Heme oxygenase-1-mediated autophagy protects against pulmonary endothelial cell death and development of emphysema in cadmium-treated mice

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Surolia R, Karki S, Kim H, Yu Z, Kulkarni T, Mirov SB, Carter AB, Rowe SM, Matalon S, Thannickal VJ, Agarwal A, Antony VB. Heme oxygenase-1-mediated autophagy protects against pulmonary endothelial cell death and development of emphysema in cadmium-treated mice. Am J Physiol Lung Cell Mol Physiol 309: L280–L292, 2015. First published June 12, 2015; doi:10.1152/ajplung.00097.2015.—Pulmonary exposure to cadmium, a major component of cigarette smoke, has a dramatic impact on lung function and the development of emphysema. Cigarette smoke exposure induces heme oxygenase-1 (HO-1), a cytoprotective enzyme. In this study, we employed a truncated mouse model of emphysema by intratracheal instillation of cadmium (CdCl2) solution (0.025% per 1 mg/kg body wt) in HO-1+/−, HO-1−/−, and overexpressing humanized HO-1 bacterial artificial chromosome (hHO-1BAC) mice. We evaluated the role of HO-1 in cadmium-induced emphysema in mice by analyzing histopathology, micro-computed tomography scans, and lung function tests. CdCl2-exposed HO-1−/− mice exhibited more severe emphysema compared with HO-1+/+ or hHO-1BAC mice. Loss of pulmonary endothelial cells (PECs) from the alveolar capillary membrane is recognized to be a target of emphysema. PECs from HO-1+/−, HO-1−/−, and hHO-1BAC were employed to define the underlying molecular mechanism for the protection from emphysema by HO-1. Electron microscopy, expression of autophagic markers (microtubule-associated protein 1B-light chain 3 II, autophagy protein 5, and Beclin1) and apoptotic marker (cleaved caspase 3) suggested induction of autophagy and apoptosis in PECs after CdCl2 treatment. CdCl2-treated HO-1−/− PECs exhibited downregulation of autophagic markers and significantly increased cleaved caspase 3 expression and activity (~4-fold higher). Moreover, hHO-1BAC PECs demonstrated upregulated autophagy and absence of cleaved caspase 3 expression or activity. Pretreatment of HO-1+/+ PECs with rapamycin induced autophagy and resulted in reduced cell death upon cadmium treatment. Induction of autophagy following CdCl2 treatment was found to be protective from apoptotic cell death. HO-1 induced protective autophagy in PECs and mitigated cadmium-induced emphysema.

CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) is a chronic progressive lung disease with major global health impacts and is one of the leading causes of morbidity and mortality in the world (1). It is now ranked as the third highest cause of death in the USA (29), with a prevalence of 9–10% among adults aged >40 yr (16). Pulmonary emphysema is a clinical phenotype of COPD and is characterized by irreversible enlargement of airspaces distal to the terminal bronchiole, accompanied by irreversible loss of alveolar structures. In humans, cigarette smoke (CS) exposure is the most commonly identified risk factor for the development of COPD. Each cigarette is reported to contain about 2 μg of cadmium among other toxic entities (3).

Cadmium is an important toxic heavy metal released into the environment from mining, coal-fired power plants, metallurgy, and plastic industries and remains an important contaminant in our ecosystem. Although CS is the main source for cadmium toxicity in humans, other sources such as water, food, and air contamination also serve as modes of exposure. Cadmium has an extended half-life of ~15 yr in humans (22). It accumulates in tissues, primarily in kidney, liver, and lungs, and contributes to the pathogenesis of a variety of disorders, including but not limited to COPD (18, 65). This includes the role of cadmium as one causative agent of acquired Cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction from smoking (51), which may contribute to the pathogenesis of COPD (49, 63).

Chronic CS exposure results in severe lung injury by initiating a complex inflammatory cascade (46, 64). There is activation and influx of inflammatory cells with seclusion of disease-specific mediators such as cytokines and growth factors (21). Neutrophils and macrophages release proteolytic enzymes and generate oxidants, resulting in tissue damage (69). Heme oxygenase-1 (HO-1), a cytoprotective enzyme, is a major inducible stress protein and a key regulator of several important biological processes. HO-1 and its products released from its interactions have antioxidant, anti-inflammatory, and...
antiapoptotic properties (61). Multiple studies have demonstrated that HO-1 responses are impaired during the development of COPD and contribute to the etiology of this disease (7, 37, 62, 75).

Endothelial cells are a critical component of the blood gas barrier and are essential for alveolar gas exchange. Loss of integrity of the alveolar capillary membrane has been shown to contribute to the development of emphysema (25, 26, 54). Importantly, loss of endothelial cells by programmed cell death leads to structural damage and development of anatomic defects in this membrane (41). Autophagy is a highly regulated cellular response of stress signaling and homeostasis. It has been recognized as an important moderator of fundamental cellular processes such as redox balance, inflammation, proliferation, and apoptosis in lung diseases (38, 43, 44). Elevated levels of autophagy have been shown in epithelial cells in patients with COPD compared with healthy individuals (42–44). An imbalance of protease/antiprotease molecules, traditionally regarded as the prime factor in the pathogenesis of emphysema, can induce apoptosis in endothelial cells (2, 41).

It is unclear whether autophagy enhances or mitigates apoptosis and cell death (43, 45, 53). In this study, we demonstrate that cadmium induces emphysema in mice through apoptosis of pulmonary endothelial cells (PECs). HO-1 overexpression protects mice from the development of emphysema by induction of cellular autophagy with decreased and delayed apoptosis, thus preserving the integrity of the alveolar capillary membrane and suggesting a potential therapeutic target.

MATERIALS AND METHODS

Chemical and reagents. Cadmium chloride (catalog no. 529575) was purchased from Sigma-Aldrich.

Experimental animals and treatment. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham. HO-1−/− and HO-1+/+ mice [8 to 12 wk of age, (C57BL/6xFVB)Fn] were used for both in vitro and in vivo studies. The humanized HO-1 bacterial artificial chromosome (hHO-1BAC) mice are transgenic mice harboring the human HO-1 gene. An 87-Kb human BAC containing DNA and three regulatory regions were bred to HO-1−/− to generate hHO-1BAC transgenic mice. Further details are described elsewhere (28). These mice overexpress human HO-1 RNA and protein. The human HO-1 gene has a specific transcriptional internal enhancer conjugated to the HO-1 promoter, which recapitulates steady-state level expression of HO-1 by cadmium exposure (20). Littermates of the HO-1−/− and hHO-1BAC mice were used as controls. Test mice received 1 mg/kg body wt of 0.025% cadmium chloride (CdCl2) in sterile saline intratracheally, whereas control mice received sterile saline. The mice were then subjected to microcomputed tomography (micro-CT) imaging on postadministration day 21. The animals were then killed at different time points, and lungs were harvested for histology.

Bronchoalveolar lavage preparation and characterization of inflammatory response. The lungs were lavaged by using a 24-gauge cannula (Insyte-N Autoguard, catalog no. 381411; BD Biosciences) inserted into the trachea, and the lungs were washed with 2×0.75 ml aliquots of cold, sterile 1× PBS. The recovery of lavage was 80–90% of the total volume. The lavage fluid was pooled and centrifuged at 400 g for 5 min at 4°C. Bronchoalveolar lavage fluid (BALF) was used for estimation of permeability, as protein leaked into the lungs and inflammatory cells were counted using a differential count based on the morphology. For cytospin analysis, cells were spun down at 400 g and resuspended in RPMI 1640 medium containing 10% (vol/vol) FBS. Total cell counts were determined by hemocytometer.

Micro-CT imaging and calculation of total lung volume. To study the progression of cadmium-induced emphysema and changes in lung volume, the experimental mice were subjected to micro-CT imaging. Control and the test animals were imaged under anesthesia in supine position at 21 days. The raw files were used to create 3D models of lung, and calculation of lung volume was done using Image J software.

Histology and lung morphometry. Lung morphometry was performed following previously described methods (76). Briefly, mice lungs were inflated with 0.5% low-melting agarose at a constant pressure of 25 cmH2O, fixed in 10% formalin for 48 h, and paraffin embedded by standard techniques. Sections (5 μm) were stained with hematoxylin and eosin (H and E). Images were acquired with a Carl Zeiss AxioCam color camera (Carl Zeiss Vision) and analyzed using AxioVision LE Imaging System software (Carl Zeiss Vision). Ten random lung fields per tissue section were captured at a ×100 magnification, and then AxioVision H Imaging System software was used to measure the mean alveolar airspace areas (MAAs) in pixels per square millimeter.

Respiratory mechanics. Mice were mechanically ventilated and challenged with increasing concentrations of methacholine, as described elsewhere (19). Briefly, mice were anesthetized with diazepam (17.5 mg/kg) and ketamine (450 mg/kg), intubated and connected to a ventilator (flexiVent, SCIREQ), and ventilated at a rate of 160 breaths/min at a tidal volume of 0.2 ml, with a positive end-expiratory pressure of 3 cmH2O. Total compliance was recorded as described previously (19). Increasing concentrations of 0–40 mg/ml methacholine chloride (Sigma-Aldrich) were administered via aerosolization within an administration time of 10 s. Airway responsiveness was recorded every 15 s for 3 min, after each aerosol challenge.

Immunoblotting and antibodies. Cells were collected from 35-mm culture dishes by scraping and quantitated by hemocytometer counting and solubilized in sample loading buffer (93.75 mM Tris-HCl, pH 6.8, 3% SDS, 15% glycerol, and 0.015% bromophenol blue) supplemented with 5% DTT. Samples were sonicated on ice in 10-s bursts twice at 50 W and heated to 95°C for 5 min, and proteins were separated by SDS/PAGE and transferred to a nitrocellulose membrane. Immunoblotting of transferred samples was performed using incubations with anti-β-actin (dilution 1:2,000, Sigma), anti-microtubule-associated protein 1B-light chain 3 (LC3B) (dilution 1:1,000, Cell Signaling), anti-LC3 II antibody (dilution 1:1,000, EMD Millipore), anti-autophagy protein 5 (ATG5) (dilution 1:1,000, Millipore), anti-Beclin1 (dilution 1:1,000, Cell Signaling), anti-cleaved caspase 3 (dilution 1:1,000, Cell Signaling), and horseradish peroxidase-conjugated secondary antibodies (dilution 1:5,000, Sigma) as previously described (72). Bands were visualized by enhanced chemiluminescence (Millipore).

Primary PEC isolation. Four to six mice (6–8 wk old) were used for isolation of PECs. Each mouse initially received a 25-μl intramuscular injection of heparin (1,000 USP U/ml). The mice were anesthetized (ketamine/xylazine, 140/14 mg/kg), followed by exposure of the thoracic cavity. Five milliliters of cold DMEM was then injected via the right ventricle to flush the lung of blood. One milliliter of collagenase A (1.5 mg/ml) was instilled through the trachea into the lungs, and the trachea was then tied off. The lungs were incubated with 5 ml of collagenase A in a 50-ml tube for 30 min in a 37°C water bath. The tube was gently agitated for a few seconds every 10 min during this incubation. PBS (20 ml) was added to the tube after 30-min incubation. The tube was then vigorously shaken for 30 s to dissolve the lung, and the resulting suspension was filtered through a 70-μm strainer. The filtered cell suspension was centrifuged for 4 min at 900 revolution/min. The cell pellet was washed with complete DMEM and then resuspended in 10 ml of complete DMEM and plated into a gelatin-coated T-75 tissue culture flask. The medium was changed to endothelial basal medium 2 (EBM2) (Lonza) after 24 h.
and the cells were cultured for an additional 1–2 days. Cells were washed with PBS and incubated with Alexa Fluor 647 rat anti-mouse CD31 (BD Pharmingen) for 1 h at room temperature in the dark. Cells were washed twice with PBS, trypsinized, and pooled together into one suspension for the fluorescence-activated cell sorting (FACS). The sorted cells were collected and plated in EBM2 medium. All experiments were performed within six to eight cell passages. In some experiments, PECs were plated at a density of 2 × 10^5 viable cells/well in six-well plates. PECs were treated with CdCl2 (10 μM) for 24 h, and chloroquine (CQ) 20 μM was added to the cells in the final 2 h before harvest. For autophagy induction experiments, PECs were treated with rapamycin (100 nM) for 24 h, and the media was replaced with or without CdCl2 (10 μM). Cells were then harvested for Western blot analysis.

Transmission electron microscopy. Transmission electron microscopy of primary PECs was performed in the High Resolution Imaging Shared Facility in University of Alabama at Birmingham. Cells grown on plastic dishes were rinsed in PBS and then fixed overnight at 4°C in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Cells harvested by scraping were pelleted by centrifugation and osmicated for 60 min at 4°C in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Following the wash with Na cacodylate buffer to remove osmium, samples were stained en bloc with 2% aqueous uranyl acetate for 30 min. Following dehydration in graded alcohol, pellets were embedded in Epon 812 and cured at 60°C overnight. Sections (80 nm thick) were generated with a Leica UC6 ultramicrotome (Leica Microsystems) and collected on 200 mesh copper grids, stained in 50% alcoholic uranyl acetate and Reynolds’s lead citrate. Air-dried grids of both fixed cell lines and clinical biopsy samples were subsequently examined on an FEI Tecnai-T12 electron microscope. Images were collected using a Hamamatsu CCD camera (Hamamatsu Photon).

DEVD-pNA cleaved caspase-3 activity assay. Caspase 3 proteolytic activity was measured using caspase-3 colorimetric protease assay kit (Invitrogen) according to the manufacturer’s instructions. Briefly, HO-1^+/+, HO-1^−/−, and hHO-1BAC PECs treated or untreated with CdCl2 were pelleted. To extract total protein, cell pellets were resuspended and lysed with 50 μl of cold lysis buffer and incubated on ice for 10 min. Cell lysates were then centrifuged at 10,000 g for 1 min at 4°C. The concentration of proteins was measured by BCA assay. 4 mM caspase-3 substrate (DEVD-pNA) was added to 200 μg protein from each sample and incubated at 37°C for 4 h. The p-NA light emission was quantified using ELISA plate reader at 405 nm. Comparison of the absorbance of p-NA from an apoptotic sample with an uninduced control allowed determination of the fold increase in caspase-3 activity.

Statistical analysis. Data are presented as means ± SE. The t-test was used for comparisons between two groups. For the comparisons that involved more than two groups, ANOVA and the Newman-Keuls test were used for analysis, with statistical significance considered at P < 0.05.

RESULTS

Cadmium-induced lung inflammation and emphysema. To study the progression of cadmium-induced lung injury and the development of emphysema, control and CdCl2-treated mice were euthanized on days 7, 15, or 21 following a single dose of CdCl2, and histological analyses were performed (Fig. 1A). H and E staining of the lungs of the wild-type mice treated with CdCl2 revealed an influx of inflammatory cells on day 7. The intensity of inflammation partially subsided by day 15, and H and E sections of the lungs demonstrated enlargement of alveolar spaces compared with controls. The development of centrilobular emphysema was evident by day 21. At that time, there was significant increase in the MAA (more than 2-fold, *P < 0.05) in the alveolar airspaces of CdCl2-treated mice lungs compared with control mice lungs (Fig. 1B).

We also evaluated the influx of inflammatory cells in the BALF of the mice at different time points. There was a time-dependent increase of the number of inflammatory cells in the BALF of CdCl2-treated mice between days 7 and 21 (Fig. 1C). At 21 days after exposure, a significant increase in the absolute number of neutrophils, macrophages, and eosinophils was evident in the BALF of CdCl2-treated mice compared with saline-treated littermate controls; here, *P < 0.05, **P < 0.005, control vs. CdCl2-treated mice (Fig. 1D). We also evaluated protein leak as an indicator of increased permeability of lungs in control and CdCl2-treated mice. We found increase in the protein concentration in the BALF of CdCl2-treated mice on days 7, 15, and 21 (Fig. 1E). The observed increase in MAA and the number of neutrophils in BALF of CdCl2-treated mice suggest the development of airspace enlargement and persistent inflammation.

HO-1^−/− mice demonstrated increased susceptibility to cadmium-induced emphysema, whereas hHO-1BAC were protected. Anti-inflammatory interventions have been shown to be beneficial in mice exposed to CS (9). Because we postulated that cadmium would initiate lung toxicity through induction of oxidative stress (4–6), we evaluated the role of HO-1 as a key to protect against cadmium-induced emphysema. We employed HO-1^+/+, HO-1^−/−, and hHO-1BAC mice that overexpress HO-1 to test this hypothesis (Fig. 2, A–F). Analysis of lung tissue sections of saline-treated HO-1^+/+, HO-1^−/−, and hHO-1BAC mice (controls) suggested that these mice did not develop emphysema spontaneously (Fig. 2, A–C). Upon CdCl2 treatment, HO-1^−/− mice showed significant enlargement of alveolar area space (Fig. 2E) compared with HO-1^+/+ mice (Fig. 2D), whereas the hHO-1BAC mice had essentially normal lung architecture (Fig. 2F). We measured MAA in these mice and found that HO-1^−/− mice had a significant increase in MAA compared with HO-1^+/+ (2.5-fold increase, *P < 0.05) and with hHO-1BAC mice (7-fold increase, **P < 0.005). MAA in hHO-1BAC mice was similar to mice treated with saline (Fig. 2G). Total protein in BALF from HO-1^−/− mice was higher than that of HO-1^+/+ (⁎P < 0.05) and hHO-1BAC (⁎P < 0.05) 21 days after CdCl2 treatment (Fig. 2H). Also, the infiltration of inflammatory cells shifted from a predominance of macrophages to neutrophils in the BALF of HO-1^−/− mice compared with HO-1^+/+ (Fig. 2J) and hHO-1BAC mice (Fig. 2J). The number of neutrophils was significantly increased in HO-1^−/− mice, whereas no significant increase was noted in hHO-1BAC CdCl2-treated mice compared with their respective saline-treated littermate controls (Fig. 2, I and J). Collectively, these data demonstrate that cadmium-induced emphysema is potentiated by lack of HO-1 and mitigated by HO-1 overexpression in vivo.

Quantitative and qualitative assessment of aerated lung volumes by in vivo micro-CT imaging and flexiVent analysis. To assess the qualitative and quantitative assessment of aerated lung volumes, we performed micro-CT imaging and lung function dynamics after cadmium treatment. All tomograms obtained in vivo from HO-1^+/+, HO-1^−/−, and hHO-1BAC mice 21 days following CdCl2 treatment presented distinguishable emphysema compared with their respective controls (saline-treated mice) (Fig. 3A). Micro-CT of HO-1^+/+CdCl2-

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treated mice demonstrated enlargement of airspaces, whereas HO-1−/− mice lungs exhibited bullous emphysema. CdCl2-treated hHO-1BAC mice lungs had less enlargement of air space. In addition to the qualitative visual data, calculated 3D parameters of the in vivo micro-CT images resulted in a quantitative volume output, expressed as the change in total voxel number (Fig. 3B). CdCl2-treated HO-1−/− mice showed highest lung volume compared with CdCl2-treated HO-1+/+ and hHO-1BAC mice (Fig. 3B). We next tested the lung function of mice administered saline and CdCl2 at different time points. Pressure-volume (PV) loops were measured in anesthetized mice. Figure 3 shows the average quasistatic PV loop of HO-1+/+, HO-1−/−, and hHO-1BAC animals. Consistent with micro-CT observations, CdCl2-treated mice always showed an upward and leftward shift in the PV relationship compared with saline-treated HO-1+/+ and HO-1−/− mice demonstrating reduced elastic recoil (Fig. 3C). Interestingly, hHO-1BAC mice demonstrated no significant shift in PV loop compared with saline-treated hHO-1BAC mice (Fig. 3C). Collectively, qualitative and quantitative assessment of aerated lungs demonstrated that HO-1 overexpression inhibited cadmium-induced emphysema, whereas lack of HO-1 exacerbated the effect.

Cadmium-induced autophagy in PECs. Maintenance of alveolar structure is dependent on the integrity of the epithelial and capillary membranes. The capillary membrane is constituted predominantly of alveolar endothelial cells. Endothelial cell injury leads to alterations and loss of integrity of the alveolar capillary membrane. Initially, we investigated the effect of cadmium treatment in mice lung morphology and function. Furthermore, we checked expression of proteins considered to be reliable autophagy markers to assess the status of autophagy following cadmium treatment. Increased levels of Beclin1 were found in protein lysates of mice lung on posttreatment days 7, 12, 14, 18, and 21. LC3B II expression was also upregulated on days 7 and 12 after CdCl2 treatment. LC3B II is one of the...
autophagy proteins that specifically interacts only with the autophagic vesicles and remains associated until vesicle breakdown; here, *P < 0.01, **P < 0.05, day 0 vs. posttreatment days 7, 12, 14, 18, and 21 (Fig. 4, A–C). Immunofluorescence staining of lung tissue sections from saline-treated and CdCl2-treated mice (day 12) was performed for LC3B (red) and platelet endothelial cell adhesion molecule 1 (PECAM1)-positive PECs (green). Lung sections from CdCl2-treated mice demonstrated autophagy in PECs (white arrows). Saline-treated mice lung endothelial cells showed no autophagy (Fig. 4D). Quantification of PECAM1- and LC3B II-positive cells (red and green positive) demonstrated significantly increased autophagy in CdCl2-treated mice (*P < 0.001 compared with control, Fig. 4E).

We then isolated PECs from mice and exposed them to CdCl2 as described in MATERIALS AND METHODS. Immunofluorescence staining with CytoID autophagosome-specific stain demonstrated the presence of autophagosome (green puncta)-positive cells in the cytoplasm of CdCl2-treated cells (Fig. 4F). These labeled cells were used for FACS analysis, which confirmed the results with an increase in autophagosomes in CdCl2-treated cells compared with control (Fig. 4G). Transmission electron microscopy indicated the presence of newly formed vacuoles and mature vacuoles, suggesting increased autophagic flux. Untreated cells demonstrated no vacuolization in cytoplasm (Fig. 4H). Western blots of whole cell lysates from CdCl2-treated PECs confirmed the induction of autophagy-related proteins, namely LC3B, Beclin1, and ATG5. CdCl2 treatment also caused upregulation of HO-1 (Fig. 4I). To determine autophagic flux, we treated cells with CQ, which inhibits autophagy by raising lysosomal pH, which leads to inhibition of both fusion of autophagosome with lysosome and lysosomal protein degradation. Enhanced LC3B II formation was noted in CdCl2-treated cells compared with control (Fig. 4D). Western blots of whole cell lysates from CdCl2-treated mice confirmed the results with an increase in autophagosomes in CdCl2-treated cells compared with control (Fig. 4G). Transmission electron microscopy indicated the presence of newly formed vacuoles and mature vacuoles, suggesting increased autophagic flux. Untreated cells demonstrated no vacuolization in cytoplasm (Fig. 4H). Western blots of whole cell lysates from CdCl2-treated PECs confirmed the induction of autophagy-related proteins, namely LC3B, Beclin1, and ATG5. CdCl2 treatment also caused upregulation of HO-1 (Fig. 4I). To determine autophagic flux, we treated cells with CQ, which inhibits autophagy by raising lysosomal pH, which leads to inhibition of both fusion of autophagosome with lysosome and lysosomal protein degradation. Enhanced LC3B II formation was noted in CdCl2- and CQ-cotreated murine PECs (Fig. 4J), demonstrating significant increase in autophagic flux induced by CdCl2; *P < 0.05, PBS-treated control PECs vs. CQ- and CdCl2-treated PECs; #P < 0.05, CQ-treated PECs vs. CQ- and CdCl2-treated PECs; ¥P < 0.05, CdCl2-treated PECs vs. CQ- and CdCl2-treated PECs (Fig. 4K).
Cadmium induced more autophagy in PECs from hHO-1BAC mice compared with HO-1−/− mice. PECs isolated from HO-1−/− and hHO-1BAC mice were exposed to 10 μM CdCl₂ for 24 h and were analyzed for autophagy by electron microscopy. Vacuoles were observed in untreated PECs (control) from HO-1−/− mice, which demonstrated the presence of a basal level of autophagy. Electron micrograph of CdCl₂-treated HO-1−/− PECs demonstrated cytoplasm condensation (green arrow), organelle packaging (yellow arrow), shrinkage and membrane blabbing (red arrow), and chromatin condensation (white arrow) with the presence of few autophagic vacuoles. This suggested the presence of apoptotic cell death in HO-1−/− PECs after CdCl₂ treatment (Fig. 5A). In contrast, hHO-1BAC cells did not show basal autophagy but had increased autophagic vacuoles in the cytoplasm after CdCl₂ treatment (Fig. 5B). We also analyzed protein lysates for autophagic markers in CdCl₂-treated HO-1−/− and hHO-1BAC PECs at different time intervals up to 48 h. HO-1−/− PECs did not exhibit an increase above basal levels. Also, basal levels of LC3B II, ATG5, and Beclin1 were downregulated in HO-1−/− PEC (Fig. 5C). However, hHO-1BAC PECs demonstrated increased autophagy with upregulated Beclin1 and ATG5 levels. Increased LC3B II expression was also noted (Fig. 5C). During autophagy, soluble LC3B I is lipidated with the addition of phosphatidyl-ethanolamine to LC3B II that is an insoluble form. The LC3B I/LC3B II ratios were calculated by densitometry from Western blots for LC3B expression levels in CdCl₂-treated HO-1+/+ (Fig. 4I), HO-1−/−, and hHO-1BAC mice (Fig. 5C). Figure 5D demonstrates the time-dependent increase of autophagy in HO-1+/+ and hHO-1BAC PECs compared with HO-1−/− PECs at 24 and 48 h (**p < 0.01 and ***p < 0.05, respectively).

Cadmium-induced apoptosis in PECs from HO-1−/− and HO-1+/+ mice but not in hHO-1BAC mice. CdCl₂-treated HO-1−/+, HO-1−/−, and hHO-1BAC PECs were subjected to Western blot to determine cleaved caspase-3 expression levels. HO-1−/− cells treated with CdCl₂ had higher levels of cleaved caspase-3 compared with HO-1−/− endothelial cells, indicating increased apoptosis. The hHO-1BAC endothelial cells demonstrated low expression of cleaved caspase-3 at 48 h after CdCl₂ exposure (Fig. 6A). Caspase-3 activity was also measured via DEVD-pNA colorimetric assay, which showed an ~1.8-fold increase in the induction of cleaved caspase-3 functional activity compared with the untreated HO-1−/− controls after 24 h of incubation with CdCl₂. The caspase-3 activity in CdCl₂-treated HO-1−/+ PECs was increased approximately twofold (Fig. 6B). HO-1−/− PECs treated with cadmium show approximately fourfold higher caspase-3 activity than CdCl₂-treated HO-1+/+ PECs. hHO-1BAC PECs treated with CdCl₂ showed no significant caspase-3 activity compared with untreated hHO-1BAC cells (Fig. 6B). The immunofluorescence staining of lung sections from CdCl₂-treated HO-1+/+, HO-1−/−, and
Fig. 4. Cadmium induces autophagy in primary pulmonary endothelial cells (PECs) in mice. A: Western blot analysis of Beclin1, microtubule-associated protein 1 light chain-3 (LC3), and β-actin in whole lung lysates from CdCl2-treated mice on days 0, 7, 12, 14, 18, and 21. B and C: densitometries of Western blots of Beclin1 (B) and LC3B II (C) were analyzed by histogram after being normalized with β-actin. Here, *P < 0.01, **P < 0.05, day 0 vs. posttreatment days 7, 12, 14, 18, and 21. D: immunofluorescent staining of saline (control) and CdCl2-treated mice (killed on 14th day posttreatment) for platelet endothelial cell adhesion molecule 1 (PECAM1) (green) and LC3 (red). E: quantification of PECAM1- and LC3B II-positive cells in mice lung sections. **P < 0.001, control mice vs. CdCl2-treated mice. F: representative image of the punctated CytoID autophagosome stain in murine PECs treated with 10 μM CdCl2 for 24 h (magnification, ×40; scale bar = 100 μm). G: autophagy detection by CytoID Green (Enzo) labeling in saline-treated and CdCl2-treated murine PECs by fluorescence-activated cell sorting analysis. GFP, green fluorescence protein. H: formation of autophagic vacuoles in murine PECs treated with CdCl2 for 24 h as shown by transmission electron microscopy (magnification, ×1,100; scale bar = 2 μm) Black arrow shows immature autophagic vacuoles; red arrow shows degradative autophagic vacuole. J: cells were treated with CdCl2 for indicated time points and analyzed for ATG5, Beclin1, HO-1, and LC3B. β-Actin was used as loading control. Blots shown are representative of 3 independent experiments. J: autophagic flux in PECs after CdCl2 treatment in presence and absence of chloroquine (CQ), an inhibitor of autophagosome-lysosome fusion. Protein lysates were analyzed for LC3B. β-Actin was used as loading control. K: densitometry of Western blots of LC3B II was analyzed by histogram after being normalized with β-actin. Here, *P < 0.05, PBS-treated control PECs vs. CQ- and CdCl2-treated PECs; #P < 0.05, CQ-treated PECs vs. CQ- and CdCl2-treated PECs; ¥P < 0.05, CdCl2-treated PECs vs. CQ- and CdCl2-treated PECs.
hHO-1BAC mice demonstrated high expression of cleaved caspase-3 in CdCl2-treated HO-1\(^{-/-}\) lung sections compared with CdCl2-treated HO-1\(^{+/+}\) and hHO-1BAC mice (Fig. 6C). To determine whether the higher autophagy in hHO-1BAC caused less apoptosis in PECs, autophagy was induced using rapamycin in HO-1\(^{-/-}\) PECs before CdCl2 treatment. The phase-contrast microscopy demonstrated that PECs underwent appreciably more cell detachment upon exposure to CdCl2 than untreated or rapamycin-treated cells, whereas rapamycin pretreatment before CdCl2 exposure reduced the rate of cell detachment (Fig. 6D). To determine whether the observed cell detachment represented cell death via apoptosis, the cells lysates were analyzed for cleaved caspase-3 expressions. LC3B II levels were also determined. It is notable that the level of LC3 I remained almost unchanged when compared with only rapamycin-treated cells, but LC3B II expression was upregulated in CdCl2-treated and rapamycin-pretreated PECs exposed to CdCl2. The expression of cleaved caspase-3 was reduced in rapamycin-pretreated PECs exposed to CdCl2 when compared with only CdCl2-exposed PECs (Fig. 6E).

**DISCUSSION**

CS is mixture of over 5,000 chemicals (52), including cadmium, which accumulates in tissues including the lung, has an extended half-life of over 15 yr in humans, and is one of 20 potentially harmful CS constituents highlighted by the FDA (http://www.fda.gov/TobaccoProducts/GuidanceCompliance-RegulatoryInformation/default.htm) as potentially concerning. Smokers have up to an eightfold increase in lung cadmium levels compared with nonsmokers (47). The extended half-life of cadmium in humans may explain, in part, the persistence of inflammation and continued lung damage in subjects who stop smoking and have not smoked in several years (66). We established a simple truncated mouse model of emphysema using a single administration of cadmium that generated airspace enlargement within 21 days. We also demonstrated that HO-1, a cytoprotective, anti-inflammatory, and antiapoptotic protein, protected mice against cadmium-induced emphysema, whereas its absence exacerbated disease.

Cadmium-treated mice demonstrated a robust inflammatory response in the lungs compared with saline-treated animals, similar to previous models of emphysema. Cadmium-treated mice further demonstrated a protein leak into the lung as measured in BALF (74), similar to other studies (4, 11, 17). Infiltration of neutrophils with release of proteolytic enzymes including elastases and metalloproteinases in response to CS plays a pivotal role in progression of lung destruction in mice models of emphysema (11, 17, 58). Existence of a similar mechanism in patients with COPD has been reported (4, 58, 59). Although the influx of inflammatory cells decreased by day 7, inflammation persisted up to 21 days with development of airspace enlargement in cadmium-treated mice, compared with controls. Yamada et al. (74) demonstrated increased neutrophil influx with subsequent release of elastase via degranulation, using a beagle dog model of CdCl2-induced acute inflammation (34). Elastases, along with other proteinases, contribute to the proteolytic cleavage and remodeling of extracellular matrix, resulting in airspace enlargement (55, 59, 78). The widely used models of emphysema including exposure to CS take several months to demonstrate emphysematous lesions (32, 52). In comparison, our model uses a single dose of cadmium to demonstrate development of emphysema with histological analysis, CT imaging, and lung mechanics, 21 days posttreatment. These manifestations of COPD following a single dose of cadmium are similar to and consistent with those obtained from exposing mice with CS over a period of 6 mo (32, 55).
Cellular and molecular mechanism for the development and progression of emphysema can be attributed to injury followed by proteolysis, inflammation, or cell death (32). Several reports demonstrate increased numbers of apoptotic cells in the lungs of patients with COPD (44, 53). Interestingly, recent reports suggest that CS-induced autophagy can predate apoptosis in lung epithelial cells (8, 27, 53). The pathogenesis of CS-induced emphysema involves apoptosis triggered by oxidative stress (27), but the regulation of cell death pathways in response to cadmium exposure is incompletely understood. Exposure to prolonged low concentrations of cadmium generates a diverse range of cellular events such as inflammation, proliferation, and differentiation (31). Cadmium also induces autophagy in several cell types (14, 35, 72). Autophagy may help cell survival in cells damaged by toxic metabolites and intracellular pathogens by enabling breakdown of intracellular organelles for biosynthesis and energy metabolism (5, 12, 23, 67). However, autophagy may also promote type II programmed cell death through excessive self-digestion and degradation of essential cellular constituents (39). We demonstrate that cadmium exposure increased autophagy in whole lung tissue, as indicated by LC3B II B, Beclin1, and ATG5 upregulation. Immunofluorescence stain-

Fig. 6. Overexpression of HO-1 inhibits cadmium-induced caspase-3-mediated cell death. A: HO-1+/+ , HO-1−/−, and hHO-1BAC PECs were treated with 10 μM CdCl₂ for indicated time, and cell lysates were analyzed for cleaved caspase-3 levels. The blots were stripped and probed for β-actin to confirm loading. B: caspase-3 activity was measured using a DEVD-pNA calorimetric assay. After treatment with CdCl₂ for 4 h, PECs were lysed, and 50 μg of protein was incubated with DEVD-pNA for 2 h at 37°C. Absorbance measurements were taken at a wavelength of 405 nm, and the fold induction of caspase-3 activity relative to the untreated HO-1+/+ control was shown. *P < 0.05 and **P < 0.01 vs. control by a 1-way ANOVA with HSD test. #P < 0.05, CdCl₂-treated HO-1+/+ PECs vs. CdCl₂-treated HO-1−/− PECs. C: representative immunohistochemical staining for cleaved caspase-3 (green) and DNA (blue) in HO-1+/+ , HO-1−/−, and hHO-1BAC lung sections from mice treated with CdCl₂ (magnification, ×40; scale bar = 10 μm). D: phase-contrast microscopy of PEC cultures exposed with or without rapamycin (100 nM) followed by CdCl₂ (10 μM) treatment for 24 h. Rapamycin protected against CdCl₂-induced cell death in PECs. E: Western blot analysis of LC3 and cleaved caspase-3 of the PECs exposed with or without rapamycin (100 nM) followed by CdCl₂ (10 μM) treatment for 24 h. Blots shown are representative of 3 independent experiments.

to COPD pathogenesis by inducing chronic bronchitis (10, 15, 49, 63) although this cannot be readily assessed in mice because they are protected from bronchitis even following prolonged smoke exposure or complete knockout of the CFTR gene.

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ing of cadmium-treated mice lung sections demonstrated that PECs undergo autophagy. Previous studies have suggested that vascular endothelial cells treated with cadmium can undergo both autophagy and apoptosis or apoptosis alone (6, 14, 33). Cadmium-induced autophagy resulted in cell death in skin epidermal and mesangial cells (68, 72), but autophagy was protective in fibroblasts (35) and kidney cells (5). It has been recognized that cadmium exposure results in reactive oxygen species (ROS) generation and oxidative stress (23). Recent work has suggested that increased ROS is one of the cytotoxic mechanisms by which cadmium causes autophagy and cell death (67). A study in ROS-exposed human chondrocytes highlighted the role of autophagy as a critical protective mechanism against mitochondrial dysfunction (36). ROS-mediated autophagy has a protective role in radiation-induced bystander effect on hepatoma cells (73). Lim et al. (50) demonstrated that induction of autophagy interrupted apoptosis by counterbalancing endoplasmic reticulum stress after cadmium exposure in lung epithelial fibroblasts. Upon cadmium exposure in renal cells, autophagy is induced as a housekeeping process to get rid of damaged proteins (51).

The loss of endothelial cells might occur through the process of autophagy-mediated programmed cell death. According to the vascular hypothesis of CS-induced emphysema, endothelial cell death initiates disappearance of alveolar cells in emphysema (25, 34, 57). To address whether autophagy acts as a cell survival mechanism or cell death pathway, PECs were isolated from mice lung and exposed to cadmium in vitro. We showed that cadmium induces autophagy in PECs and subsequently assessed autophagy flux by treatment with both CQ and cadmium. Autophagic flux was accompanied by elevated levels of LC3 II (40, 56) compared with cells treated with CQ alone and CQ and cadmium both.

The lung elicits adaptive and protective mechanisms to inhibit injury. Induction of HO-1 is a key and prominent defense mechanism in this process (13). HO-1 is an antioxidant, anti-inflammatory, and cytoprotective enzyme; however, its role in COPD has not been extensively studied. Yamada et al. (75) reported that patients with COPD have tandem repeat polymorphisms in the HO-1 promoter region, which results in lower expression of HO-1 (75). In addition to this, alveolar macrophages from patients with COPD have been documented to have a lower expression of HO-1 compared with alveolar macrophages from patients without COPD (71). In vitro studies by Kim et al. (27) demonstrated that overexpression of HO-1 in BEAS-2B cells resulted in cell survival after CS exposure. Adenovirus-mediated HO-1 overexpression in mice lungs demonstrated protection in elastase-induced pulmonary emphysema (60). To gain a better understanding of the role of HO-1 in cadmium-induced emphysema, we employed HO-1−/− mice and mice that specifically overexpress human HO-1 gene (hHO-1BAC mice) (28). The human HO-1 gene has a specific transcriptional internal enhancer in conjugation with HO-1 promoter, which recapitulates the steady-state level expression of HO-1 by cadmium exposure (61).

HO-1 regulates both autophagy and apoptosis (26, 54). We sought to evaluate the role of HO-1 in cadmium-induced autophagy in PECs from HO-1−/− and hHO-1BAC mice. Electron micrographs of cadmium-treated HO-1−/− PECs demonstrated apoptotic cell morphology, whereas hHO-1BAC PECs showed presence of autophagic vacuoles, with no apoptotic cells. The expression of cleaved caspase-3 in HO-1−/− but not in hHO-1BAC PECs following cadmium exposure further strengthened the findings in electron micrographs. We thus postulated that overexpression of HO-1 activated autophagy upon cadmium exposure and resulted in a decreased susceptibility to apoptosis in hHO-1BAC PECs. Contrary to our results, Kim et al. (27) demonstrated that HO-1 inhibited autophagy in CS-exposed BEAS-2B cells (27). This discrepancy may be attributed to the morphological and physiological difference in the cell type used in our study.

The induction of autophagy following cadmium treatment may efficiently eliminate damaged proteins and cell organelles. Autophagic elimination of mitochondria and endoplasmic reticulum contributes to protection attributable to interruption of apoptosis (12). In a model of hyperoxia-induced lung injury, Zhang et al. (77) demonstrated that inhibition of autophagy led to apoptosis in endothelial cells in which HO-1 expression was abolished. Furthermore, a recent report suggested that induction of autophagy is protective in vascular endothelial cells exposed to oxygen-glucose deprivation (70). Also, a recent study showed that upregulation of autophagy by administration of aerosolized Trehalose decreased alveolar injury following exposure of mice to chlorine gas (24). Our data in PECs show that lack of HO-1 is associated with downregulation of autophagy with cadmium treatment and early onset of apoptosis.

High carbon monoxide (CO), a reaction product of HO-1, may also play an important role in autophagy induction and cell survival. Raval et al. (50) demonstrated that CO generation following exposure to CS extract results in upregulation of LC3B and inhibition of apoptosis. It has also been demonstrated that autophagy induces apoptosis resistance in endothelial cells after CS exposure (48). Our findings on PEC viability with autophagy inducer (rapamycin) also support our hypothesis that autophagy induced by cadmium (low concentration, 10 μM) is a survival mechanism.

Animal models have proven to be exceptionally useful to gain insight into the mechanisms of disease development. Whereas anatomic changes are an important feature of emphysema, these alterations also affect functional status. Snider et al. (65) demonstrated the development of lung emphysema by aerosolized CdCl2 inhalation, but they did not determine the effect of the observed morphometric changes on lung physiology. Therefore, we recapitulated and further characterized the cadmium-induced emphysema model. It was important to determine whether cadmium-associated emphysema recapitulated relevant changes in lung function seen in patients with COPD. Micro-CT provided topographical distribution and qualitative evaluation of the severity of cadmium-induced emphysema in aerated lungs of HO-1−/−, HO-1−/−, and hHO-1BAC mice by a noninvasive method. Micro-CT images exhibited enlarged air spaces, a hallmark of emphysematous lungs. PV curves are also in agreement with micro-CT demonstrating that the PV curve for HO-1+/− and HO-1−/− mice was shifted upward, whereas the cadmium-treated hHO-1BAC mice deflation PV curve was not significantly different from the saline-treated hHO-1BAC mice PV curves. A previous study by Shinohara et al. (60) demonstrated reduced levels of proinflammatory cytokines and suppressed inflammation attributable to HO-1 overexpression that mitigated progression of elastase-induced emphysema. Additionally, a recent report
demonstrated that oxidative stress-induced HO-1 activation leads to reduced neutrophil migration into the lung and decreased tissue destruction. This is consistent with our results demonstrating less neutrophil influx and tissue destruction in cadmium-treated hHO-1BAC mice (30).

In summary, we present a modified and truncated model of emphysema where administration of cadmium recapitulates CS-induced emphysema with mice developing airspace enlargement within 21 days. Unlike CS-induced emphysema in mice where inflammation decreases following cessation of CS exposure, a single dose of cadmium treatment induces persistent inflammation even though the mice are no longer exposed to cadmium, similar to continued inflammation in patients with COPD. Importantly, these data are the first to demonstrate the etiological and protective role of HO-1 in PECs in cadmium-induced lung emphysema by regulation of the balance between autophagy and apoptosis. Our data suggest that expression of HO-1 in the lung is a promising therapeutic target for intervention in preventing the development of emphysema.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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HEMENOGENASE-1 PROTECTS FROM CADMIUM-INDUCED EMPHYSEMA


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