Inhibition of the Expression of Lysyl Oxidase and Its Substrates in Cadmium-Resistant Rat Fetal Lung Fibroblasts

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Copper (Cu)-dependent lysyl oxidase (LO) catalyzes cross-linking of collagen and elastin stabilizing the extracellular matrix (ECM). Chronic inhalation of cadmium (Cd), a toxic metal, induces emphysema. To probe mechanisms of Cd injury to the lung, we developed Cd-resistant (CdR) cells from rat fetal lung fibroblasts (RFL6) by chronic exposure to CdCl2 from 1 to 40 μM and further examined their expressions of LO, LO substrates, and Cu-scavenging thiols. Levels of cellular thiols, metallothionein, and glutathione in CdR cells were elevated to 13.0- and 3.2-fold of parental controls, respectively, whereas LO mRNA and protein levels were markedly reduced in these cells, with catalytic activity declining to only 16% of the parental control. A conspicuous 52 kDa species rather then the normal 50 kDa proenzyme appeared in the CdR cell extract but not in the conditioned medium, which was codistributed with the endoplasmic reticulum marker [DI0C5(3)] within the cell, implying the Cd-induced 52 kDa species as a product of an abnormal LO-processing defect in secretion. Addition of Cu into CdR cell cultures enhanced the expression of LO mRNA, protein and catalytic activities reflecting limitation of Cu bioavailability for LO in these cells. With inhibition of LO, CdR cells also displayed downregulation of collagen and elastin, substrates of LO. Restoration of collagen synthesis by exposure of CdR cells to purified LO or Cu suggests that inhibition of LO and limitation of Cu cofactor by Cd, as key phenotype changes, accelerated collagen and elastin damage, a critical event pertinent to emphysema pathogenesis.

Key Words: lysyl oxidase; cadmium; copper; collagen; elastin; cellular thiols.

INTRODUCTION

Lysyl oxidase (LO) (E.C.1.4.3.13) is a copper (Cu)-dependent enzyme secreted by fibrogenic cells such as fibroblasts. This enzyme catalyzes crosslinking of collagen and elastin, the major structural proteins of the extracellular matrix (ECM) by oxidizing specific peptidyl lysine residues within these proteins to peptidyl α-aminoadipic-δ-semialdehyde leading to the formation of condensation products and stabilization of the ECM. Therefore, lysyl oxidase displays a critical role in ECM morphogenesis and tissue repair (Kagan and Li, 2003; Fibroblasts originally synthesize LO as a 46 kDa proenzyme. After signal peptide cleavage and N-glycosylation, the resulting 50 kDa proenzyme is secreted and proteolytically cleaved to the 32 kDa functional species in the extracellular space (Li et al., 1995).

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 United States (IARC, 1993). In addition to the occupational exposure, cigarette smoke constitutes a major source of Cd exposure for humans. Tobacco leaves naturally accumulate Cd, and each cigarette contains Cd 1–2 μg (IARC, 1993). The lung as a major Cd-target organ not only absorbs but also accumulates Cd with a biological half-life of 9.4 years because inhaled Cd binds to cellular thiols such as metallothionein (MT) and glutathione (GSH) with high affinity (IARC, 1993; Post et al., 1984). Long-term exposure to Cd can result in emphysema. Fatal emphysema developed in Cd-poisoned patients who had survived acute lung injuries in industrial accidents (Lane and Campbell, 1954; Snider et al., 1986). Smokers with severe emphysema have higher Cd levels in their lungs than non-smokers (IARC, 1993; Paakko et al., 1989).

The lung ECM, a dynamic structure, is composed of a number of functionally diverse elements that are integrated mainly by interstitial cells, e.g., fibroblasts. The overall pattern of the lung ECM results from an intricate balance between synthesis and degradation of its major structural proteins, e.g., collagen and elastin, the substrates of LO. Because fibroblasts are a major population in the pulmonary interstitium, abnormalities in their population or functions may lead to pathogenesis of the lung such as fibrosis and emphysema. Emphysema is a pathological lesion characterized by abnormal enlargement of the respiratory airspaces with destruction of...
alveolar walls (Snider et al., 1986). The critical role of LO in emphysema development was reflected in the disruption of the lung structure in chicks and rats following diet-induced deficiency of Cu, a cofactor for LO. The lung lesions in these animals resembled panlobular emphysema in humans (Dubic et al., 1985; Harris, 1986). Feeding of β-aminopropionitrile (BAPN), an irreversible inhibitor of LO, markedly enhanced elastase-induced emphysema in hamsters (Kuhn and Sturcher, 1980). Moreover, with simultaneous feeding of hamsters with BAPN, intratracheally instilled Cd resulted in lesions of emphysema but not fibrosis in the lung (Niewoehner and Hoidal, 1982). Additional evidence correlating LO deficiency with emphysema derives from studies of genetic variants of animals, e.g., blotchy mice, which are deficient in LO due to abnormal Cu transport, developing emphysema (Fisk and Kuhn, 1976).

To probe the molecular mechanisms of Cd injury to the lung ECM, we have isolated Cd-resistant (CdR) cells from rat fetal lung fibroblasts (RFL6) by incubation in graded concentrations of CdCl₂. Using CdR cells as a long term-Cd exposure model, we have examined their relative capacities to synthesize LO, LO substrates, i.e., collagen and elastin and metal scavenging thiols, i.e., MT and GSH. We found that CdR cells exhibited a serious reduction of LO at protein, mRNA, and catalytic levels associated with inhibition of collagen type I and tropoelastin production, but with enhancement of cellular Cu scavenging agents, MT and GSH. Furthermore, we found that the deficiency of LO in the CdR phenotype played a crucial role in inhibition of the production of substrates, collagen and elastin, a critical event in evolution of emphysema.

MATERIALS AND METHODS

General experimental design. The overall goal of this study was to explore molecular mechanisms of Cd damage to the lung ECM. Considering the chronic course of Cd exposure to humans and animals, we developed Cd-resistant (CdR) cells from parental rat fetal lung fibroblasts incubated in the presence of graded concentrations of Cd for a long time. Using CdR cells with Cd exposure markers, i.e., presence of graded concentrations of Cd for a long time. Using CdR cells with resistant (CdR) cells from parental rat fetal lung fibroblasts incubated in the absence or presence of CdCl₂ for 16 h at indicated doses, fixed in 3.7% formaldehyde/0.2% Triton X-100/ PBS, and stained with an anti-tubulin antibody conjugated with FITC to visualize microtubules, with the primary rabbit anti-LO antibody and with the secondary goat anti-rabbit IgG coupled with rhodamine to visualize LO and with DIOCS(3) (Yang et al., 1997) to visualize the ER. All photographs were taken under the Nikon fluorescence microscope at the same magnification with a 40X Planapochromat objective.

Determination of cellular MT and GSH. Cellular MT was assessed by a modified cadmium-hemoglobin affinity assay as described elsewhere (Li et al., 1994). Briefly, growth-arrested parental CdS and CdR cells on coverslips were incubated in the absence or presence of CdCl₂ for 1 h at indicated doses, fixed in 3.7% formaldehyde/0.2% Triton X-100/ PBS, and stained with an anti-tubulin antibody conjugated with FITC to visualize microtubules, with the primary rabbit anti-LO antibody and with the secondary goat anti-rabbit IgG coupled with rhodamine to visualize LO and with DIOCS(3) (Yang et al., 1997) to visualize the ER. All photographs were taken under the Nikon fluorescence microscope at the same magnification with a 40X Planapochromat objective.

Materials. Cadmium chloride and cupric chloride, each 99.9% pure, were from Aldrich Chemicals (Milwaukee, WI) and Fisher Scientific (Springfield, NJ), respectively. Glutathione, GSH reductase, reduced nicotinamide adenine dinucleotide phosphate (NADPH), hemoglobin, and β-aminopropionitrile (BAPN) were obtained from Sigma Chemical Company (St. Louis, MO). [35S]-methionine (673 Ci/mmol), [14C]-proline (250 mCi/mmol), [3H]-L-lysine (100 Ci/mmol), [32P]-dCTP (3,000 Ci/mmol) and carrier-free [109Cd (1.83 mCi/mg) were purchased from New England Nuclear Corporation (Boston, MA). The endoplasmic reticulum (ER) marker [DIOCS(3)] was from Molecular Probes Inc. (Eugene, OR). Goat anti-rat lung elastin was from Elastin Products Co. (Owensville, MI). Horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG and protein A agarose beads were from Santa Cruz Biotech. (Santa Cruz, CA). Rabbit anti-LO antibody was prepared and employed as a probe of LO protein as previously described (Li et al., 1995). Rabbit anti-tubulin antibody was from Polyscience (Warrington, PA), and rhodamine-conjugated goat antirabbit IgG was from Organon-Teknika (Malvern, PA). Protein assay reagent was from Bio-Rad (Richmond, CA). All tissue culture products were from GIBCO (Grand Island, NY).

Cell culture and isolation of Cd-resistant cells. Rat fetal lung fibroblasts (RFL6) from ATCC were incubated in graded concentrations of CdCl₂ from 1 µM to 40 µM in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) over a 6-month period. Surviving cells were grown to subconfluence and subcultured at least 4 times before they were exposed to the next higher dose of Cd. Selection was continued until cells were able to survive in culture containing 40 µM Cd. To obtain growth-arrested cultures, both Cds and CdR cells were incubated in 0.3% FBS/DMEM for 3 days, changed to fresh medium, and used for experiments (Li et al., 1995). Under growth-arrested conditions, CdR cells remained viable in the presence of 30 µM CdCl₂.

Fluorescence microscopy. Microtubules, LO, and the ER were revealed fluorescently in control and treated cells as described elsewhere (Li et al., 1994). Briefly, growth-arrested parental CdS and CdR cells on coverslips were incubated in the absence or presence of CdCl₂ for 16 h at indicated doses, fixed in 3.7% formaldehyde/0.2% Triton X-100/ PBS, and stained with an anti-tubulin antibody conjugated with FITC to visualize microtubules, with the primary rabbit anti-LO antibody and with the secondary goat anti-rabbit IgG coupled with rhodamine to visualize LO and with DIOCS(3) (Yang et al., 1997) to visualize the ER. All photographs were taken under the Nikon fluorescence microscope at the same magnification with a 40X Planapochromat objective.

For assay of LO activity. Lysyl oxidase activity levels in the conditioned medium and the cell extract were assessed by determining [1H]-H₂O₂ release using [1H]-lysine-labeled recombinant human tropoelastin as a substrate as described (Li et al., 1995). Briefly, growth-arrested CdS and CdR cells were
incubated in 0.3% FBS/DMEM for 16 h. The conditioned media were collected and cell layers extracted with 4 M urea in 16 mM potassium phosphate buffer, pH 7.8. In a typical assay, samples (e.g., 700 μl conditioned medium) were incubated with 350,000 cpm of tritiated substrate for 3 h at 37°C in a 0.15 M NaCl/0.1 M sodium borate buffer, pH 8.0. In a total volume of 750 μl in the presence or absence of 0.5 mM BAPN, an active site inhibitor of LO. Tritiated water released during the incubation was isolated by vacuum distillation and counted by liquid scintillation spectrometry. Enzyme activities were normalized to total cell protein.

Assay for LO synthesis and processing. Isotope labeling coupled with immunoprecipitation was used to assess LO synthesis and processing as described (Li et al., 1995). Briefly, growth-arrested CdS and CdR cells were labeled with 35S-methionine (30 μCi/ml) for 5 h in methionine and serum-free DMEM. The conditioned media of 10 ml for each sample were collected, immediately supplemented with one tablet of the protease inhibitor cocktail (Roche, Mannheim, Germany) and 1 mg bovine serum albumin as a carrier. Proteins were precipitated by addition of 10% TCA. After centrifugation, pellets were washed with cold acetone, air-dried, resuspended in TBS (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 4% sodium dodecyl sulfate (SDS) with the protease inhibitor cocktail), and incubated at 100°C for 5 min before use for immunoprecipitation. Cell layers were lysed in the TBS buffer by forcing the suspension through 21-gauge needles 10 times. Cell lysates were heated at 100°C, centrifuged and supernatants collected. Aliquots of samples containing equal amounts of radioactivity were diluted with the nonionic detergent buffer (50 mM Tris-HCl, pH 7.4, 0.19 M NaCl, 6 mM EDTA, 2.5% Triton X-100), mixed with a rabbit anti-LO antibody (1:3000) and 30 μl of a 1:1 suspension of protein A-Sepharose CL-4B and incubated for 2 h at room temperature with shaking. The beads were washed, resuspended in SDS-gel sample buffer, and boiled for 5 min. The resulting supernatants were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. Densities of protein bands were measured with the 1 D Scan EX software (Scanalytics, Fairfax, VA) as described elsewhere (Chen et al., 2005).

Northern blot for LO steady-state mRNA. Total RNA was isolated from CdS and CdR cells by TRIzol reagent (Invitrogen, Carlsbad, CA). The steady-state concentrations of LO mRNA were determined by Northern blot, using the 32P-labeled LO cDNA probe as described elsewhere (Li et al., 1995). Briefly, equal amounts of RNA (15 μg), quantified by spectrophotometry at 260 nm, were size-fractionated by electrophoresis on a 1% (wt/vol) agarose gel. RNA was transferred to a GeneScreen nylon membrane (NEN, Boston, MA) and immobilized by ultraviolet crosslinking. The equal loading of RNA sample was revealed by ethidium bromide fluorescent staining and UV irradiation. Approximately 25 ng of the cDNA probe for LO or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an internal control, was labeled with 32P using Ready-to-Go DNA labeling beads (dCTP) from Amersham Biosciences (Piscataway, NJ). The RNA blots on the membrane were hybridized with 32P-labeled cDNA at 68°C in an ExpressHyb hybridization buffer (Clontech, Palo Alto, CA). The membrane was washed with 2× SSC at room temperature, then with 0.2× SSC at 50°C and exposed to Kodak XAR-5 film at −80°C with an intensifying screen. Autoradiograms were analyzed with the 1 D Scan EX software for densities of mRNA bands (Scananalytica, Fairfax, VA) (Chen et al., 2005).

Assay for collagen type I synthesis. The pepsin digestion assay was used to determine collagen type I synthesis as described (Lim et al., 1994). Briefly, growth-arrested CdS and CdR cells were labeled for 16 h with 14C-proline (20 μCi/ml) in serum-free DMEM supplemented with ascorbic acid. The labeled collagen in the conditioned medium was precipitated with 25% ammonium sulfate at 4°C, and precipitates were resuspended in 0.5 N acetic acid (pH 2.0). The cell layers were scraped into 0.5 N acetic acid and homogenized with a polytron. Aliquots of medium precipitates or cell homogenates containing the same amount of protein were then treated with 0.1 mg/ml pepsin at 4°C. After lyophilization, pepsin-resistant protein was processed for SDS-PAGE analysis followed by autoradiography.

Assay for total collagen production. Total collagen production in CdS and CdR cells under different conditions was determined by isotope-labeling and collagenase digestion assay as described elsewhere (Diegelmann and Peterkofsky, 1972; Derdak et al., 1992). Briefly, growth-arrested cells were labeled with 14C-proline (20 μCi/ml) for 16 h in serum-free DMEM supplemented with ascorbic acid in the presence or absence of modulators (exogenously added LO purified from bovine aorta or Cu). After incubation, cells were scraped, combined with conditioned media, counted for total cell numbers, and heated to 90°C to destroy protease activity. The radiolabeled proteins were separated from the unincorporated isobyes by repeated precipitation with 5% TCA and centrifugation, and finally resuspended in 0.2 N NaOH. Aliquots of protein suspension were then incubated with 100 U/ml ultrapure Clostridial collagenase (Advance Biofactures, Lynbrook, NY) at 37°C. The solubilized collagen was then separated from the noncollagen fraction by 5% TCA precipitation in the presence of 0.25% tannic acid. Radioactivity associated with collagen and noncollagen fractions were monitored by scintillation counting. Collagen production in CdS and CdR cells was expressed as collagen-digestible counts normalized by total cell numbers.

Assays for elastin protein levels. Tropoelastin levels in CdS and CdR cells were determined by Western blot with a primary goat anti-elastin antibody (1:1000) and a secondary anti-goat IgG conjugated with HRP (1:2000) as described elsewhere (Chen et al., 2005). Blots were developed with an enhanced chemiluminescence system (PerkinElmer Life Sciences, Boston, MA). Molecular weights were determined by comparison with Benchmark pre-stained protein ladder (Invitrogen, Carlsbad, CA). Protein bands were quantitated by the 1 D Scan EX software (Scananalytica, Fairfax, VA).

To measure insoluble elastin production, CdS and CdR cells were growth-arrested for 21 days, scraped in cold water, homogenized, and treated with 0.1 N NaOH at 98°C for 45 min. The residual protein recovered is defined as elastin and its purity as determined by amino acid analysis showed a characteristic composition high in nonpolar amino acids as described elsewhere (Stone et al., 1997). The amount of elastin was calculated as the sum of the amino acids in the hot alkali residue times the average residue mass for elastin of 85.

Statistical analysis. In MT, GSH, LO activity, and collagen synthesis assays, data are presented as mean ± standard deviation (SD) of three separate experiments in which each determination was assessed in triplicate dishes. A one-way analysis of variance (ANOVA), followed by Dunnett’s test was performed. Differences were considered significant at p < 0.05.

RESULTS

The Stable Cytoskeleton and Elevated Levels of Cellular Metallothionein and Gluthionine as Markers for Cd-Resistance

To probe the molecular mechanisms of Cd injury to the lung ECM, we have isolated Cd-resistant (CdR) cells from rat fetal lung fibroblasts (RFL6) by incubation in graded concentrations of CdCl2 from 1 μM to 40 μM in DMEM containing 10% FBS. Because Cd caused characteristic injury to microtubules (Li et al., 1994), alteration in the cytoskeleton as revealed by immunofluorescence staining was selected as one of the markers for Cd-resistance. As shown (Fig. 1A, a–d), exposure of CdS cells to 6 μM Cd for 16 h under the growth-arrested condition induced disruption of microtubules (compare Fig. 1A, b to Fig. 1A, a: CdS-control), whereas treatment of CdR cells with 30 μM Cd under identical conditions did not change the
Microtubule organization (compare Fig. 1A, d to Fig. 1A, c: CdR-control), indicative of true Cd resistance of these cells. Metallothionein and GSH are major cellular thiols involved in heavy metal detoxification (Kagi and Schaffer, 1988; Meister, 1984). Cadmium exposure is known to induce elevation of cellular MT and GSH (Li et al., 1994, 1995). To compare the relative capacities of CdS and CdR cells to synthesize these thiol-containing molecules, we performed the 109Cd-hemoglobin affinity assay and the Tietze assay (Eaton and Toal, 1982; Tietze, 1969) to reveal levels of MT and GSH in these cells. As shown (Fig. 1B), CdR cells exhibited markedly elevated levels of cellular thiols, reaching 13.0- and 3.2-fold of the CdS controls for MT and GSH, respectively. Thus, elevated levels of MT and GSH collectively protected CdR-cells from toxicity of high concentrations of Cd in the development process of Cd resistance. Using such CdR cells with stable microtubules and enhanced levels of cellular thiols as a chronic Cd exposure model, we further defined characteristics of ECM component injuries by Cd.

**Inhibition of the LO Expression at Protein and Catalytic Levels in CdR Cells**

To characterize alterations of LO expression in CdR cells, we assessed LO catalytic activity and proenzyme synthesis and processing in both CdS and CdR cells. Lysyl oxidase activity in the conditioned medium and the cell extract from parental and CdR cultures was assayed by determining 3H-H2O2 release using 3H-lysine–labeled tropoelastin as a substrate (Li et al., 1995). As shown (Fig. 2A), the LO activity was present mainly in the conditioned medium. The radioactivity released by LO action in the CdR cell-conditioned medium was much less than that in the CdS cell-conditioned medium with the former, amounting to only 16% of the latter. Moreover, LO activity in the CdR cell extract (i.e., membrane and ECM bound LO activity) was also decreased by 66% relative to the CdS-control. Thus, the total LO activity was severely diminished in CdR cells.

To assess for possible alterations in LO biosynthesis and processing, growth-arrested CdS and CdR cells were labeled for 5 h with 35S-methionine. Radioactive proteins from the cell extract and the conditioned medium were immunoprecipitated and analyzed by 12% SDS-PAGE and autoradiography (Li et al., 1995). As shown (Fig. 2B), immunoreactive proteins in CdS cells include a 46 kDa band, a densely labeled 50 kDa band, and a 32 kDa band in the conditioned medium, consistent with a typical protein profile of LO synthesis and processing by fibrogenic cells; i.e., CdS cells initially synthesized a 46 kDa preproenzyme. Then, after signal peptide cleavage and N-glycosylation, the resulting 50 kDa proenzyme was secreted and proteolyzed to the 32 kDa functional species extracellularly. In contrast, similar analysis of CdR cells revealed a marked decrease in the 46 kDa and the 50 kDa proteins, amounting to 20% and 7% of CdS controls, respectively.
instead, a conspicuous 52 kDa band appeared in the cell extract, but not in the conditioned medium. Moreover, the mature 32 kDa species including cell-associated and medium-released forms in CdR cells was essentially undetectable (only 3.1% of the CdS-control). These results suggest that CdR cells exhibited an abnormal LO processing in addition to a decreased synthesis of the 46 kDa preproenzyme (20% of the CdS control). The 52 kDa protein may represent a variant of LO precursor which was not secreted extracellularly, but instead accumulated inside the cell. Thus, the appearance of the 52 kDa LO variant protein may represent an important characteristic for the Cd resistance phenotype of cells after long-term Cd exposure.

**Cellular Localization of the 52 kDa Species in CdR Cells**

As shown (Fig. 2B), there was a 52 kDa protein, instead of the 50 kDa LO proenzyme normally secreted by CdS cells, appearing predominantly in the CdR cell extracts but not in the conditioned media. The fact that it is recognized by an LO antibody suggests that this 52 kDa protein is a variant species of the LO precursor that could not be secreted from the cell. Because secreted proteins commonly undergo post-translational modifications in the ER, we fluorescently double-stained the ER (Yang *et al.*, 1997) and LO (Li *et al.*, 1997) in the same cell to determine the localization of the 52 kDa protein in CdR cells. Figure 3A shows the well-spread CdS cells containing numerous LO-positive fragments, which are generally concentrated in the perinuclear area with some intermittently extending to the cell periphery. Further, these LO-positive fragments were mostly co-localized with the ER (Fig. 3B). In contrast, CdR cells exhibited a distinctly different distribution of LO protein. They often lacked the typical, short LO-positive fragments; instead, numerous irregular patches of LO-positive staining appeared around the nuclei, or asymmetrically distributed in the cytoplasm (Fig. 3C). As expected, these irregular LO patches overlapped with the ER staining (Fig. 3D), pointing to the accumulation of the 52 kDa protein in this organelle. Notably, Figure 3 also shows LO-positive staining in the nuclei consistent with previously published data by us (Li *et al.*, 1997).

**Downregulation of Collagen and Elastin in CdR Cells**

To answer the question whether downregulation of LO in CdR cells is accompanied by altered metabolism of its substrates, we examined the expression of collagen type I, selected as a representative of the collagen protein family, and elastin in these cells. As shown (Fig. 4A), CdR cells exhibited reduced levels of 14C-proline–labeled, pepsin-resistant α1(I) and α2(I) chains of collagen type I in both the cell extract and the conditioned medium in comparison to corresponding CdS controls. The densitometry assay indicated that the total collagen type I level in CdR cells was decreased by 68% in comparison to the CdS control. Furthermore, the Cd-resistant phenotype also displayed a decreased level of tropoelastin (27% of the CdS control), as illustrated by Western blot analysis (Fig. 4B), and produced a very low level of insoluble...
elastin amounting to 2% of the CdS control as determined by amino acid analysis of hot–alkali-resistant protein (Fig. 4C). These results showed that in parallel to inhibition of LO, CdR cells exhibited downregulation of collagen type I and elastin, substrates of LO.

Effects of Addition of Exogenous Cu on LO Expression in CdS and CdR Cells

The deficiency of LO in CdR cells may result from the lack of availability of Cu, a cofactor of LO, due to increased cellular thiols in these cells. To assess this possibility, enzyme activities were examined in growth-arrested CdS and CdR cells incubated in the presence or absence of various doses of CuCl₂. As shown (Fig. 5A), a 16-h incubation of CdS cells with 10 μM Cu induced a maximal LO activity level in the conditioned medium, reaching 142% of the basal level. Higher doses (>30 μM) of Cu decreased enzyme activity, likely due to toxicity under these conditions. Notably, exposure of CdR cells to Cu at 30, 100, and 300 μM also elevated LO activity levels to 381%, 882%, and 521% of the basal level, respectively, although low doses of Cu did not alter the depressed levels of LO catalysis in these cells. The lack of effects on LO activity at low doses of Cu in CdR cells may reflect the titration of the elevated level of metal binding sites in the markedly increased cellular MT and GSH to which the added Cu ions were liganded until the saturation level of binding was approached. It is also important to note that the peak increase in LO levels of CdR cells treated with 100 μM Cu (i.e., 882% of the basal level) reached 88 ± 6% of the CdS-control without Cu treatment (100 ± 8%). Furthermore, Northern blot (Fig. 5B) showed that a 16 h-incubation with Cu also enhanced steady-state LO mRNA levels in both CdS and CdR cells. Consistent with prior observations, two LO transcripts were seen in the blot of CdS cells incubated in the absence of Cu (lane 1) at approximately 5.8 and 4.5 kb, presumably due to alternative polyadenylation of the transcript (Li et al., 1995). In contrast, CdR cells without Cu treatment (lane 6) exhibited only a 4.5-kb LO mRNA species, and the total mRNA level in these cells was decreased to 23% of the CdS control (lane 1). Thus, downregulation of LO in CdR cells was evident not only at catalysis and protein levels (Figs. 2A and 2B) but also at the transcriptional level (compare lane 6 with lane 1 in Figure 5B). The lower MW species (4.5 kb) of LO mRNA was apparently more sensitive to Cu treatment in CdR cells. For example, 100 and 300 μM Cu enhanced mRNA expression in these cells, reaching 2.9- and 2.2-fold of the basal level. In addition, the immunoprecipitation assay demonstrated that Cu ions stimulated LO expression at the protein level in both CdS and CdR cells (Fig. 5C). For example, 10 μM Cu increased the level of the 50 kDa species in CdS cells to 129% of the control without Cu treatment. Notably, more 32 kDa mature LO was produced by and associated with CdR cells exposed to 100 μM Cu, reaching 63% of the CdS basal level. In sharp contrast, no detectable 32 kDa mature enzyme was associated with CdR cells without Cu treatment (Fig. 5C). These results indicated that addition of exogenous Cu at least in part restored the LO expression at catalytic activity, protein, and mRNA levels in the CdR phenotype.

FIG. 3. Lysyl oxidase localization in CdS and CdR cells. Growth-arrested cells on coverslips were double stained for visualization of LO and ER in the same cell using a purified rabbit anti-LO antibody and a secondary antibody coupled with rhodamine (red color), and a fluorescent ER-labeling dye [DiOC₆(3)] (green color). A. Lysyl oxidase staining in CdS cells. B. Endoplasmic reticulum staining in CdS cells. C. Lysyl oxidase staining in CdR cells. D. Endoplasmic reticulum staining in CdR cells.
Modulation of Collagen Synthesis by Exogenously Added LO or Cu in CdS and CdR Cells

Lysyl oxidase has a potentially regulatory effect on its substrate expression (Diegelmann and Peterkofsky, 1972; Jackson et al., 1991). To assess the role of LO restoration in its substrate production, we examined 14C-proline–labeled collagen synthesis (Diegelmann and Peterkofsky, 1972; Derdak et al., 1992) in CdS and CdR cells incubated in the presence of ascorbic acid without or with purified LO or Cu. As shown...
(Fig. 6A), exogenously added LO (purified from bovine aorta) at 10 and 30 nM induced dose-dependent increases of collagenase-released radioactivity of 14C-proline–labeled proteins, amounting to 203% and 238% of the basal level in CdS cells and 264% and 327% of the basal level in CdR cells, respectively. Notably, such increases in collagen synthesis by exogenous LO were fully inhibited by 100 μM BAPN, indicating that regulatory effects of this enzyme on its substrate production occurred at the catalytic level, requiring active LO. Furthermore, Figure 6B shows that Cu stimulated collagen synthesis in both CdS and CdR cells despite marked difference in doses, as seen in its effects on LO expression in these cells (Fig. 5). The maximum increase amounting to 205% of the basal level occurred at 10 μM Cu in CdS cells, whereas the significant increases reaching 295% and 355% of the basal level in CdR cells required 30 and 100 μM Cu, respectively. Importantly, collagen synthesis in CdS and CdR cells incubated with Cu was dependent in large part on Cu-enhanced LO activity because BAPN significantly reduced the elevation in collagen synthesis elicited by this metal ion. Thus, LO deficiency due to the limitation of Cu availability plays a critical role in downregulation of its substrates in CdR cells.

**DISCUSSION**

In this article, we report the phenotype conversion of rat fetal lung fibroblasts (RFL6) from CdS to CdR after long-term Cd exposure accompanied by elevated levels of cellular MT and GSH, two metal-scavenging agents, but reduced levels of LO, a Cu-dependent enzyme essential for the ECM crosslinking, as well as collagen and elastin, substrates of LO and major components of the lung ECM. CdR cells expressed an apparent 52 kDa variant of LO in the cell extract, but it was not secreted. The expression of LO was, at least in part, restored at mRNA, protein, and catalysis levels in CdR cells by incubation with exogenously added Cu, a cofactor of LO. Furthermore, collagen synthesis was recovered in these cells by exposure to purified LO or Cu. These results suggest the critical role of LO and Cu homeostasis in stabilization of the lung ECM, directly pointing to the molecular mechanisms for Cd damage to the lung relevant to the pathogenesis of Cd-induced emphysema.

Cadmium is a toxic metal and a health concern. The lung is a major Cd target organ. Injury of the lung in humans by Cd are mainly manifest as emphysema (Coultas and Samet, 1989; Davison et al., 1988; Kazantzis and Blacks, 1992). The critical role of LO in the pathogenesis of emphysema was supported by evidence that inhibition of LO by BAPN or by a Cu-deficient diet induced the disruption of the lung ECM in experimental animals resembling emphysematous lesions in human (Dubic et al., 1985; Harris, 1986). Repeated inhalation of Cd in rats resulted in a biphasic response of lung LO synthesis, i.e., a elevation at the early phase, followed by diminution at the late phase. Reduced LO at the late phase correlated with marked increases of MT (Sampson et al., 1984). To further investigate molecular mechanisms of Cd damage to the lung ECM, stimulated by the implications of these findings, we developed Cd-resistant sublines from Swiss 3T3 fibroblasts, as reported previously (Li et al., 1995), and rat fetal lung fibroblasts, as described in this article, by long-term exposure of parental cells to increasing concentrations of Cd. Both CdR cell types exhibited markedly decreased levels of LO coupled with strongly enhanced levels of cellular thiols closely similar in pattern to alterations in the lung of rats chronically exposed to Cd (Sampson et al., 1984). It should be noted that the parental RFL6 (CdS) cells are an ideal model for the lung ECM study,
because these cells displayed key characteristics of the pulmonary fibroblasts expressing collagen type I, tropoelastin, and LO (Absher, 1989; Campagnone et al., 1987), unlike the Swiss 3T3 cells, which produced collagen and LO only, without elastin production (data not shown). This is a major reason to develop this new CdR-RFL6 cell model for our Cd-lung toxicity research. The pulmonary ECM is composed of approximately 60–65% collagen. The major portion of interstitial collagens consists of type I and type III, with the ratio 3–6:1 in the normal lung (Absher, 1989; Tremblay et al., 1995). In addition to collagens, another essential component of the lung ECM is the elastin fiber, which is involved in the pulmonary mechanical action during breathing and also in the mechanism of emphysema pathogenesis (Campagnone et al., 1987; Tremblay et al., 1995). Tropoelastin expression and insoluble elastin production are unique properties of pulmonary fibroblasts with which an in vitro model of emphysema has been established (Foster et al., 1990). Emphysema is a chronic pulmonary disease manifested by dilatation of the alveolar spaces, disruption of the alveolar walls, and loss of lung elasticity. Among these alterations, destruction of the alveolar walls is a key pathological event that results in enlargement of the air sacs and weakening of the lung’s ability to stretch and recoil. Different mechanisms for disruption of the alveolar walls have been reported, such as the elastase–antielastase imbalance and apoptosis of interstitial cells, e.g., fibroblasts, inhibition of LO, etc. (Carnevali et al., 2003; Snider et al., 1986). Notably, unpublished data from our animal studies indicated that elevated levels of MT coupled with decreased LO activity and collagen synthesis were observed in the emphysematous lung tissue of rats intratracheally instilled with Cd for 6 weeks (30 μg CdCl₂ in 100 μl physiological saline once a week), resembling closely the phenotypes of CdR cells. Thus, the alterations in LO, collagen, and elastin in the CdR phenotype as shown here suggest that CdR-RFL6 cells provide a valuable cell model for investigation of Cd emphysema and the lung ECM injury by long-term exposure to Cd.

Cadmium damages LO at different levels from the gene transcription and translation to the catalytic activity. Northern blot analysis showed that CdR cells expressed a very low level of steady-state mRNA (Fig. 5B). This may result from inhibition of nascent mRNA synthesis and reduction of mature mRNA stability (Parent et al., 2004). The data published from this lab have shown that Cd-containing cigarette smoke condensate inhibited initiation of LO transcription and enhanced LO mRNA instability, collectively leading to down-regulation of LO mRNA (Gao et al., 2005). Cadmium has been reported to inhibit activities of various transcription factors such as SP1, hypoxia-inducible factor-1 (HIF1), etc. (Gong et al., 2000; Chun et al., 2000). These corresponding cis-elements have been found in the rat LO promoter region (Gao et al., 2005).

Lysyl oxidase is synthesized by cultured fibroblasts as a 46 kDa preproenzyme with N-terminal signal sequences which direct nascent proteins into the lumen of the rough ER. The proprotein resulting from cleavage of the N-terminal signal peptide undergoes N-linked glycosylation in the ER and Golgi and is then secreted as a 50 kDa proenzyme. In the ECM, the proLO is further proteolyzed into the mature 32 kDa species by proteinases (Kagan and Li, 2003). At the protein level, the major difference between CdR and parental RFL6 cells is the presence of the variant 52 kDa LO precursor in the former (Fig. 2B). The 52 kDa protein existed in the cell extract fraction but not in the conditioned medium, reflecting deficiency of secretion. As revealed by double immunofluorescent staining, a number of large LO-positive patches colocalized with the ER structure in CdR cells (Fig. 3), indicating an abnormal processing of LO precursor with its product accumulated in this organelle. Because this variant LO precursor is apparently known to be a total of 20 amino acids, i.e., Met-Ala-Phe-Ala-Trp-Thr-Val-Leu-Phe-Leu-Gly-Gln-Leu-Gln-Phe-Cys-Pro-Leu-Leu-Arg (Trackman et al., 1990). Another possibility to account for the variant LO proenzyme with a higher molecular weight in CdR cells may be a different degree of N-glycosylation (Trackman et al., 1990). It should be noted that the same 52 kDa protein recognized by the anti-LO antibody was also found in CdR-3T3 cells derived from Swiss 3T3 fibroblasts by means of the same Cd exposure regimen (Li et al., 1995). These results suggest that the variant proenzyme derived from abnormal LO processing at translational and post-translational levels may represent an important characteristic of long-term Cd insult to LO.

It is well established that LO is a metalloenzyme requiring 1 mole of Cu at its active site per mole of enzyme (Kagan and Li, 2003). Histidine-containing sequences provide the Cu-binding motifs for LO. Because unprocessed LO precursor appears to bind Cu (Kosonen et al., 1997), the ER and the Golgi apparatus in the secreted pathway presumably are potential organelles for incorporation of Cu into LO. Dietary deprivation of Cu reduced LO activity, resulting in lathyritic injuries in experimental animals (Dubic et al., 1985; Harris, 1986). Moreover, the deficiency in Cu transport found in the Menkes syndrome, a genetic disorder, was associated with downregulation of LO (Harris, 1993). Thus, Cu homeostasis is closely implicated in the LO functional integrity. It should be noted that inhibition of the LO expression was coupled with enhancement of cellular MT and GSH, two metal-scavenging agents, in the CdR phenotype (Fig. 1B). Studies in this lab have illustrated that the LO expression was inhibited in RFL6 cells, either by exogenously added MT or by increase of cellular GSH by using glutathione monoethyl ester (GME), a GSH delivery system, in culture (Chen et al., 2005). The thiol groups provide the metal binding sites of MT and GSH, exhibiting higher affinity for Cu than Cd (Kagi and Schaffer, 1988; Meister, 1984). Thus, Cd-induced upregulation of MT and GSH is expected to limit Cu bioavailability for LO, subsequently inducing
downregulation of LO. This expectation was supported in this study by the fact that exogenously added Cu in CdR cell cultures restored, at least in part, the LO expression not only at the catalytic level but also at mRNA and protein levels (Fig. 5).

Binding to apo-LO with supplemental Cu confers the enzyme catalytic activity, possibly by orientation of the amine substrate with the quinone cofactor of LO (Kagan and Li, 2003). However, the mechanisms for Cu enhanced transcription of LO remain to be understood. This metal ion may stimulate LO promoter activity, thus upregulating LO mRNA. Recent studies have shown Cu-inducible transcription of several genes via pathways for activation of metal and antioxidant response elements (MRE and ARE) (Mattie and Freedman, 2004). These cis-elements (MRE and ARE) are present in the promoter region of the rat LO gene (Gao et al., 2005). In addition, the restoration of the LO expression in CdR cells by incubation with Cu also indicated that downregulation of LO in these cells is not due to a selective cell isolation process in which LO-expressing cells were killed while those intrinsically deficient in the LO gene were retained. There is clear evidence for the presence of functional genes of LO in CdR cells, as is true in its parental CdS cells.

Our studies demonstrated downregulation of collagen type I and elastin (Fig. 4) in association with a marked reduction of LO at protein, mRNA, and catalytic levels (Fig. 2 and 5B) in CdR cells. These results are consistent with other reports in which Cd was shown to selectively inhibit procollagen production in lung fibroblasts (Chambers et al., 1994) and dietary Cd to suppress Cu-mediated synthesis of collagen and elastin in chicks (Hill et al., 1963). Notably, findings in this report further pointed out that decreased levels of LO due to abnormal Cu homeostasis as a result of upregulation of cellular thiols may be a key mechanism for inhibition of collagen and elastin biosynthesis in long-term Cd-exposed cells since exogenously added LO (purified from bovine aorta) or Cu restored collagen synthesis in the CdR phenotype to the normal basal level of the parental RFL6 control (Fig. 6). Lysyl oxidase regulation of its substrate expression is strongly supported by different studies in which administration of lathyrogens such as BAPN, an inhibitor of LO, or Cu-deficient diets in experimental animals resulted in inhibition of LO accompanied by the loss of the lung ECM, inducing emphysematous lesions (Dubic et al., 1985; Harris, 1986; Kuhn and Starcher, 1980). Treatment of rat neonatal aortic smooth muscle cells with BAPN induced the specific downregulation of tropoelastin mRNA levels (Jackson et al., 1991). Moreover, overexpression of LO in COS-7 cells enhanced promoter activity of human collagen III (Giampuzzi et al., 2000). Inhibition of crosslinking, destabilization and eventual degradation of substrates due to the deficiency of LO in CdR cells may trigger a feedback regulation mechanism to inhibit their own synthesis (Diegelmann and Peterkofsky, 1972; Jackson et al., 1991) further aggravating ECM damages.

The regulatory effect of LO on collagen synthesis could be mediated by direct modification of membrane-bound substrate because the interaction of collagen with its receptors, i.e., integrins on the cell surface, initiates a major pathway for transduction of extracellular signals into the cells, which can, for example, activate feedback regulation of collagen synthesis (Ivarsson et al., 1993; Riikonen et al., 1995). Recent studies by us and others have confirmed nuclear localization of LO (Giampuzzi et al., 2000; Li et al., 1997). Exogenous LO was internalized by cells and further transported into the nucleus (Nellaiappan et al., 2000). Enhancement of collagen promoter activity by intracellular LO as reported (Giampuzzi et al., 2000) may represent an alternative mechanism for regulation of collagen synthesis by this enzyme.

In brief, our studies indicate that long-term Cd exposure induced inhibition of Cu-dependent LO and its substrates, by upregulation of Cu scavenging thiols in pulmonary fibroblasts. Downregulation of LO resulted from limited Cu bioavailability as a central event contributing to Cd-induced ECM damage. Restoration of LO levels in Cd-insulted lung cells or tissues by replenishing Cu may have a protective or therapeutic effect on Cd-induced emphysema.

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REFERENCES


