppressors (nineth group)						
Transcription termination factor TTF1	11446 ± 1103	5614 ± 2085	0.49	521 ± 515	779 ± 308	1.49
Tumor susceptibility protein TSG101	3440 ± 840	1844 ± 380	0.54	3836 ± 836	2879 ± 1610	0.75
c-myc oncogene	464 ± 105	1145 ± 110	2.47	537 ± 299	774 ± 176	1.44
Erin; Villin 2	9229 ± 1202	5792 ± 1039	0.63	7936 ± 1436	6553 ± 1582	0.83
c-met oncogene	10622 ± 3924	6705 ± 1681	0.63	8104 ± 945	8506 ± 1464	1.05
Retinoblastoma-like protein RBL2	1263 ± 149	2851 ± 1053	2.26	1330 ± 525	2125 ± 855	1.60
Ion channels and transporters (tenth group)						
Glutamate receptor, ionotropic NMD2A	618 ± 77	4697 ± 1691	6.31	2533 ± 1059	1636 ± 721	0.65
P-glycoprotein (MDR1)	1237 ± 50	3277 ± 952	2.65	3052 ± 674	2055 ± 844	0.67
Receptors (eleventh group)						
IL-6 receptor alpha	553 ± 141	4217 ± 1750	7.62	1891 ± 393	1841 ± 120	0.99
5-hydroxytryptamine receptor	1066 ± 824	11149 ± 9102	10.5	N.D.	N.D.	
Serine protease inhibitor 1-2	4486 ± 610	17023 ± 1151	3.79	10910 ± 5711	13300 ± 5171	1.22

*The data are mean ± SEM of four separate experiments from MT-null and WT mice treated with lead.
N.D., not detected.

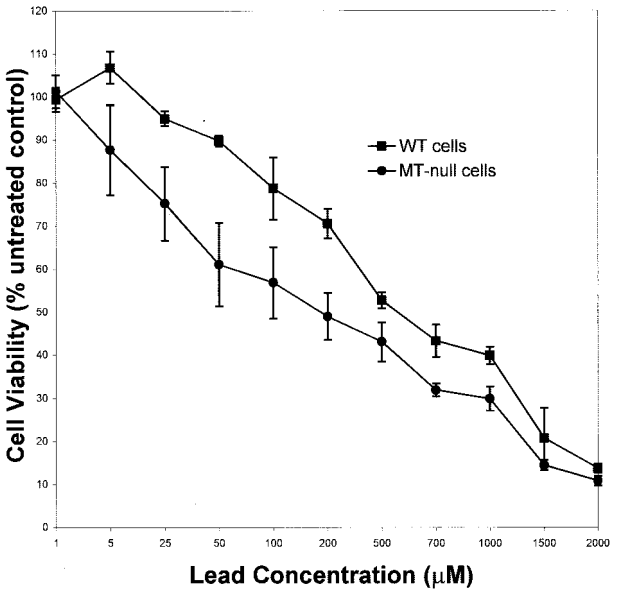

Figure 3. Acute cytotoxicity of lead in MT-null and WT cells. MT-null and WT cells were treated with the lead glutamate at the concentration indicated for 24 hours. Cytotoxicity was measured by the MTS assay. Data are expressed as percentage of untreated control that is set at 100%. Results are presented as the mean ± SEM ($n = 4$).

ing and are particularly common in the kidney epithelial cells, although they can occur in the brain and elsewhere.[14] Several studies have shown that inclusion bodies are composed of a lead-protein complex, and it has been hypothesized that inclusion bodies play an important role in the mitigation of lead toxicity.[14] The present results indicate that the inability to produce MT predisposes animals or cells to lead toxicity and is associated with an inability to produce lead inclusion bodies. In this regard, MT-deficient mice were more sensitive than WT mice to lead-induced nephromegaly, depression of renal function, and molecular lesions resulting in aberrant gene expression. *In vitro*, cells incapable of MT production were clearly hypersensitive to lead-induced cytotoxicity. MT deficiency was associated with the inability to produce lead inclusion bodies in both the *in vivo* and *in vitro* system. Thus, it seems that MT is somehow required for inclusion body production because the MT-null genotype does not allow for the formation of inclusion bodies after lead exposure. Precisely how MT may facilitate inclusion body formation is not known, but because MT deficiency enhances lead toxicity as well as preventing inclusion body formation, assuming there is a connection between these events, these results seem to support the concept that inclusion bodies may mitigate lead toxicity.[7,12] What is perhaps more important is that this study predicts that individuals less able to produce MT may be hypersensitive to lead intoxication, indicating a potential genetic basis for lead sensitivity. It is clear that genetic polymorphisms exist in the human MT genes,[40] although how these polymorphisms might affect lead toxicity is unknown. In addition, based on the current results, we would predict that MT-null animals would be more sensitive to lead-induced neurotoxicity. Subtle neurotoxicity of lead is a major issue in child health in the United States and identifying sensitive subpopulations would be a considerable advance in this important public health issue.

Exactly how the inability to produce MT may enhance lead toxicity is unclear. MTs contain numerous thiol groups because of their very high cysteine content, which provides the basis for high-affinity binding of many metals.[16–18] It is likely that a major purpose of these proteins is detoxification of metals.[16] The mitigation of the adverse effects of many toxic metals, including cadmium and mercury, by MT is quite well established and probably occurs through sequestration of the toxic metal in a nonbioavailable, and thus, toxicologically inert form.[16] However, the role of MT in lead toxicity has been only poorly defined. Lead can stimulate MT production *in vitro* and *in vivo* in some cases[18,19,23] but not in others.[25] Our results indicate that *in vitro* lead exposure induces MT in the WT cells used in the present study. The finding that lead induces MT does not, in and of itself, establish that it plays a role in reduction of lead toxicity. However, the results of the present study clearly show that the ability to express the major forms of MT reduces the toxic impact of lead *in vivo* and *in vitro*. It is important to note that MT-null animals or cells were more sensitive to lead despite accumulating significantly less of the metal. Even in the face of favorable biokinetics, the MT-null genotype is more sensitive to lead toxicity, as manifested *in vivo* as nephromegaly, impaired renal function, and aberrant gene expression and *in vitro* as acute cytotoxicity. Therefore, the present results implicate MT as an important factor in lead toxicity, through an as yet undefined mechanism.

It is also seems from the present results that MT may play a role in lead-induced inclusion body formation. In fact, there was a total absence of inclusion body formation in the MT-null genotype both *in vitro* and *in vivo*, even at toxic levels of lead. Exactly how MT may facilitate inclusion body formation is, at present, unknown. Perhaps the simplest explanation for the absence of inclusion body formation with the MT-null phenotype would be biokinetic in nature. In essence, the hypothesis here would be that, because MT-null animals or cells accumulate less lead, the levels of the metal in MT-null mice would be below that needed to stimulate inclusion body formation. However, an examination of the present data (Table 2) shows that renal lead levels in MT-null animals given 2000 ppm lead (11.0 μg/g wet weight), where no inclusion bodies were found (Table 1), actually exceed lead levels in the kidneys of WT mice given 1000 ppm lead (10.9 μg/g wet weight), where inclusion bodies were quite common. On this basis, it would seem that levels of lead sufficient to stimulate inclusion body formation in WT mice did in fact reach the kidneys in MT-null animals without producing any inclusion bodies. This does not entirely exclude biokinetics as an aspect of the inability to form lead inclusion bodies associated with the MT-null genotype. For instance, it is possible that MT may act as a temporary intracellular transport biocomplex with lead to facilitate localization of the metal to the appropriate cellular point for production of inclusion bodies. A variety of high-affinity renal-binding proteins have come to light.[41] Chemical analysis of inclusion bodies indicates a

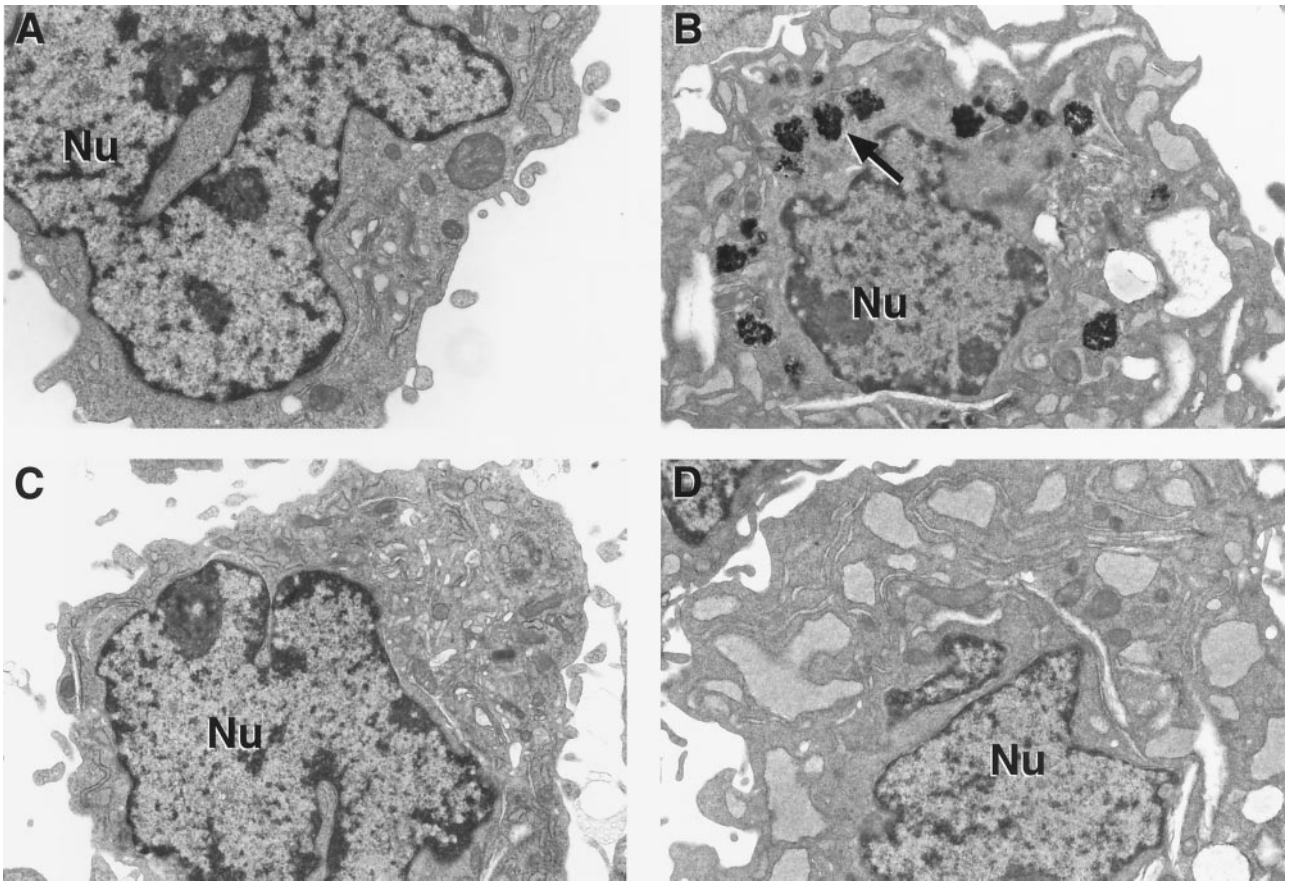

Figure 4. Lead-induced inclusion bodies as detected by EM. MT-null and WT cells were treated with lead for 48 hours; after fixation and staining they were viewed by EM (original magnification, ×15,000; scale bar, 1 μm). **A:** Control WT cells. **B:** Lead-treated WT cells with groups of irregularly shaped inclusion bodies around the nucleus present in the cytoplasm. **C:** Control MT-null cells. **D:** Lead-treated MT-null cells (note absence of inclusion bodies).

relatively constant protein-to-lead ratio, suggesting an orderly process,[7] which would be consistent with a facilitory role for MT in this process. Defining the exact nature of inclusion bodies has been problematic,[42,43] but it is clear they contain both lead and protein.[7,42,43] Immunohistochemical analysis clearly showed MT was not prominently associated with inclusion bodies, but this is only after formation of the inclusion bodies. MT could still possibly be within inclusion bodies, but in an immunologically changed form that would not be detected by the antibody used in this study. Further research will be required to more fully define the role of MT in lead-induced inclusion body formation, but the present results indicate MT is required for such formation, perhaps in a facilitative or temporary transport role.

GSTs are a family of phase II detoxification enzymes involved in the conjugation of a diverse group of electrophilic substrates with glutathione followed by excretion of the conjugate.[44] Wright and colleagues[44] first reported that increases in GSTs are closely linked to tissue damage resulting from lead exposure. These data suggest that increases in GST precede cellular and physical changes induced by lead, and thereby provide an extremely sensitive tissue biomarker of lead exposure.[44] Moser and colleagues[45] and Oberley and colleagues[46] have also reported that acute or chronic inorganic lead exposure during development produces cell-type-specific increases in GST expression in the rat kidney. However, whether these increases in GSTs are a result of lead-induced injury or serve as a protective adaptation is not clear.[44] Regardless of whether this is a toxic response or an adaptive response to intoxication, the

Table 4. Lead Uptake in WT and MT-Null Cells

Cell type	Lead uptake
WT	8.7 ± 1.1
MT-null	4.9 ± 1.2*

WT and MT-null cells were treated with lead for 48 hours, and intracellular lead levels (μg lead/10^6 cells) were measured by AAS. Data given as the mean ± SEM (n = 3).
*Significant ($P < 0.05$) difference from WT cells.

Table 5. The Levels of MT in the WT Cells Treated with Lead

	Lead exposure concentration, μmol/L		
	0	100	200
Metallothionein (μg/10^6 cells)	10.9 ± 1.1	86.1 ± 8.2*	112.5 ± 12.2*

WT cells were treated with lead for 48 hours. MT was measured by the Cd-hemoglobin method. Data are given as the mean ± SEM (n = 3).
*Significant ($P < 0.05$) difference from untreated cells.

present study demonstrates that the expression of genes encoding for GSTs (including μ, θ, π) were significantly increased in MT-null mice by lead treatment but not in WT mice. This indicates that the molecular responses to lead-induced toxic insult are exaggerated by the inability to produce MT. It is thought that MT may also serve as a scavenger for reactive oxygen species,[47] although this is not clearly established. Thus, the up-regulation of defense-related genes, such as those encoding GSTs, because of lead exposure in MT-null mice, may act as a cellular adaptive mechanism in the absence of MT. Therefore, it appears in the present study that up-regulation of GST may serve as a subtle indicator of lead toxicity as previously suggested.[44] The exact role of the alterations in defense-related genes, as well as the relationship of these genes to lead toxicity, are worth further investigation. Furthermore, how overexpression of heat shock proteins HSP27 and HSP84, as well as cell signaling and transducers in MT-null mice, might contribute to lead toxicity requires additional study.

In summary, the MT-null phenotype does not allow inclusion body formation after lead exposure and predisposes to lead toxicity both *in vivo* and *in vitro* despite reducing accumulation of the metal. Thus, MT seems to play an important role in lead toxicity and in inclusion body formation. These results indirectly support a role for inclusion bodies as a potential element in cellular lead tolerance. From these results it is possible to conclude that individual variation in the ability to express MT may dictate sensitivity to lead toxicity in exposed populations, which may have important public health implications.

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