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THE CRITICAL ROLE OF THE CELLULAR THIOL HOMEOSTASIS IN CADMIUM PERTURBATION OF THE LUNG EXTRACELLULAR MATRIX

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Abstract

Cadmium (Cd) inhalation can result in emphysema. Cd exposure of rat lung fibroblasts (RFL6) enhanced levels of metal scavenging thiols, e.g., metallothionein (MT) and glutathione (GSH), and the heavy chain of γ-glutamylcysteine synthetase (γ-GCS), a key enzyme for GSH biosynthesis, concomitant with downregulation of lysyl oxidase (LO), a copper-dependent enzyme for crosslinking collagen and elastin in the extracellular matrix (ECM). Cd downregulation of LO in treated cells was closely accompanied by suppression of synthesis of collagen, a major structure component of the lung ECM. Using rats intratracheally instilled with cadmium chloride (30 μg, once a week) as an animal model, we further demonstrated that although 2-week Cd instillation induced a non-significant change in the lung LO activity and collagen synthesis, 4- and 6-week Cd instillation resulted in a steady decrease in the lung LO and collagen expression. The lung MT and total GSH levels were both upregulated upon the long-term Cd exposure. Emphysematous lesions were generated in lungs of 6-week Cd-dosed rats. Increases of cellular thiols by transfection of cells with MT-II expression vectors or treatment of cells with GSH monoethyl ester, a GSH delivery system, markedly inhibited LO mRNA levels and catalytic activities in the cell model. Thus, Cd upregulation of cellular thiols may be a critical cellular event facilitating downregulation of LO, a potential mechanism for Cd-induced emphysema.

Keywords

cadmium; metallothionein; glutathione; lysyl oxidase; collagen; emphysema

1. Introduction

Lysyl oxidase (LO, E.C.1.4.3.13), a copper (Cu)-dependent enzyme, catalyzes crosslinking of collagen and elastin essential for extracellular matrix (ECM) morphogenesis and tissue repair

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(Kagan and Li, 2003). The critical role of LO in the lung physiology and pathology is evidenced by findings that inhibition of LO induces emphysematous lung in the animal model (Snider et al, 1986)

Cadmium (Cd) is a toxic metal but still widely used in industries. Occupational exposure to Cd occurs mainly in the form of airborne dusts and fumes affecting an estimated 510,000 workers in the US (IARC, 1993). In addition to the occupational exposure, cigarette smoke constitutes a major source of Cd exposure for humans since tobacco leaves naturally accumulate Cd. Since it binds to cellular thiols such as metallothionein (MT) and glutathione (GSH) with high affinity, Cd can be absorbed by and accumulated in the lung with a biological half-life of 9.4 years (IARC, 1993). Long-term exposure to Cd results in emphysema. Fatal emphysema developed in Cd-poisoned patients who had survived acute lung injuries in industrial accidents (Lane and Campbell, 1954; Snider, 1992). Smokers with severe emphysema have higher Cd levels in their lungs than non-smokers (Paakko et al., 1989; Post et al., 1984).

Elevation of lung metallothionein (MT) and glutathione (GSH) as a defense response is a resultant marker for Cd exposure (Hart et al., 2001; Paakko et al., 1989). Mammalian MT consists of 61-62 amino acids of which 20 are cysteines (Cys). These Cys-SH groups provide metal binding sites for MT. One mole of MT protein can bind a total of 7 moles of Cd or 11 or 12 moles of Cu (Kagi and Schaffer, 1988; Vasak, 2005). GSH is a thiol-containing tripeptide playing a critical role in cellular metal transport, metabolism and protection (Meister, 1984). Our previous studies have shown that downregulation of LO was associated with upregulation of cellular MT and GSH in Cd-resistant cells isolated from long-term Cd exposure (Zhao et al., 2006). To probe the molecular mechanisms of Cd injury to the lung ECM, we further examined the role of cellular thiol homeostasis in LO regulation in Cd-pulsed rat fetal lung fibroblasts (RFL6) and in Cd-dosed lungs of rats. We found that Cd enhanced levels of metal scavenging thiols, e.g., MT and GSH in pulse-treated cells and in the animal model concomitant with downregulation of LO. Cd downregulation of LO was closely coupled with suppression of synthesis of collagen, a major structure component of the lung ECM. Moreover, the *in vitro* assays further indicated that Cd upregulation of cellular thiols played a critical role in facilitating downregulation of LO, a potential mechanism for Cd-damage to the lung ECM.

2. Materials and Methods

2.1. Materials

Cadmium chloride, 99.9% pure, was from Aldrich Chemicals (Milwaukee, WI). Glutathione (GSH) monoethyl ester (GME), GSH, GSH reductase, reduced nicotinamide adenine dinucleotide phosphate (NADPH), hemoglobin, diaminopentane, and β-aminopropionitrile (BAPN) were obtained from Sigma Chemical Company (St. Louis, MO). [^{3}H] or [^{14}C]proline (250 mCi/mmol), [^{3}H]lysine (100 Ci/mmol), ^{32}P-d-CTP (3000 Ci/mmol) and carrier-free ^{109}Cd (1.83 mCi/mg) were purchased from PerkinElmer Life and Analytical Sciences, Inc. (Waltham, MA). A rabbit anti-LO antibody was prepared and employed as a probe of LO protein as previously described (Li et al., 1995). A mouse anti-MT antibody (Dako-MT, E9) against both MT-I and MT-II was from Dako Inc. (Fort Collins, CO). A rabbit antibody against the heavy chain of γ-glutamylcysteine synthetase (γ-GCS) was from NeoMarkers (Fremont, CA). Horseradish peroxidase (HRP)-conjugated goat anti rabbit or mouse IgG was from Santa Cruz Biotech. (Santa Cruz, CA). Ultrapure clostridial collagenase was from Advance Biofactures (Lynbrook, NY). Amplex red was from Molecular Probes, Inc. (Eugene, OR). Protein assay reagent was from Pierce (Rockford, IL). All tissue culture products were from Invitrogen Co. (Carlsbad, CA)

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2.2. Cell culture and treatments

RFL6 cell line derived from the normal Sprague-Dawley rats was obtained from ATCC and maintained in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM). To obtain growth-arrested cultures, cells were incubated in 0.3% FBS/DMEM for 3 days then changed into the fresh medium (Li et al., 1995) and exposed to agents such as $CdCl_2$ at different doses over a 24 h period. Cells with such different treatments were then assayed for cellular MT, GSH and LO levels.

2.3. Determination of cellular MT and GSH

Cellular MT was assessed by a Cd-hemoglobin affinity assay as described (Eaton and Toal, 1982). This assay was originally created as a rapid, easy and sensitive methodology to determine MT levels regardless of its isoform types in biological systems in response to Cd. Briefly, growth-arrested control and Cd-treated cells were harvested by scraping and sedimentation, and disrupted in ice cold 10 mM Tris-HCl buffer (pH 7.4) by sonication. After denaturing in boiling water for 2 min to release originally bound Cd from MT and centrifugation at 2,000 × g for 10 min, the supernatant was collected for immediate determination of MT content by incubation at room temperature with ^{109}Cd (1 μCi/ml, mixed with cold Cd to the final concentration of 2 μg/ml), then 2% bovine hemoglobin both in 10 mM Tris-HCl, pH 7.4. The mixture was heated in a boiling water bath, cooled on ice, and then spun. Hemoglobin solution was again added into the supernatant. The heating and centrifugation steps were repeated. The amount of radioactivity in the supernatant fraction was determined by γ-counting. Results were expressed as nanograms MT per milligram total cell protein based on 7 g-atoms of Cd bound to 1 mole of MT and the molecular weight of MT = 6 kDa as described in our previous publication (Li et al., 1994).

Cellular GSH was assessed by the Tietze enzymatic assay as described (Li et al., 1994). Briefly, growth-arrested control and Cd-treated cells were harvested and lysed in 0.2% Triton X-100 in PBS. Cell protein was precipitated by addition of cold 50% TCA to a final concentration of 2.5% followed by centrifugation. The supernatant was then assayed for total cellular GSH by measuring the change in color of the reaction mixture at A412 nm in the presence of GSH reductase and NADPH. Results were expressed as nanograms of GSH per mg total cell protein calculated by using a GSH standard curve as described (Li et al., 1994).

2.4. LO catalytic activity assay

Fluorometric assays for H_2O_2 release in the LO-substrate reaction were carried out to assess effects of Cd, MT and GSH on LO catalytic activities in the cell model using diaminopentane as a substrate and Amplex red as a hydrogen peroxide probe as described by Palamakumbura and Trackman (2002) and in our previous publication (Chou et al., 2007). All enzyme activities were continuously monitored for at least 300 seconds at excitation and emission wavelengths of 563 and 587 nm, respectively, at a constant temperature of 37°C, as specified in the thermostatted cuvette chamber of an LS 55 Luminescence Spectrometer (PrekinElmer Instruments, Shelton, CT). Results were expressed as fluorescence values at 300 seconds after the reaction, corrected for background levels of H_2O_2 release determined in the reaction mixture supplemented with BAPN, an active site inhibitor of LO, and normalized to total cell protein. For the animal model, 0.2 g lung tissues were homogenized in 16 mM potassium phosphate buffer, pH 7.8, containing 4 M urea. LO activities in lung tissue extracts were assessed by a standard protocol using a recombinant human tropoelastin substrate labeled with [^{3}H]-lysine as described (Li et al., 1995). Enzyme activities were expressed as cpm of [^{3}H]-H_2O release/mg total tissue protein. In enzyme activity assays, purified bovine aorta LO (0.2 μM) was used as a positive control (data not shown). Calf aorta LO (32 kDa) was isolated as described (Bedell-Hogan et al., 1993).

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2.5. Western blot analysis

Control and treated cells or tissues were homogenized in the RIPA buffer composed of 1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 2 M urea, pH 7.4 (Chen et al., 2005). One tablet of the protease inhibitor cocktail (Roche, Mannheim, Germany) was freshly added to 10 ml of RIPA. After microcentrifugation, supernatants were collected and stored at −80 °C. Protein concentration in each sample was determined by the BCA protein assay reagents (PIERCE, Rockford, IL). Cell lysates containing equal amounts of protein (25 μg) were analyzed by SDS-PAGE. LO, MT or γ-GCS protein levels in control and treated cells were determined by Western blot with a primary rabbit anti-LO (1:1000), mouse anti-MT, i.e., Dako-MT E9 (1:500), or rabbit anti-γ-GCS heavy chain (1:500), and a secondary anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (HRP) (1:2000) as described (Chen et al., 2005). Protein bands were quantitated by the 1 D Scan EX software (Scananalytics, Fairfax, VA).

2.6. Reverse transcription (RT)–PCR

MT, γ-GCS heavy chain and LO mRNA expression was determined by RT-PCR as described (Chen, et al., 2005). Briefly, total RNA was extracted from control and treated cells using a TRIzol Reagent Kit (Invitrogen, Carlsbad, CA). Using 50-100 ng of total RNA as a template, MT-II, γ-GCS heavy chain and LO cDNAs were created and amplified with the SuperScript One-Step RT-PCR with Platinum *Taq* Kit (Invitrogen, Carlsbad, CA) under the following conditions: reverse-transcript at 50°C for 30 min and pre-denaturation at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 90 sec for a total of 35 repetitive cycles. Final extension was performed at 72°C for 5 min. The forward and reverse primers were synthesized for cDNA amplification by PCR of LO, MT-II, γ-GCS heavy chain and glyceradehyde 3-phosphate dehydrogenase (GAPDH), an internal control, as described in our previous publication (Chen et al., 2005). Such PCR products as a 216 bp for MT-II, a 1.8 kb for γ-GCS heavy chain, a 1.3 kb for LO and a 500 bp for GAPDH were respectively separated on 2-2.2% agarose gels, stained with ethidium bromide and visualized on a UV transilluminator. Bands of cDNA were quantitated with 1 D Scan analysis as described (Chen et al., 2005).

2.7. Northern blot

In some experiments, LO mRNA expression in control and Cd-treated cells was also analyzed by Northern blot using ^{32}P-labeled LO cDNA probe as described (Li et al., 1995).

2.8. Assays for collagen synthesis

Total collagen synthesis in cultured cells and lung tissues was assessed by the [^{14}C] or [^{3}H] proline labeling and collagenase digestion assay as described (Zhao et al., 2006). For cell culture, growth-arrested control and treated cells were labeled with [^{14}C]proline (20 μCi/ml) for 24 h in serum-free DMEM supplemented with 25 μg/ml ascorbic acid. After incubation, labeled cells were counted for numbers or collected and homogenized on ice followed by centrifugation. For tissue culture, the physiological saline-perfused lung was minced into 1 mm^3 pieces, and aliquots of minced tissue (50 mg/ml) were preincubated in DMEM at 37°C for 10 min. The tissues were transferred to a fresh DMEM supplemented with 100 μCi/ml of [^{3}H]proline in the presence of ascorbic acid and incubated for 24 h with gentle shaking at 37°C in a 5% CO_2 atmosphere. The pulsed tissues were washed and stored in DMEM at −80°C until further processing. The frozen samples were thawed, homogenized on ice with a polytron and centrifuged. Aliquots of tissue extracts were saved for the protein assay (Li et al., 1994). TCA was then added to cell or tissue extracts to a final concentration of 5% on ice and the mixture is centrifuged at 10,000 × g at 4°C to remove unincorporated isotopes. Pellets are resuspended in 0.2 N NaOH and incubated with 100 units/ml ultrapure clostridial collagenase

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for 90 min at 37°C. The solubilized collagen is separated from noncollagen fraction by 5% TCA precipitation in the presence of 0.25% tannic acid. Radioactivities associated with each fraction (collagen and noncollagen) were determined by β-counting. Collagen synthesis was expressed as collagenase-digestible counts normalized by total cell numbers or tissue protein (Zhao et al., 2006).

2.9. Increase of cellular GSH by exposure of cells to GME

To increase cellular GSH, growth-arrested RFL6 cells were exposed to GME at indicated doses for 24 h followed by incubation of cells in the GME-free medium for an additional 24 h period (Chen et al., 2005). Levels of cellular GSH and LO mRNA and catalytic activity in control and treated cells were determined as described above.

2.10. Increase of cellular MT-II by transfection of cells with MT-II expression vectors

For transfection of MT-II expression vectors, rat MT-II cDNA was prepared by RTPCR. Approximate 500 ng of total RNA extracted from RFL6 cells by TRIzol were converted to cDNA which was then amplified using the SuperScript One-Step RT-PCR with Platinum *Taq* Kit (Invitrogen, Carlsbad, CA) under conditions as described (Gao et al., 2007). The forward (F) and reverse (R) primers each contained two restriction enzyme sites (labeled with underlines) allowing us to create the sense and antisense MT-II cDNAs. They were 5′-GATGGTACCCTCGAGATGGACCCCAACTGCTCCTGTGCCACAGATGGATC-3′ (F) and 5′-GATAAGCTTTCTAGATCAGGCGCAGCAGCTGCACTTGTCCGAAGCCTCTTT-3′ (R). The mammalian MT-II expression vectors pcDNA3.1-MT-II sense and pcDNA3.1-MT-II antisense were respectively constructed by ligating the rat MT-II coding sequence with the pcDNA3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA) in their KpnI/XbaI sites for the sense and HindIII/XhoI sites for the antisense. After cloning, all expression vectors were sequenced to ensure fidelity. Subconfluent RFL6 cells grown on 100 mm dishes were respectively transfected with vectors at indicated doses containing MT-II sense and antisense cDNA as well as the pcDNA3.1 vector without MT-II cDNA insert, a negative control, by lipofectamine reagents as described (Gao et al., 2007). After 24 h incubation, cells were washed with PBS, and grown in the fresh 0.3% FBS/DMEM for an additional 36 h. MT-II and LO expression in control and transiently transfected cells were determined as described above.

2.11. Animal studies

Male Sprague-Dawley Rats were from Charles River Labs (Wilmington, MA; 10 rats/group). Body weights of rats were measured at the first time of Cd instillation (= 150 ± 8 g/rat). Animals were anesthetized with isoflurane. A constant dose of $CdCl_2$ at 30 μg (containing 18.4 μg Cd/rat, i.e., 0.123 ± 0.007 mg Cd/kg body weight) in 100 μl physiological saline were intratracheally instilled into the lung once a week for 2, 4 and 6 weeks. Total Cd doses ranged from 36.8 μg to 110.4 μg Cd/rat. Control rats received saline only. Since each time point contained both the control and the Cd-dosed rats, a total of 6 animal groups were included in this study. Rats were killed 1 week after the last instillation. This exposure regimen was designed for rat stepwise adaptation to Cd to mimic chronic exposure conditions in humans based on Cd absorption and deposition coupled with less elimination by the lung with the half-life of 9.4 year (IARC, 1993). Notably, the same Cd administration protocol has been widely used for carcinogenesis studies (Pott et al., 1987; IARC, 1993). For morphological studies (5 rats/group), lungs were fixed by filling with 0.2% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at a pressure of 25 cm of fixative and then processed for slice preparation and HE staining (Kuhn III and Starcher, 1980). For electron microscope (EM) examination, lung tissues from control and Cd-treated rats were fixed in 2% paraformaldehyde, 0.1% glutaraldehyde and 5% sucrose in PBS to maintain the integrity of the original tissue

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morphology. Small pieces of samples were dehydrated with graded alcohols and embedded in Epon 812. The thin sections were cut, mounted on nickel grids, stained with a saturated aqueous solution of uranyl acetate and lead citrate, and then observed under a Philips CM 12 transmission electron microscope. All EM images were recorded on SO-163 film. For biochemical assays (5 rats/group), lungs were perfused with physiological saline *via* the pulmonary artery. The left lung was minced into 1 mm^3 pieces and labeled with [^{3}H]proline for assaying collagen synthesis while the right lung was homogenized to determine levels of LO, MT, and GSH as described above.

2.12. Statistical analysis

Data were expressed as mean ± SD of at least three independent experiments. Statistical differences between means were determined using one-way ANOVA followed by Bonferroni's post hoc test or two-tailed Student's test when appropriate (Chen et al., 2005). A p value < 0.05 was considered significant.

3. Results

3.1. Cd enhancement of cellular levels of MT and GSH concomitant with inhibition of LO catalytic activity

MT and GSH are major cellular thiols regulating the metal metabolism and heavy metal detoxification (Kagi and Schaffer, 1988; Meister, 1984; Vasak, 2005). Cd is an effective inducer of cellular MT and enhancer of cellular GSH. LO is a critical ECM crosslinking enzyme using Cu as cofactor (Kagan and Li, 2003). To evaluate the critical role of cellular thiols in Cd damage to the lung ECM, we first examined Cd effects on cellular levels of MT and GSH, and LO catalytic activity in conditioned media of lung fibroblasts incubated in the presence or absence of Cd at various doses (Fig. 1A). As determined by Cd-hemoglobin affinity and GSH enzymatic assays, Cd markedly elevated levels of cellular thiols in a dose-dependent manner, reaching 3.0, 5.0, and 8.1-fold of the control for MT and 1.5, 2.0 and 3.0-fold of the control for GSH, respectively (100% MT level in the control = 120 ± 19 ng/mg total cell protein, 100% GSH level in the control = 850 ± 60 ng/mg total cell protein) in cells treated with Cd at 1, 3, and 6 μM for 24 h. In contrast, the identical Cd treatments induced a dose-dependent inhibition of LO activities in cell conditioned media probed by the H_2O_2 release assay using diaminopentane as a substrate and Amplex red as a hydrogen peroxide probe, amounting to 56, 30 and 15% of the control (100% LO activity in the control = 6,520 ± 580 fluorescence units/mg total cell protein), respectively. Furthermore, the time course study showed that 6 μM Cd resulted in a biphasic response in cellular GSH levels, i.e., an initial decrease at 3 h followed by a steady increase upon long term exposure, in association with a marked enhancement of cellular MT. Notably, the same Cd treatment elicited a constant reduction of LO activities in cell culture media (Fig. 1B). These results demonstrate Cd enhancement of cellular levels of MT and GSH concomitant with inhibition of LO catalytic activity.

3.2. Cd stimulation of MT-I, MT-II, and g-GCS heavy chain expression at protein and mRNA levels

To further elucidate Cd effects on MT and GSH expression, Western blot and RT-PCR were conducted to assess protein and mRNA levels of MT and the heavy chain of γ-GCS, a rate-limiting enzyme for GSH biosynthesis (Yan and Meister, 1990), in control and Cd-treated cells. As noted, the Dako-MT, E9, antibody recognizes both MT-I and MT-II proteins. Western blot (Fig. 2A) showed that control RFL6 cells without Cd treatment expressed very low level of MT-I and MT-II proteins, major isoforms of MT in the lung (Hart et al., 1996). Cd-induced MT protein production in treated cells was evidenced by appearances of conspicuous MT-I and MT-II fused bands on the gel in cells exposed to 1, 3, and 6 μM Cd for 24 h amounting to 50, 67 and 61-fold of the control, respectively. In parallel, the identical Cd treatments elevated

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the steady-state mRNA levels of MT-II to 2.3, 4.5 and 5.0-fold of the control, respectively (Fig. 2B). Furthermore, both protein (Fig. 2C) and steady-state mRNA (Fig.2D) levels of the γ-GCS heavy chain were also enhanced in a dose-dependent manner in Cd treated cells consistent with increased levels of cellular GSH in these cells. For example, 6 μM Cd enhanced protein and mRNA levels of the γ-GCS heavy chain to 2.3 and 10.9-fold of the corresponding controls, respectively. These results provide the strong evidence for Cd enhancement of the expression of MT-I, MT-II, and GSH, the major cellular thiol components. It should be noted that upregulation of γ-GCS by Cd conferred high levels of cellular GSH on treated cells as a result of activation of GSH biosynthesis.

3.3. Cd inhibition of LO expression at mRNA and protein levels and collagen synthesis

Cd effects on the expression of LO at mRNA and protein levels were examined in RFL6 cells treated with Cd at various doses for 24 h. As determined by RT-PCR (Fig. 3A), cells exposed to Cd exhibited dose-dependent decreases in levels of LO cDNA (1.3 kb) in comparison to the internal control, GAPDH cDNA (500 bp). One-D Scan EX analysis showed that Cd at 1, 3, and 6 μM reduced LO cDNA to 61, 14 and 4%, respectively, of the control without Cd treatment indicating very high sensitivity of LO mRNA for Cd inhibition. Furthermore, Western blot analysis indicated that Cd decreased levels of all LO protein species including the 46 kDa preproenzyme, the 50 kDa proenzyme and the extracellular, but cell membrane-associated 32 kDa mature enzyme, in a dose-dependent manner (Fig. 3B). For example, the 46 kDa preproenzyme, the 50 kDa proenzyme and the 32 kDa mature enzyme were reduced to 33, 42 and 48%, respectively, of their corresponding controls in cells exposed to 1 μM Cd while the 50 kDa proenzyme was further decreased to only 5% of the control associated with nondetectable preproenzyme and mature enzyme in cells treated with 6 μM Cd. Notably, in parallel with inhibition of the LO catalytic activity, Cd exposure suppressed synthesis of collagen, a substrate of LO (Kagan and Li, 2003), in treated cells in a dose (Fig. 3C) and time (Fig. 3D) dependent manner as probed by the [^{14}C]-proline labeling assay. Cells treated with Cd at 1, 3, and 6 μM reduced isotope incorporation with collagens to 75, 38, and 20% of the control (100% collagen synthesis level in the control = $(6.2 \pm 0.7) \times 10^4$ cpm/10^6 cells), respectively. These results suggest that Cd may damage to the lung ECM by perturbing its major components, i.e., LO and its substrates.

3.4. Elevated levels of MT and GSH coupled with decreased levels of LO expression and collagen synthesis in the lung tissue of repeatedly Cd-dosed rats

To evaluate Cd injury to the lung, 10 rats per group were intratracheally instilled with 30 μg $CdCl_2$ in 100 μl physiological saline weekly for 2, 4 and 6 weeks. Control rats received saline only. Animals were killed 1 week after the last instillation. Five rats per group were used for morphological studies. At postmortem examination, the lungs from 2 week-Cd instilled rats were 1.5-fold heavier than the control (the lung wet weight in the control = 1.1 ± 0.3 g) and showed areas with red and white patches on the pleural surface while those from 6 week-Cd instilled rats exhibited only few white patches on the pleural surface with a wet weight close to the normal control (Table 1). It should be noted that Cd-dosed rats displayed gradual loss of body weights in comparison to controls. After filling with the fixing solution, the 6-week Cd-instilled lungs had increased lung volumes (1.6 ± 0.2-fold of the control). Microscopically, in comparison to the control (Figs 4A, a and b; Fig 4B, a and Fig 4C, a), an increased cellularity characterized by infiltration of inflammatory cells, leakage of red blood cells and proliferation of epithelial and interstitial cells in alveolar spaces and walls was observed in the 2-week Cd-instilled lungs (arrowheads in Figs 4A, c-e; Fig 4B, b and Fig 4C, b) accounting for gross pulmonary alterations in these rats. In contrast, less inflammatory response occurred in the lungs of long-term Cd exposed rats suggesting their gradual adaptation to this metal insult. However, extensive enlargement of airspace and multiple pulmonary bullae with or without small areas of interstitial fibrosis (arrowheads) were found in 6-week Cdinstilled rats (Figs 4A,

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f-j; Fig 4B, c and Fig 4C, c). Furthermore, EM photographs also showed the thin alveolar wall with less deposition of the matrix components such as elastin and collagen (arrowheads) in these long-term Cd-dosed animals (compare Fig 4C, c with control of Fig 4C, a).

For biochemical assays of 5 rats/group, the left lung was minced into 1 mm^3 pieces and labeled with [^{3}H]-proline to assess collagen synthesis by the collagenase-digestion assay while the right lung was homogenized to determine levels of LO, MT and GSH as described in Methods. As shown (Fig. 4D), although a non-significant change in the lung LO activity and collagen synthesis respectively amounted to 127 ± 18% and 144 ± 25% of the controls (100% LO activity in the control = 2,395 ± 579 cpm of [^{3}H]H_2O release/mg lung tissue protein, 100% collagen synthesis in the control = 6,833 ± 1,377 cpm/mg lung tissue protein), found in rats instilled with Cd for 2-weeks, a marked decrease in the lung LO activity and collagen synthesis respectively amounted to 25% and 38% of the control, detected in rats dosed with Cd for 6 weeks. This response of the lung to Cd suggests that the crosslinking of collagen and elastin was inhibited upon long-term Cd exposure due to downregulation of LO in conjunction with the airspace enlargement (see Figs. 4A-4C). Since polymeric collagen and elastin deposited at the early stages of Cd exposure possibly due to slightly increase of LO activity and collagen synthesis were more stable and resistant to proteolysis than non-crosslinked forms, Cd-induced emphysema at the late phase of Cd exposure was associated with or without the fibrotic deposits formed earlier in some areas as shown in Figs.4A.f-j. However, the lung MT and total GSH levels were increased almost linearly over the 6-week Cd instillation period, reaching 5.2 and 3.2-fold of the controls (100% MT level in the control = 381 ± 133 ng/mg lung tissue protein, 100% GSH level in the control = 758 ± 96 ng/mg lung tissue protein). Furthermore, Western and Northern blots also confirmed decreased LO at protein and mRNA levels in lungs of 6-week Cd exposed rats (Fig. 4E). These results indicate upregulation of MT and GSH was accompanied by downregulation of LO and its substrates in the emphysematous lung of long term Cd-dosed rats consistent with the phenotype change of lung fibroblasts in response to Cd *in vitro* (Figs. 1-3).

3.5. Evidence for cellular thiol modulation of LO expression

To assess the modulating effects of cellular thiols on LO expression, we treated cells with GME, a GSH delivery system, and transiently transfected cells with the MT-II coding region containing vectors, respectively, to increase cellular GSH and MT, and then examined LO expression at activity and mRNA levels. To increase cellular GSH, growth-arrested RFL6 cells were exposed to GME at indicated doses for 24 h followed by washing and incubating in the absence of GME for additional 24 h (Chen et al., 2005). As shown (Fig.5), the GME at 2 and 4 μM elevated cellular GSH levels to 2.3- and 3.6-fold of the control (100% GSH level in the control = 962 ± 77 ng/mg total cell protein) which were associated with reductions of LO activities by 45% and 88%, respectively, in conditioned media of treated cells (100% LO activity in the control = 5,840 ± 771 fluorescence units/mg total cell protein). Moreover, the RT-PCR assays showed that increased levels of cellular GSH by 2 and 4 μM GME inhibited steady-state LO mRNA levels to 74 and 55% of the control ($p < 0.05$), respectively (see Fig. 5 insert). It is known that the enhancement of cellular GSH brought about by incubation of cells with GME is due to uptake of GME followed by rapid hydrolysis and efficient release of intact GSH inside the cell (Anderson et al., 1985). Therefore, the decrease of LO activity and mRNA levels by GME appears to be mediated by elevated levels of cellular GSH.

To increase cellular MT, growth-arrested RFL6 cells were transfected with MT-II coding cDNA vectors at various doses as described (Gao et al ., 2007), using the pcDNA3.1 vector without the MT-II cDNA insert and an MT-II antisense as negative controls. As shown (Fig. 6), cells tranfected with MT-II expression vectors effectively increased cellular MT levels as revealed by the Cd-hemoglobin affinity assay reaching 5 and 12-fold of the control (100% MT

level in the control = 140 ± 28 ng/mg total cell protein) in cells transfected with 2 and 6 μg MT-II cDNA vectors, respectively (column 4 and 5). In contrast, there were very low levels of MT in control cells without transfection (column 1) and in cells tranfected with the pcDNA3.1 vector without MT-II cDNA insert or with MT-II antisense cDNA (columns 2 and 3). These MT-II overexpressed cells exhibited very low levels of LO catalytic activity in the conditioned media reducing to 33% and 21% of the control (100% LO activity in the control = 6,260 ± 814 fluorescence units/mg total cell protein) in cells transfected with 2 and 6 μg MT-II cDNA vectors, respectively (Fig. 6). Furthermore, cells transfected with 2 and 6 μg/ml MT-II cDNA vectors also exhibited marked suppression of LO mRNA expression amounting to 74% and 55% of the control, respectively (compare columns 4 and 5 with column 2 in Fig 6 insert). These results directly linked the upregulation of cellular thiols, e.g., MT and GSH, to the downregulation of LO at transcriptional and catalytic levels.

Discussion

Using rat lung fibroblasts (RFL6) and rat lungs as *in vitro* and *in vivo* models, we studied Cd effects on cellular thiol homeostasis and lung ECM metabolism. Results indicated that Cd exposure enhanced cellular levels of MT-I, MT-II, and GSH, major metal scavenging agents, as well as the heavy chain of γ-GCS, an enzyme for GSH biosynthesis, but inhibited the expression of LO and collagen, two major components of the lung ECM. Changes in levels of MT, GSH, LO and collagen synthesis were associated with the development of emphysematous lesions in lungs of intratracheally Cd-instilled rats. Furthermore, increases of cellular thiols by treatment of cells with GME, an intracellular GSH delivery system, and transfection of MT-II expressing vectors reduced catalytic activities and steady-state mRNA levels of LO in the cell model. These results suggest that cellular thiols may, at least in part, mediate Cd damage to the lung ECM leading to emphysema pathology.

Cd is a heavy metal with high toxic effects on humans. Inhalation of Cd either from occupational exposure or from cigarette smoke can result in emphysema (IARC, 1993; Paakko et al., 1989). Although the “elastase-antielastase imbalance” has been shown as a critical mechanism for emphysema pathogenesis (Snider, 1992), the molecular basis for Cd-induced lung ECM injury is still not very clear. LO is a Cu-dependent enzyme secreted by fibrogenic cells such as fibroblasts (Kagan and Li, 2003). This catalyst initiates the crosslinking of collagen and elastin, the major structural components of the ECM, by oxidizing peptidyl lysine residues within these proteins to form peptidyl α-aminoadipic-δ-semialdehyde, thus stabilizing polymeric collagen or elastin as insoluble fibers. The lung ECM, a dynamic structure, is composed of a number of functionally diverse elements which are integrated mainly by interstitial cells, e.g., fibroblasts (Davidson, 1990). The overall pattern of the lung ECM results from an intricate balance between the synthesis and the degradation of its major structural components, e.g., collagen and elastin. LO as a critical effector in emphysema development was evidenced by the disruption of the lung structure in chicks, hamster and rats following diet-induced Cu deficiency. Markedly reduced levels of LO associated with reduction of elastin deposition in the lung accounted for the role of Cu as a cofactor for LO (Dubic et al., 1985; Harris, 1986). The lung lesions in these animals were similar with panlobular emphysema in humans (Soskel et al., 1984). With simultaneous feeding of hamsters with β-aminoproprionitrile (BAPN), an irreversible inhibitor of LO, intratracheally instilled Cd resulted in emphysematous lung without fibrosis (Niewoehner and Hoidal, 1982). Furthermore, blotchy mice with alleles at Mottled locus of the X-chromosome exhibited an abnormal Cu transport and a deficiency of LO, developing emphysema-like injuries in the lung (Fisk and Kuhn, 1976). Our previous studies investigating phenotype changes in Cd-resistant lung fibroblasts and in cells exposed to Cd-containing cigarette smoke condensate (CSC) illustrated Cd downregulation of LO at multiple levels accompanied by elevation of cellular MT and GSH (Chen et al., 2005; Zhao et al., 2006). Pursuing these findings in this study, we further

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demonstrated that enhancement of cellular thiols by Cd, a critical cellular defense mechanism against metal toxicity, may play an important role in Cd emphysema pathogenesis by inhibition of pulmonary LO.

MT is a cysteine-rich metalloprotein involved in many cellular functions such as essential metal transport and storage, heavy metal detoxification, protection against oxidative stress, etc. (Kagi and Schaffer,1988; Vasak, 2005). In mammals, there are four MT isoforms, of which only MT-I and MT-II are expressed in the lung (Hart et al., 1996; Vasak, 2005). MT-I and MT-II molecules differ in a small number of amino acids and are encoded by separate genes (Hart et al., 1996). Mammalian MT protein sequences contain 20 cysteine (Cys) residues. These Cys-SH groups provide metal binding sites for MT. The metal binding *in vivo* involves mainly Zn (II), Cu(I), Cd(II) and Hg(II) , while *in vitro* additional metals such as Ag(I), Au(I), Bi(III), Co (II), Fe(II), Pb(II and Tc(IV) may be bound to apothionein. However, mammalian MTs mostly bind zinc under physiological conditions (Penkowa, 2006). The chemical structure of MT is partitioned into two separate metal-thiolate clusters in which cluster α in the C-terminus contains 11 Cys able to bind 4 divalent metal ions such as Cd(II) and Zn(II) or 6 monovalent metal ions such as Cu(I) while cluster β in the N-terminus contains 9 Cys able to bind 3 divalent metal ions such as Cd(II) and Zn(II) or 6 monovalent metal ions such as Cu(I) (Penkowa, 2006) . The affinity of the metal ions for the MT binding sites follows the order of thiolate metal complexes, i.e., Zn(II)<Pb(II)<Cd(II)<Cu(I), Ag(I), Hg(II), Bi(III) (Kagi and Schaffer, 1988). Cd, Cu and Zn are effective inducer of MT. In smokers, 56% of pulmonary Cd accumulated was bound to MT (Post et al., 1984). Exposure to Cd activated MT genes, resulting in elevated levels of MT in RFL6 cells (Fig. 2) and lung tissues (Fig. 4) as shown in this study in favor of metal storage and detoxification. Metal-responsive transcription factor-1 (MTF-1) plays a central role in transcription activation of the MT genes which contain at least 6 metal response elements (MRE) at its promoter regions (Giedroc et al., 2001). MTF-1 is a Zn-finger transcription factor. Cd and oxidative stress (such as H_2O_2) enhance MTF-1 binding to the MT-I gene promoter by binding to or oxidation of cellular thiols releasing bound Zn that in turn increases MTF-1 affinity for DNA (Giedroc et al., 2001).

GSH is a thiol-containing tripeptide playing a critical role in cellular metabolism, essential metal transport and heavy metal detoxification (Meister, 1984). Cellular concentrations of GSH range from 0.1 to 10 mM, accounting for 90% of total cellular non-protein thiols (Morris and Bernard, 1994). GSH participates in protecting airspace epithelium of the lung from oxidant and inflammation injuries. GSH levels in epithelial lining fluid (ELF) of the human lung reach > 400 μM, thus establishing its significance in lung diseases with implications for therapy. Marked increases of total GSH concentration in ELF were observed in cigarette smokers and patients with acute respiratory distress syndromes (Morris and Bernard, 1994). Through catalysis of GSH peroxidase, GSH reduces H_2O_2 and lipid hydroperoxides, but itself is converted to the oxidized form, GSSG. GSSG can be reduced back to GSH by GSH reductase (Meister, 1984). Thus, GSH is an important cellular scavenger for reactive oxygen species. The synthesis of GSH requires the consecutive actions of γ-GCS and glutathione synthetase (Meister, 1984). γ-GCS is a rate-limiting enzyme for GSH biosynthesis. This enzyme consists of a catalytic (heavy) subunit and a modifier (light) subunit encoded by different genes and dissociated each other under reduced conditions. Since γ-GCS heavy chain exhibits both the enzyme activity and the feedback inhibition by GSH, the catalytic unit is widely investigated and its levels determine the rate of GSH synthesis (Yan and Meister, 1990). Various agents can modulate γ-GCS expression and GSH levels including oxidants, inflammatory mediators, etc. Our study showed Cd enhancement of GSH levels in cultured cells and lung tissues of the animal model (Figs. 1, 2 and 4D). As determined by Western blot and RT-PCR (Figs. 2C and 2D), upregulation of the γ-GCS catalytic unit at protein and mRNA levels in treated cells indicated the activation status of GSH biosynthesis under Cd exposure conditions.

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LO is synthesized by fibroblasts as a 46 kD preproenzyme. Following signal peptide cleavage and N-glycosylation, the resulting 50 kD proenzyme is secreted and then proteolytically cleaved to the 32 kD functional species in the extracellular space (Kagan and Li, 2003). The sequences within the mature enzyme at which the active site is located are highly conserved (95%) in rat, mouse, chick and human. LO specifically binds 1 g-atom of Cu(II) at its active site per mole of enzyme (Kagan and Li, 2003). Five histidine residues located at the sequence of His 283-His 297 constitute the copper-binding motif. Cu binding to proenzyme occurs in the secreted pathway such as the Golgi apparatus (Chou et al., 2007; Kosonen et al., 1997). In addition to Cu, LO also contains a covalently bound carbonyl cofactor identified as lysine tyrosylquinone (LTQ) deriving from Lys 314 and Tyr 349 based on the sequence of rat LO (Wang et al., 1996). This peptidyl orthoquinone functions as a transient electron sink during amine oxidation. The precise role of Cu in the LO action remains poorly understood. This metal ion may be essential for maintaining the structural integrity of LTQ and/or the protein (Kagan and Li, 2003). The activity of purified LO can be severely inhibited by metal chelators, e.g., α,ά-dipyridyl (Gacheru et al., 1990). Dietary deprivation of Cu induced lathyritic injuries in animals due to suppression of LO activity (Dubic et al., 1985; Harris, 1986). Decreased LO activity is associated with disorders of Cu metabolism, e.g., Menkes syndrome (Harris, 1993). Because of the high affinity of Cu for MT and GSH, Cd elevation of cellular thiols such MT and GSH may change cellular Cu homeostasis and thus limit Cu bioavailability for LO which uses Cu as a cofactor. Our previous studies have showed that an extremely low level of Cu was associated with the LO fraction and an extremely high level of Cu was bound to the MT fraction in long-term Cd exposed cells as revealed by ^{64}Cu-metabolic assays (Chou et al., 2007). High affinity of Cu for MT was also evidenced by that the addition of exogenous MT, led to a shift of labeled ^{64}Cu from Cu-binding protein fractions to the MT-containing fraction in cell extract (Farrell, et al., 1993). Thus, scavenging of cellular Cu cofactor for LO by cellular thiols such as MT and GSH may represent a critical mechanism for inactivation of LO catalytic activity in Cd treated cells.

MT and GSH may regulate gene transcription via modulation of the homeostasis of intracellular metals and reactive oxygen species (ROS) and thus affect the active status of metal and redox-sensitive transcription factors such as Zn-finger containing MTF-1 and SP1, and the nuclear factor-E2-related factor 2 (Nrf2) which interacts with the antioxidant response element (ARE) sensitive to ROS (Penkowa, 2006; Hayes et al., 2000; Rahman and MacNee, 2000; Narayan et al., 1997). The rat LO gene contains at least two MREs, two SP1 binding sites and one ARE in the promoter region −804/−1 upstream of ATG which displayed the maximal promoter activity (Gao et al., 2007). Marked increased levels of cellular thiols by MT-II cDNA transfection and GSH intracellular delivery are expected to trap cellular essential metals such as Zn preventing from its use for transcription factors and to reduce cellular ROS levels thus inactivating LO MRE, ARE and SP1-binding sites and inhibiting LO gene transcription as shown in Figs 5 and 6. Cu has been shown to regulate transcription of several genes by activation of MRE and ARE consistent with its redox-active property for catalyzing the formation of reactive oxygen species in the biological system (Mattie and Freedman, 2004). Our previous studies have shown that exogenously added Cu elevated LO mRNA levels in cultured RFL6 cells (Zhao et al., 2006). In contrast, scavenging cellular Cu or reactive oxygen species by increasing cellular MT and GSH may act as a potential factor leading to inactivation of LO MRE and ARE, and the final reduction of LO mRNA. It should be noted that downregulation of LO by Cd was coupled with upregulation of MT. MT-I and MT-II gene promoters each contain at least 6 MREs. Although the LO promoter also contains MREs, in contrast, Cd exposure inhibited the LO mRNA expression. This sharp difference between LO and MT genes in response to Cd may result from Cd perturbing the interaction of MREs with MTF-1 in the LO gene but not in the MT gene. Presumably, in cell response to Cd, many MRE-related transcription factors may be recruited to bind to MREs in the MT promoters for gene

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activation thus competing with their binding to the LO promoter. However, mechanisms for Cd specific inactivation of the LO MREs remain unclear and should be further understood.

This investigation has linked the LO dysfunction coupled with elevation of cellular MT and GSH *in vivo* to important pathological consequences of Cd pulmonary toxicity, i.e., emphysema. Animal studies by others have indicated that the nature of Cd-induced lung lesions varied with the exposure protocols and species used. Both emphysema and fibrosis with and without airspace enlargement were produced in rodents (Snider et al., 1986). The Cd doses used in this animal study are relevant to environmental Cd exposure of humans. Airborne Cd concentrations reached 10,800-233,000 μg/m^3 in occupational settings such as the metal smelting area (IARC, 1993). An epidemiological study showed that estimates of Cd exposure by inhalation amounted to 40-600 μg/m^3 (average = 320 μg/m^3) in a Cd production and refining plant during 1965-1976 (IARC, 1993). According to several parameters (e.g., a person minute ventilation ≈ 6 L, 8-h work a day, 5-day work a week, 1/10 airborne Cd inhaled, $T_{1/2}$ of Cd in the human lung ≈ 10 years, wet weight of the human lung ≈ 1200 g), a person working under a 320 μg Cd/m^3 condition for 10 years could still have a lung burden of 150 μg Cd/g wet weight of the lung. In our animal studies the maximal Cd accumulation in the rat lung was about 96.8 μg Cd/g wet weight of the rat lung when animals received 6 times the dose of 30 μg $CdCl_2$ (= 18.4 μg Cd) (the wet weight of the rat lung ≈ 1.1 g). Because of its long half-life in the lung, the accumulated Cd should be active in this organ during the experiment. Cd-elevated MT and GSH protected the lung against Cd toxicity as evidenced by less inflammatory response and prevention of extensive fibrosis as a result of downregulation of LO and collagen synthesis in the long-term Cd-exposed rats in this study. It should be noted that enhanced levels of tissue MT and GSH have been reported by others in chronically Cd exposed animals (Liu et al., 2009). Furthermore, high levels of Cd in the MT bound form were also detected in smoker lung tissues with severe emphysema (Post et al., 1984). Since both collagen and elastin are substrates of LO in the ECM, downregulation of LO is anticipated to damage the crosslinking of these substrates, favoring their instability. The resulting solubilized collagen and elastin in turn could inhibit their own synthesis, possibly by a feedback mechanism (Jackson et al., 1991; Diegelmann and Peterkofsky, 1972), thus further disturbing the balance between synthesis and degradation of these proteins and enhancing ECM disruption. These biochemical impairments as shown in inhibition of collagen synthesis upon Cd action *in vitro* and *in vivo* underlied morphologic perturbation in the form of loss of the structural integrity of the lung with developing centrilobular or bullous emphysema (Figs. 4A-C).

Briefly, data presented in this study provide evidence that cellular MT and GSH play a critical role in the pathogenesis of Cd-induced emphysema by modulation of LO expression.

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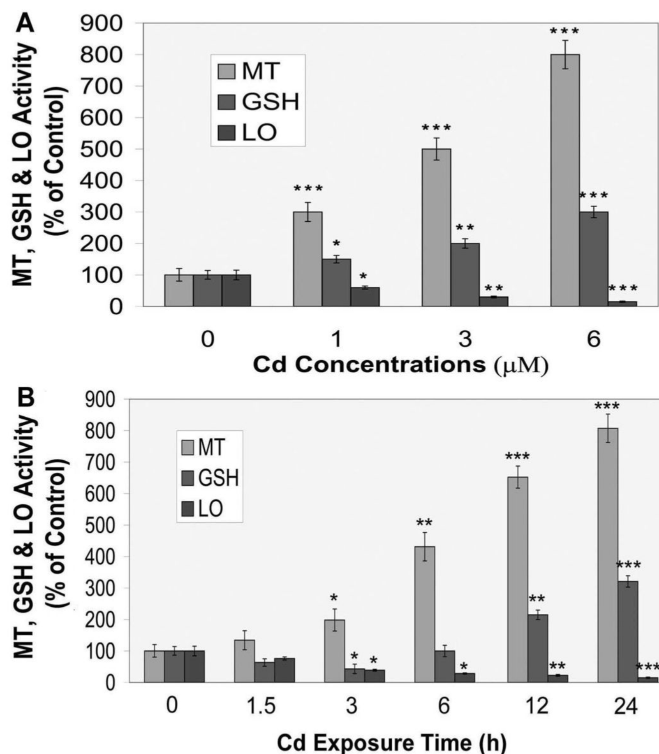

Fig. 1. Cd enhancement of cellular MT and GSH but inhibition of LO activities in cultured RFL6 cells

A. Dose response. Growth-arrested RFL6 cells were exposed to $CdCl_2$ at indicated doses for 24 h. **B. Time course.** Cells were exposed to 6 μM $CdCl_2$ for various times. The levels of cellular MT, GSH and LO activities in the conditioned media were measured respectively as described in Methods. Data are expressed as % of the corresponding controls (100% MT level in the control = 120 ± 19 ng/mg total cell protein, 100% GSH level in the control = 850 ± 60 ng/mg total cell protein and 100% LO activity in the control = 6,520 ± 580 fluorescence units/mg total cell protein. All values represent the mean ± SD of three experiments each determined with triplicate dishes. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with controls without treatment of Cd.

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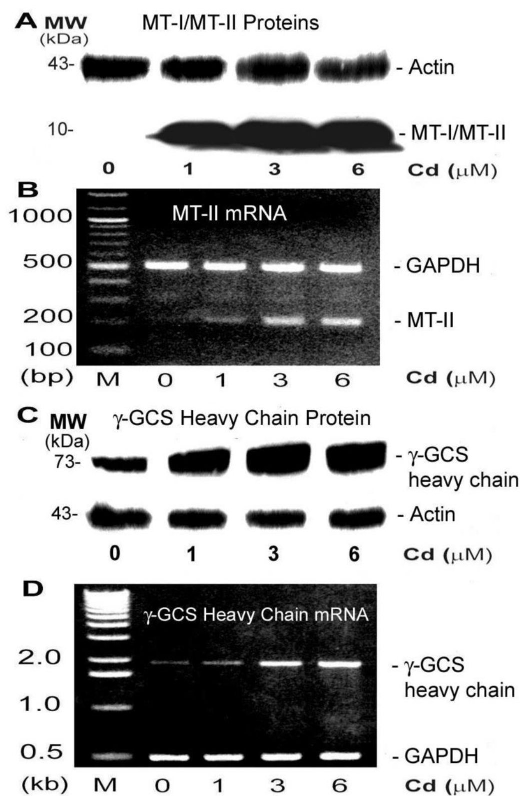

Fig. 2. Cd stimulation of MT-I, MT-II, and γ-GCS heavy chain expression at protein and mRNA levels

Growth-arrested RFL6 cells were treated with Cd at indicated doses for 24 h, and extracted for preparation of total protein and RNA. Western blot and RT-PCR assays were performed respectively to assess MT-I/MT-II (**A** and **B**) and γ-GCS heavy chain (**C** and **D**) expression at protein (**A** and **C**) and mRNA (**B** and **D**) levels. Levels of actin (**A** and **C**) and GAPDH (**B** and **D**) were determined as internal controls. M in lane 1 of mRNA assays (**B** and **D)** represents the DNA molecular marker.

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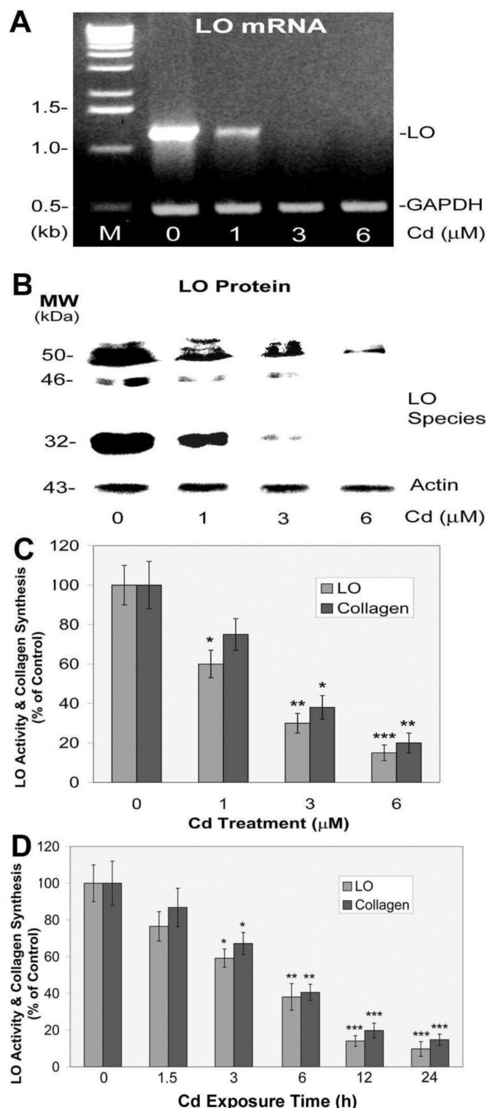
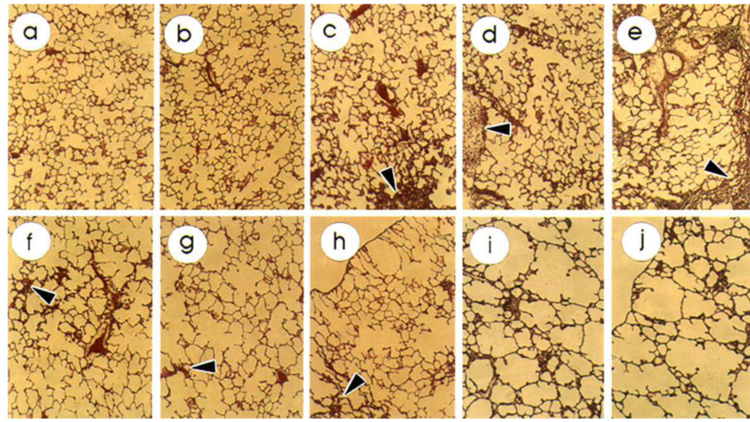
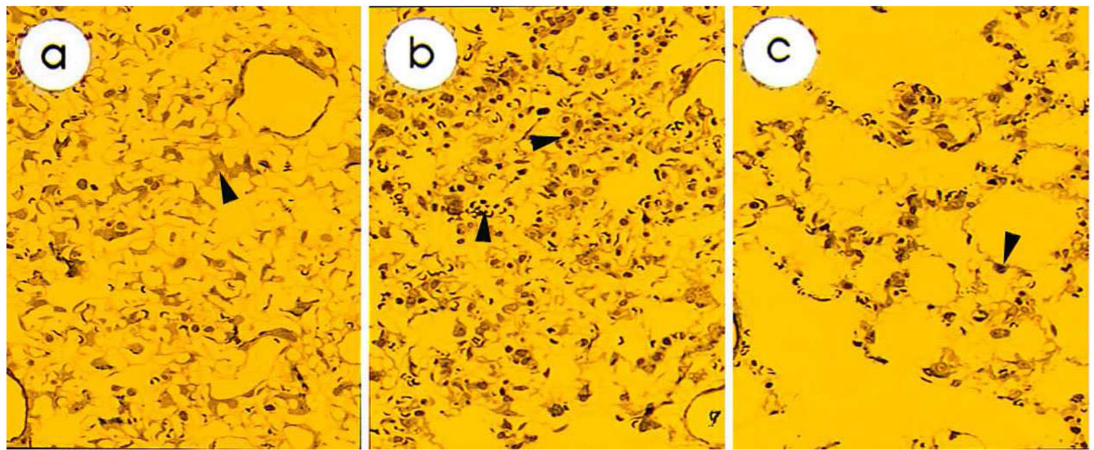


Fig. 3. Cd inhibition of LO expression at mRNA and protein levels and collagen synthesis

Total protein and RNA were extracted from control and Cd-treated cells, and assayed for the LO mRNA (**A**) and protein (**B**) expression by RT-PCR and Western blotting, respectively. For assaying the collagen synthesis, cells were labeled with [^{14}C]proline and incubated in the absence or presence of $CdCl_2$ at indicated doses for 24 h to assess the dose response (**C**) or at 6 μM $CdCl_2$ for various times to assess the time course response (**D**). Collagenase-released radioactivities from isolated proteins were assessed by β-counting. In parallel experiment with cells without isotope labeling, LO activities (**C** and **D**) in conditioned media were determined using diaminopentane as a substrate and Amplex red as a hydrogen peroxide probe. Data are expressed as % of the control (100% collagen synthesis level in the control = $(6.2 \pm 0.7) \times 10^4$ cpm/10^6 cells; 100% LO activity in the control = 5,120 ± 515 fluorescence units/mg total cell protein.). All values represent the mean ± SD of three experiments each determined with triplicate dishes. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with controls without treatment of Cd.





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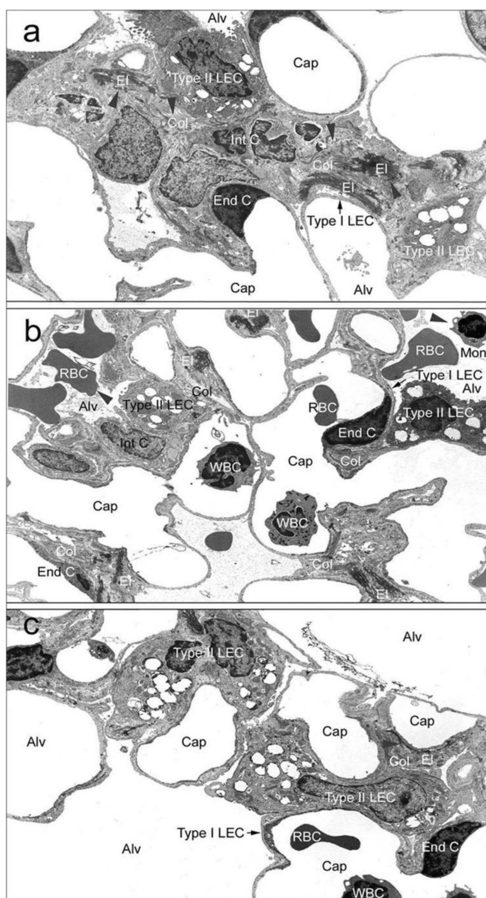

Fig. 4A-C. Emphysema-like lesions occurred in repeatedly Cd-exposed rat lungs

Rats (5 rats /group) were exposed to Cd as shown in Methods. Lungs were fixed and processed for routine slice preparation and HE staining. **A**. HE stained lung tissues under the microscopy at the low magnification (20 x). **a** and **b**, the normal lung tissue; **c**-d, serious inflammatory response in 2-week Cd-instilled lungs as evidenced by occurrence of the leakage of red blood cells in **c** (arrowhead), the infiltration of inflammatory cells in **d** (arrowhead); **e-h**, less inflammatory response in 6-week Cdinstilled lungs coupled with extensive enlargement of airspace (**e** and **f**) and multiple pulmonary bullae (**g** and **h**) with or without small areas of interstitial fibrosis (arrowheads). **B**. HE stained lung tissues under the microscopy at the high magnification (100 x). a, the normal lung tissue and the distribution of type II lung epithelial cells (arrowhead); **b**, serious inflammatory response in 2-week Cd-instilled lungs as evidenced by occurrence of the infiltration of inflammatory cells (arrowhead) and the proliferation of type II lung epithelial cells; **c**, less inflammatory response found in 6-week Cd-instilled lungs coupled with extensive enlargement of airspace (arrowhead indicates type II lung epithelial cells). **C**. Electron microscope (EM) examination at the final magnification (3,500 x). **a**, the normal lung tissues (arrowheads indicate deposition of collagen and elastin in the alveolar wall); **b**, the lung tissues from 2-week Cd-instilled rats (arrowheads indicate red blood cells and monocyte in the alveolus); **c,** the lung tissues from 6-week Cd-instilled rats (arrowhead indicates less deposition of collagen and elastin in the alveolar wall). Abbreviations: Alv, alveolus; Cap, capillary; Col, collagen; El, elastin; End C, endothelial cell; Int C, interstitial cell; Mon, monocyte; RBC, red blood cell; Type I LEC, type I lung epithelial cell (arrows); Type II LEC, type II lung epithelial cell; WBC, white blood cell.

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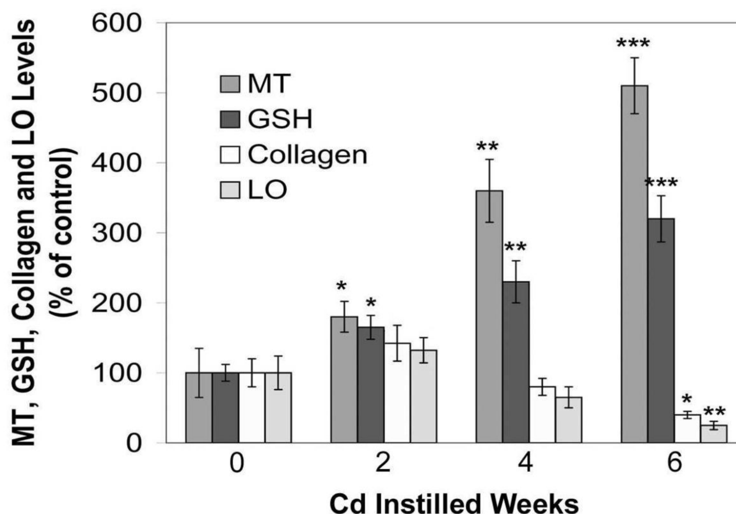

Fig. 4D. Elevated levels of MT and GSH coupled with decreased LO activity and collagen synthesis in the lung tissue of repeatedly Cd-dosed rats

Rats (5 rats /group) were exposed to Cd as shown in Methods. Physiological saline perfused lung tissues were homogenized and assayed for MT and GSH levels (100% MT level in the control = 381 ± 133 ng/mg lung tissue protein and 100% GSH level in the control = 758 ± 96 ng/mg lung tissue protein). For the LO activity assay, lung tissues were homogenized in 4 M urea and 0.016 M potassium phosphate, pH 7.8. Enzyme activity in lung tissues were assayed using a recombinant human tropoelastin substrate labeled with [^{3}H]lysine (100% LO activity in the control = 2,395 ± 579 cpm of [^{3}H]H_2O release/mg lung tissue protein). For the collagen synthesis assay, the physiological saline-perfused lungs were minced into 1 mm^3 pieces, and labeled in fresh DMEM supplemented with 100 μCi/ml of [^{3}H]proline for 24 h with gentle shaking. The pulsed tissues were homogenized and centrifuged. Aliquots of supernatants were saved for the protein assay. Pellets washed were resuspended in 0.2 N NaOH and incubated with 100 units/ml ultrapure Clostridial collagenase. The solubilized collagen was separated from noncollagen fraction by 5% TCA precipitation in the presence of 0.25% tannic acid. Radioactivities associated with each fraction (collagen and noncollagen) were determined by β-counting. Collagen synthesis is expressed as collagenase-digestible counts normalized by total protein. Data are expressed as % of the control (100% collagen synthesis level in the control = 6,833 ± 1,377 cpm/mg lung tissue protein). All values represent the mean ± SD (n = or > 3) . *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with corresponding controls.

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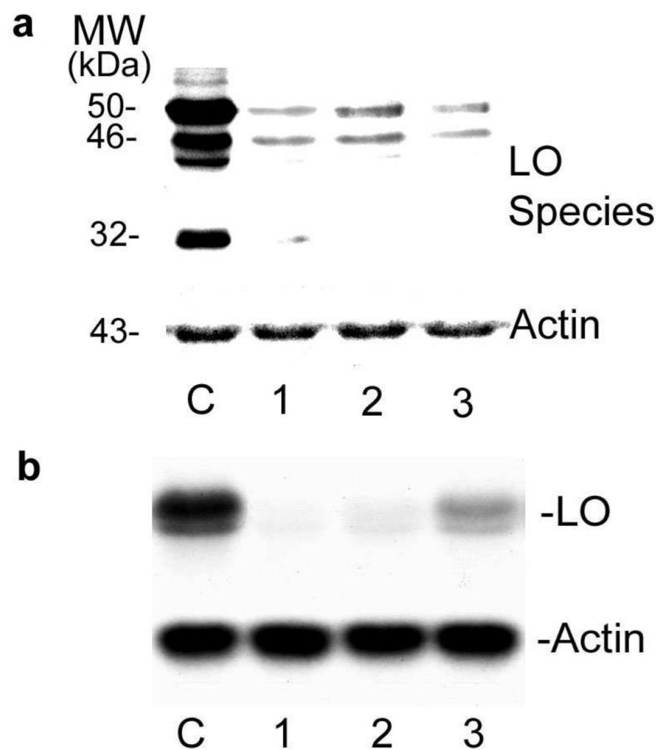

Fig. 4E. Inhibition of LO expression at protein and mRNA levels in the lung tissue of repeatedly Cd-dosed rats

Physiological saline-perfused lung tissues from control and Cd-dosed rats were extracted with the RIPA buffer and the TRIzol Reagent for preparation of total protein and RNA, respectively. LO protein and mRNA levels in the lung tissues of control (c) and 6-week Cd-instilled rats #1, #2 and #3 (1, 2 and 3) were determined by Western (**a**) and Northern (**b**) blots as described. Actin protein and mRNA levels were used as internal controls.

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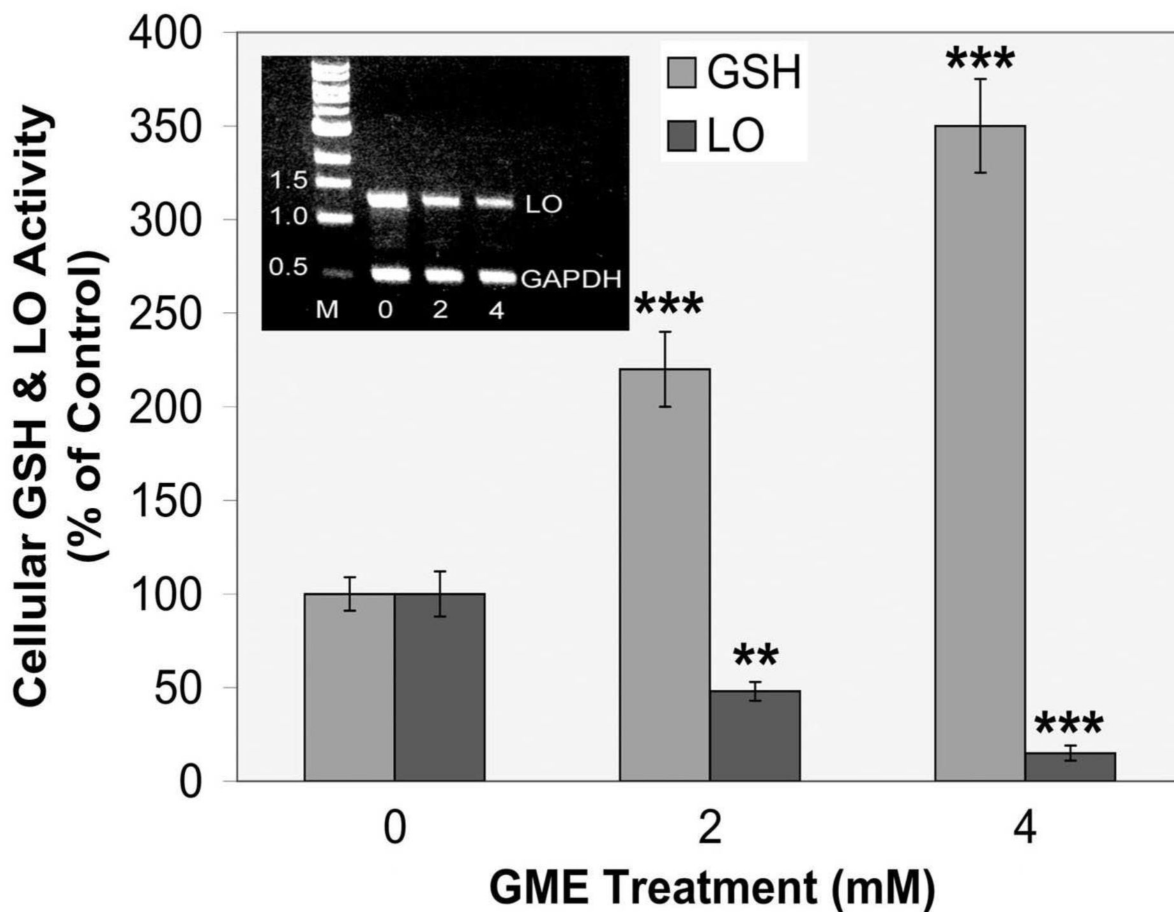

Fig. 5. Inhibition of LO mRNA expression and catalytic activities by GME, a GSH delivery system, in treated cells

Growth–arrested RFL6 cells were exposed to GME at indicated doses for 24 h followed by washing and incubating in the absence of GME for an additional 24 h period. LO activities in conditioned media and cellular GSH levels were assessed as described in Fig. 1 (100% GSH level in the control = 962 ± 77 ng/mg total cell protein, 100% LO activity in the control = 5,840 ± 771 fluorescence units/mg total cell protein). All values represent the mean ± SD of three experiments each determined with triplicate dishes. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with controls without GME treatment. In parallel, total RNA were extracted from control and GME-treated cells, and assayed for the LO mRNA expression by RT-PCR as described in Methods (see the inserted picture).

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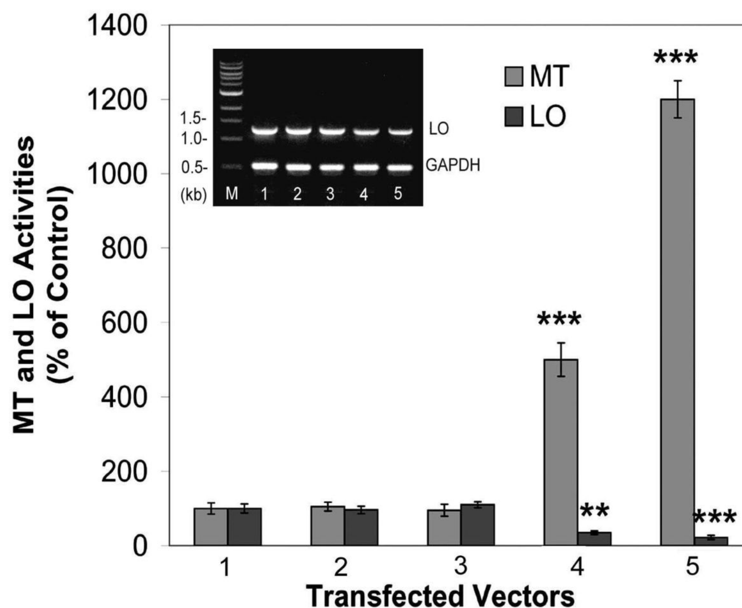

Fig. 6. Inhibition of LO mRNA expression and catalytic activities by expression of MT-II cDNA in transfected cells

RFL6 cells were transfected with MT-II expression vectors at indicated doses. Antisense of MT-II expression vectors was used as an internal control. Cells were harvested 48 h thereafter and cellular MT levels and LO activities in the conditioned media were measured as described in Methods. Data were expressed as % of the control in cells cotransfected with pcDNA 3.1 without MT-II cDNA insert (100% MT level in the control = 140 ± 28 ng/mg total cell protein, 100% LO activity in the control = 6,260 ± 814 fluorescence units/mg total cell protein). 1, control cells without transfection; 2, cells tranfected with 6 μg pcDNA3.1 vector without MT-II cDNA insert; 3, cells tranfected with 6 μg pcDNA3.1 vector with MT-II antisense cDNA; 4, cells transfected with 2 μg MT-II cDNA vectors; 5, cells transfected with 6 μg MT-II cDNA vectors. All values represent the mean ± SD of three experiments, each determined with triplicate dishes. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with controls. In parallel, total RNA was extracted from control and treated cells, and assayed for the LO mRNA expression by RT-PCR as described in Methods (see the insert picture).

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Table 1

Effects of intratracheally instilled Cd on rat body and wet lung weights

Groups	2 weeks	4 weeks	6 weeks
Control			
Body weight (g)	203 ± 10	258 ± 12	315 ± 14
Lung weight (g)	1.10 ± 0.30	1.21± 0.40	1.24 ± 0.35
Cd-dosed			
Body weight (g)	186 ± 9***	217 ± 11***	250 ± 13***
Lung weight (g)	1.69 ± 0.31*	1.50 ± 0.34	1.32 ± 0.33

A total of 60 rats with the body weight = 150 ± 8 g/rat were random divided into two groups each with 30 rats. Rats in one group were intratracheally instilled with 30 μg $CdCl_2$ in 100 μl physiological saline once a week for 2, 4, and 6 weeks (10 rats for each time point) and killed one week after the last instillation. Rats in another group as controls were instilled with physiological saline only. Values represent the mean ± SD.

* $p < 0.05$,

*** $p < 0.001$ relative to corresponding controls assessed by ANOVA analysis; in each time point group, n = 10 for the body weight measurement, and n = 5 for the wet lung weight measurement (note: no including physiological saline-perfused lungs for biochemical examinations in this assay).