Suppression of lung oxidative stress, inflammation and fibrosis following nitrogen mustard exposure by the selective farnesoid X receptor agonist obeticholic acid

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Abstract

Nitrogen mustard (NM) is a cytotoxic vesicant known that causes pulmonary injury that can progress to fibrosis. NM toxicity is associated with an influx of inflammatory macrophages in the lung. Farnesoid X Receptor (FXR) is a nuclear receptor involved in bile acid and lipid homeostasis that has anti-inflammatory activity. In these studies, we analyzed the effects of FXR activation on lung injury, oxidative stress and fibrosis induced by NM. Male Wistar rats were exposed to phosphate buffered saline (CTL) or NM (0.125mg/kg) by i.t. Penn-Century MicroSprayer® aerosolization; this was followed by treatment with the FXR synthetic agonist, obeticholic acid (OCA, 15mg/kg) or vehicle control (0.13-0.18g peanut butter), 2hr later, and then once/day, 5 days/week thereafter for 28d. NM caused histopathological changes in the lung including epithelial thickening, alveolar circularization, and pulmonary edema. Picrosirius Red staining and lung hydroxyproline content were increased indicative of fibrosis; foamy lipid laden macrophages were also identified in the lung. This was associated with aberrations in pulmonary function including increases in resistance and hysteresis. Following NM exposure, lung expression of HO-1 and iNOS, and the ratio of nitrate/nitrites in bronchoalveolar lavage fluid (BAL), markers of oxidative stress increased, along with BAL levels of inflammatory proteins, fibrinogen and sRAGE. Administration of OCA attenuated NM-induced histopathology, oxidative stress, inflammation and altered lung function. These findings demonstrate that FXR plays a role in limiting NM-induced lung injury and chronic disease, suggesting that activating FXR may represent an effective approach to limiting NM-induced toxicity.
Significance Statement

In these studies, the role of farnesoid-X-receptor (FXR) in mustard vesicant-induced pulmonary toxicity was analyzed using nitrogen mustard (NM) as a model. Our findings that administration of obeticholic acid, an FXR agonist, to rats reduces NM-induced pulmonary injury, oxidative stress, and fibrosis provide novel mechanistic insights into vesicant toxicity which may be useful in the development of efficacious therapeutics.
Introduction

Nitrogen mustard (NM) is a cytotoxic vesicant known to cause acute lung injury which can progress to chronic diseases including fibrosis, emphysema, and bronchiolitis obliterans (Balali-Mood and Hefazi 2005; Malaviya et al. 2016; Sunil et al. 2011). Like sulfur mustard (SM), a related bifunctional alkylating agent, NM was originally developed as a chemical warfare agent. Although not used in warfare, NM is still considered a high priority chemical threat agent, as it is relatively easy to synthesize and weaponize. Additionally, NM and its analogs are currently used to treat lymphoma and breast cancer (Diethelm-Varela et al. 2019; Potęga 2022). However, its efficacy in cancer chemotherapy is limited by lung toxicity. While NM and SM cause similar pathophysiological responses in the lung, SM is generally considered more potent. Currently, there are no FDA approved therapeutics to treat must lung pathology and disease. Thus, elucidating mechanisms mediating toxicity are essential for identifying targets for drug development (Sunil et al. 2011; Sunil et al. 2022; Venosa et al. 2016). In previous studies we developed a rat model of pulmonary exposure to NM to assess inflammatory mechanisms of toxicity. We found that a single intratracheal exposure of rats to NM caused progressive histopathological and inflammatory changes in the lung that resemble responses of humans to mustard vesicants (Beheshti et al. 2006). Moreover, by 28 days post-exposure in the rat model, pulmonary fibrosis is evident, a response associated with aberrant pulmonary functioning (Sunil et al. 2011; Sunil et al. 2020). Using this model, potential therapeutics have been analyzed including inhibitors of cytokines such as TNFα and antioxidants such as aminoguanidine, N-acetylcysteine and valproic acid (Malaviya et al. 2022, Malaviya et al. 2015, Malaviya et al. 2012, Venosa et al. 2017). While effective in reducing acute lung injury, their impact on chronic mustard toxicity is variable.
Lung lipids are known to play a key role in pulmonary functioning; they also regulate inflammatory responses to lung injury (Chen and Kolls 2010; Suryadevara et al. 2020). Accumulating evidence suggests that dysregulation of lung lipids contributes to NM-induced pulmonary disease pathogenesis (Cruz-Hernandez et al. 2021; Murray et al. 2022; Venosa et al. 2019). Farnesoid-X receptor (FXR) is an important regulator of lung lipid homeostasis (Fessler 2017; Francis et al. 2020; Murray et al. 2022; Shichino et al. 2019; Suryadevara et al. 2020). It has also been shown to exhibit antiinflammatory activity (Vavassori et al. 2009; Wildenberg and van den Brink 2011; Zhang et al. 2017). Primarily expressed in the liver and intestines, FXR has been identified in type I and II alveolar epithelial cells, alveolar macrophages, and lung endothelial cells and fibroblasts (Chen et al. 2016; Francis et al. 2020). In previous studies, we showed that mice lacking FXR are hypersensitive to NM-induced pulmonary injury (Murray et al. 2022). These findings suggest that promoting FXR activation may represent an effective approach for mitigating NM lung toxicity. 6α-ethyl-chenodeoxycholic acid (obeticholic acid, OCA), is a semi-synthetic derivative of the endogenous bile acid chenodeoxycholic acid (CDCA) with an ethyl substituted at the 6α-position, known to activate FXR signaling (Papazyan et al. 2018; Verbeke et al. 2016). In experimental models of nonalcoholic fatty liver disease and primary biliary cholangitis, OCA administration has been reported to reduce inflammation and fibrosis in the liver and intestines, respectively (Chapman and Lynch 2020; Vavassori et al. 2009; Verbeke et al. 2016; Wildenberg and van den Brink 2011). OCA also reduces pulmonary injury and fibrogenesis induced by bleomycin, lipopolysaccharide, or monocrotaline in rodents (Comeglio et al. 2017; Comeglio et al. 2019; Vavassori et al. 2009; Wildenberg and van den Brink 2011; Yao et al. 2014). In the present studies, we analyzed the effects of OCA on NM-induced pulmonary toxicity. Our findings that activation of FXR by OCA reduces NM-induced
pulmonary injury, oxidative stress, and fibrosis are novel as they provide new mechanistic insights into vesicant toxicity which may be useful in the development of efficacious therapeutics.

Methods and Materials

Animals and Treatments. Male Wistar rats (150-174 g; 6-8 weeks) were obtained from Envigo (Somerset, NJ). Animals were housed in filter-top microisolation cages and supplied water and food ad libitum. Animals received humane care in compliance with the institution’s guidelines outlined in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health. Animal protocols were approved by the Rutgers University Institutional Animal Care Committee. Rats were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg) and secured on an angled rodent work stand (Hallowell EMC, Pittsfield, MA) lying in a face up position while being restrained with an incisor loop. Using a cotton applicator, the tongue was extruded to allow for visualization of the larynx using a 4 mm speculum attached to an otoscope. Fresh NM (mechlorethamine hydrochloride, Sigma Aldrich, St Louis, MO; 0.125 mg/kg) or sterile PBS (0.1 mL) was administered intratracheally using a Pencentury™ Micro-sprayer® Aerosolizer (Wyndmoor, PA). The aerosolizer and speculum were immediately withdrawn, and the animals placed in a vertical position in a filter-top microisolation cage lined with ALPHA-dri® bedding and warmed with a heating pad until normal respiration was observed (~1 min). For the duration of NM instillation, appropriate personal protection was worn including safety glasses, mask, isolation gown, sleeves, and apron. Instillations were performed in a designated room under a certified chemical hood following the Rutgers University Environmental Health and Safety Guidelines. Beginning 2 hr post-exposure, and then once a day, 5 days/week thereafter for 28 days, rats were fed OCA (15 mg/kg)
(Ambeed, Arlington Heights, IL) in peanut butter (Jif Creamy Peanut Butter; Orville, OH) or with vehicle control (CTL) (0.13-0.18 g peanut butter), which was typically consumed within <5 min. Body weights were recorded daily. Rats were fed peanut butter (0.18 g/day) for 3 days prior to injury and initiating OCA administration.

**Sample Collection.** Animals were euthanized via intraperitoneal injection of ketamine (100 mg/kg) and xylazine (50 mg/kg) 28 days post NM or PBS. Bronchoalveolar lavage (BAL) fluid was collected using a 16-gauge cannula by instilling cold PBS into the trachea. BAL fluid was centrifuged (300 x g, 8 min), supernatants collected, aliquoted, and stored at −80 °C. In previous studies, we demonstrated that this procedure had no significant effect on macrophages in the lung (Venosa et al., 2016). For preparation of histological sections, following lung lavage, the left lobe of the lung was perfused via the trachea with 3% paraformaldehyde in PBS, removed and suspended in 3% paraformaldehyde. After 24 hr at 4°C, the tissue was rinsed and resuspended in 50% ethanol. The remaining lung lobes were cut into pieces (~5 mg) and flash frozen at -80°C. Alveolar macrophages (AM)s were collected as previously described (Venosa et al. 2017). Briefly, following BAL collection, the lung was removed and instilled via the cannula with 10 mL of ice-cold PBS. Fluid was withdrawn through the cannula while gently massaging the tissue and this was repeated for a total of five washes. The fluid was combined with initial BAL collection, centrifuged (400 x g, 6 min), and red bloods cells lysed using 2 mL Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma-Aldrich; St. Louis, MO). After 5 min at room temperature, 2 mL of ice-cold PBS was added, and the cells washed and counted. For differential analysis, BAL cytospin preparations were fixed in methanol and stained with Giemsa (Labchem Inc., Pittsburgh, PA). BAL cells were found to consist of 86-95% macrophages. For analysis of neutral lipids, cytospins were fixed in 10% formalin, rinsed in 60% isopropanol, and
stained with Oil Red O (Sigma-Aldrich; ST. Louis, MA) for 45 min, followed by counterstaining with Mayer’s Hematoxylin (Sigma-Aldrich; ST. Louis, MA). Slides were mounted with Vectamount (Vector Laboratories; Burlingame, CA) and images captured using a VS-120 slide scanner (Olympus Corporation; Center Valley, PA).

Protein Analysis. Total protein was quantified in BAL supernatants using a BCA Protein Assay Kit (Pierce Biotechnologies Inc.; Rockford, IL). Bovine serum albumin was used as a standard. Samples from each animal were analyzed in triplicate at 560 nm using a SpectraMax M5™ microplate reader (Molecular Devices, Sunnyvale, CA).

Histology. Longitudinally cut tissue sections (5 μm) were stained with hematoxylin and eosin or Picrosirius Red and examined by light microscopy. Lung sections were analyzed blindly by a board-certified pathologist for bronchiolar epithelial degeneration/necrosis, epithelial attenuation, metaplasia/regeneration, peribronchial edema, peribronchial inflammation, alveolar septal defects, alveolar inflammatory cells, alveolar proteinaceous fluid, fibrin, and fibrosis as previously described (Sunil et al. 2010). Alveolar epithelial thickness, alveolar circularization, and total air space were quantified blindly in 15 fields/slide using ImageJ as previously described (Golden et al. 2021). Picrosirius Red staining was assessed by brightfield fluorescent microscopy; ImageJ was used to quantify relative fluorescence intensity.

Immunohistochemistry. Lung sections were analyzed for HO-1 and iNOS protein expression as previously described (Sunil et. al. 2012). Briefly, sections were deparaffinized and peroxidase quenched using 3% hydrogen peroxide in methanol. Antigen retrieval was performed by warming the samples for 20 min in 10 mM sodium citrate buffer (pH 6.0). To block nonspecific binding, sections were incubated for 2 hr at room temperature with PBS containing 5–50% goat
serum. Sections were then incubated overnight at 4°C with rabbit-polyclonal antibodies against heme oxygenase-1 (HO-1; Enzo Life Sciences; Farmingdale, NY; 1:500; ADI-SPA-896-J), iNOS (Abcam; Cambridge, MA; 1:200; ab1523), or appropriate IgG controls (Supplemental Figure 1) (Prosci; Fort Collins, CO) diluted in blocking buffer. Samples were rinsed and incubated with biotinylated rabbit secondary antibody (Vectastain Elite ABC Kit; Vector Labs; Burlingame, CA) for 30 min at room temperature. Antibody binding was visualized using an avidin-biotinylated enzyme complex with 3, 3′-diaminobenzidine (DAB Substrate Kit; Vector Labs). Lung sections were imaged using an Olympus VS-120. Semi-quantitative grades (0-3) were assigned based on the intensity of macrophage staining with 0 = no staining, 1 = minimal staining, 2 = medium staining, and 3 = dark staining in 15 random fields per animal. The median staining score was calculated for each treatment group and representative images selected for presentation.

Hydroxyproline Analysis. Lung tissue hydroxyproline content was analyzed as previously described (Brown et al. 2001). Briefly, 100 mg of flash frozen lung tissue was hydrolyzed and oxidized with Chloramine-T (Acros; Morris Plains, NJ). Ehrlich’s reagent was added, and the samples analyzed spectrophotometrically at 560 nm. Total hydroxyproline was calculated relative to a standard curve and normalized to tissue weight (mg).

Real-Time PCR. Liver, ileum, and alveolar macrophages were homogenized and total RNA extracted using a QIAGEN RNeasy Mini Kit (Valencia, CA). A NanoDrop spectrophotometer (ThermoFisher Scientific; Wilmington, DE) was used to determine RNA purity and concentration. Next, cDNA was converted from RNA using a High-Capacity cDNA Reverse Transcription kit and an RNase Inhibitor (Applied Biosystems; Foster City, CA) using a thermocycler (ThermoFisher Scientific). Real-time PCR was performed in duplicates using
Power SYBR Green PCR Master Mix (Applied Biosystems; Foster City, CA) in a QuantStudio™ 6 and 7 Flex Real-Time PCR System (ThermoFisher Scientific). Primer pairs were generated and confirmed using the Basic Local Alignment Search Tool System (BLAST system; NCIB), synthesized through Integrated DNA Technologies (Coarlville, IA), and validated using a 5-point standard curve according to the manufacturer guidelines. Changes in mRNA expression were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Gapdh). Primer sequences for gapdh, cyp7a1, bsep, fxr, shp, fgf15, and expected annealing temperatures and gene bank accession numbers are shown in Supplementary Table 1.

Phospholipid analysis. BAL (8 mL) was centrifuged for 1 hr (17,000 x g, 4°C). Lipid-enriched large aggregate fractions were collected and resuspended in 35 μL PBS. Total inorganic phosphates were measured as previously described (Massa et al., 2014).

Western blotting. Fibrinogen and soluble (s) RAGE were analyzed using denaturing SDS-PAGE as previously described (Massa et al., 2014). Briefly, cell-free BAL fluid samples (fibrinogen, 1 μL/well; sRAGE, 12 μL/well) were loaded onto a 4%-12% Mini-PROTEAN® TGX™ 10-well, 50 μL gel (BioRad; Hercules, CA). Proteins were transferred onto precut BioRad Immuno-blot PVDF membranes. Blots were blocked with 5% milk and incubated for 1 hr at room temperature with primary anti-fibrinogen (1:1000; Agilent DAKO; A0080; Santa Clara, CA) or anti-S-RAGE (1:500; Abcam; ab65965; Waltham, MA) antibodies followed by goat anti-rabbit horseradish peroxidase conjugated (HRP) secondary antibody (Bio-Rad; Hercules, CA; Cat# 170-6515). Proteins were detected using BioRad ECL prime (Hercules, CA) and quantified using ImageJ.

Measurement of nitric oxide metabolites. A Sievers Nitric Oxide Analyzer 208i (Zysense; Weddington, NC) was used to assay nitric oxide metabolites as previously described (Atochina
Vanadium chloride or acetic acid/iodide (Sigma-Aldrich; Saint Louis, MA) were used to assay for nitrates and nitrites, respectively (sodium salts were used as standards).

**Measurement of pulmonary mechanics.** Rats were anesthetized by intraperitoneal injection of ketamine (90 mg/kg) and xylazine (13 mg/kg). A tracheostomy was performed, and an 18-gauge cannula inserted. The cannula was then attached to a SCIREQ flexiVent 7 small animal ventilator (Montreal, Canada). Respiratory mechanics were measured at positive end-expiratory pressures (PEEPs) ranging from 0-9 cm H2O as previously described (Groves et al., 2012; Massa et al., 2014). Data were analyzed using flexiVent software version 8. Resistance and elastance were calculated using MATLAB™ as described previously (Groves et al. 2013). Briefly, real impedance was used to calculate resistance using the equation $R_L = \frac{a+bf}{c+I}$, with $a/c$ reflecting tissue resistance and $b$, airway resistance. Elastance was calculated from imaginary impedance, the out-of-phase portion of the impedance sin wave, using the equation $E_L = E_\theta + \Delta E (1 - e)^{-\beta f}$.

**Statistical analysis.** Macrophage RNA expression, histopathology, protein, phospholipids, BAL cells, nitrates/nitrites, densitometry, and pulmonary function were analyzed using two-way ANOVA, followed by Tukey’s post-hoc analysis. Ileum and liver mRNA expression was analyzed with Shapiro-Wilk test for normality followed by an unpaired t-test; for data that were not normal, a Mann Whitney U test was used. A $p$ value of $\leq 0.05$ was considered statistically significant. Three to six rats/treatment group were used for each experimental endpoint.
Results

Effects of OCA on FXR target gene expression

FXR is known to regulate genes involved in lipid metabolism in the liver (Cyp7a1, Bsep) and ileum (Fgf-15, Shp) (Carino et al. 2019; Cui et al. 2012; Vonderohe et al. 2022). In initial studies we confirmed that treatment of rats with OCA increased FXR activity by assessing FXR target gene expression in these tissues. In both the liver and ileum, OCA was found to upregulate FXR expression (Supplemental Figure 2). This was associated with decreases in Cyp7a1 and increases in Bsep in the liver and increases in Fgf15 and Shp in the ileum, consistent with the known activity of FXR in these tissues (Vavassori et al. 2009; Verbeke et al. 2016; Zhu et al. 2016). NM had no effect on the expression of Fxr or its target genes in the liver or ileum (data not shown). We next analyzed the effects of OCA on Cyp7a1 and Shp in lung macrophages. In control animals, OCA caused an increase in Shp, with no effect on Cyp7a1 (Figure 1). Following NM exposure, Cyp7a1 mRNA levels were markedly upregulated in lung macrophages, while Shp was downregulated. The effects of NM on these genes were suppressed by OCA; thus, Cyp7a1 decreased, while Shp increased.

OCA blunts NM-induced lung injury, inflammation and oxidative stress

In line with previous studies (Malaviya et al. 2020; Malaviya et al. 2017; Sunil et al. 2020; Sunil et al. 2014), NM exposure was found to cause histopathological changes in the lung including pulmonary edema, increased alveolar epithelial thickness, inflammatory cell infiltration, and an accumulation of enlarged vacuolated macrophages in the tissue; alveolar epithelial thickness and alveolar space size were also increased, indicative of emphysematous-like disease (Figure 2). NM exposure also resulted in pulmonary fibrosis, as reflected by
increased collagen deposition, measured by Picrosirius Red staining, and total hydroxyproline content of the lung (Figure 3, Supplemental Figure 3). Increases in protein and total phospholipids were also observed in BAL after NM treatment of rats, indicating alveolar epithelial barrier dysfunction and dyslipidemia (Bhalla 1999; Malaviya et al. 2012; Sunil et al. 2020). In contrast, while total BAL cell number was unaffected by NM, the percentage of neutrophils increased (Figure 4). Treatment of rats with OCA blunted the effects of NM on histopathologic changes in the lung. This was associated with reduced inflammatory cell accumulation in the lung and numbers of enlarged vacuolated macrophages; edema, fibrosis and alterations in alveolar wall structure and alveolar space were also attenuated, along with increases in BAL protein and neutrophils (Figures 2-4). As shown in Figure 5, OCA also suppressed NM-induced increases in fibrinogen and sRAGE, biomarkers of inflammation (Figure 5 and Supplementary Figure 4) (Li et al. 2015; Oczypok et al. 2017; Sanders et al. 2019).

We next analyzed the effects of OCA on NM-induced oxidative stress. Following NM exposure, the antioxidant enzyme HO-1 was upregulated in macrophages after NM exposure (Figure 6 and Supplemental Table 2). Expression of iNOS was also upregulated in alveolar macrophages and epithelial cells (Figure 7 and Supplemental Table 2); this was associated with a significant increase in the ratio of nitrates to nitrites in BAL indicating increased oxidative metabolism of nitric oxide. Treatment of rats with OCA mitigated NM-induced oxidative stress; hence, expression of HO-1 and iNOS was reduced, as well as the ratio of nitrates to nitrites. OCA had no effect on lung injury, inflammation or oxidative stress in rats treated with PBS.

In further studies we characterized macrophages accumulating in the lung after NM exposure. In line with previous studies (Venosa et al. 2019), at 28 days post-NM, these cells were found to consist of enlarged foamy macrophages and smaller infiltrating macrophages.
(Figure 8). To determine if the enlarged macrophages were lipid laden, cells were isolated from the lung and stained with Oil Red O. NM exposure resulted in an increase in numbers of positively staining macrophages (Figure 8). OCA administration attenuated the effects of NM on foam cell formation.

**NM-induced alterations in lung function are blunted by OCA**

We next assessed the effects of OCA on NM-induced alterations in lung function by measuring respiratory mechanics at increasing PEEPs from 3 to 9 cm H₂O. In NM treated animals, hysteresis, the area between the ascending and descending portion of the pressure-volume curves, was significantly increased at PEEP 3, indicating air trapping. This change was not observed at PEEP 6 or PEEP 9 indicative of improved alveolar recruitment (Figure 9).

Respiratory impedance is considered a sensitive indicator of pulmonary pathology in lung disease (Bates 2006). In line with NM-induced lung injury, real impedance was significantly reduced at PEEP 3 and PEEP 6, while imaginary impedance, a measure of tissue elastance, was not altered (Figure 10). To calculate total resistance (R_L) (airway and tissue), we used real impedance data as previously described (Groves et al. 2013); R_L was then used to extrapolate tissue resistance (a/c) and airway resistance (b). Tissue resistance was significantly reduced after NM exposure at PEEP 3 and PEEP 6 (Figure 10). NM-induced alterations in hysteresis, total resistance, and tissue resistance were blunted by OCA administration. OCA had no effect on lung function in PBS treated animals.
Discussion

FXR is a ligand activated transcription factor that plays a central role in regulating bile acid, lipid, and glucose homeostasis; it has also been shown to exert antiinflammatory effects by reducing immune cell infiltration and activation (Comeglio et al. 2017; Vavassori et al. 2009; Verbeke et al. 2016; Wildenberg and van den Brink 2011; Zhang et al. 2017). The present studies show that FXR signaling in the lung is suppressed after NM exposure. Under homeostatic conditions, FXR activation decreases expression of Cyp7a1, the rate limiting enzyme responsible for converting cholesterol to bile acids, and increases expression of Shp, a small heterodimer protein that inhibits both inflammation and the conversion of cholesterol to bile acids (Cui et al. 2012; Stofan and Guo 2020). However, after NM administration, Cyp7a1 increased, while Shp decreased. Disruption of FXR activity was associated with exacerbated inflammation, oxidative stress and fibrogenesis in the lung in response to NM. These data are in line with our previous studies demonstrating that mice with a targeted disruption of the FXR gene are hypersensitive to NM-induced toxicity (Murray et al. 2022). These findings suggest that activating FXR might be an effective approach to blunting the pulmonary toxicity of NM, and this represented the focus of the current work. For these studies we used OCA, which has previously been shown to block inflammation and lung injury in experimental models of injury induced by bleomycin, monocrotaline or LPS (Comeglio et al. 2017; Comeglio et al. 2019; Fei et al. 2019).

Consistent with earlier studies (Malaviya et al. 2015; Sunil et al. 2011; Sunil et al. 2020; Venosa et al. 2016), NM exposure resulted in histopathologic changes in the lung including alveolar epithelial thickening, airway circularization, collagen deposition, and increases in hydroxyproline content, hallmarks of pulmonary tissue destruction and fibrosis. This was accompanied by epithelial barrier dysfunction, as evidenced by increases in BAL protein and
cells. The inflammatory biomarkers, fibrinogen and sRAGE, were also increased. Treatment with OCA reduced NM-induced structural alterations in the lung, as well as fibrosis and the accumulation of inflammatory proteins in BAL. Previous studies demonstrated that OCA blocked the development of fibrosis in a model of non-alcoholic steatohepatitis by reducing inflammatory cell infiltration into the liver and/or inflammatory macrophage activation (Goto et al. 2018; Huang et al. 2021). It remains to be determined if the ability of OCA to prevent NM-induced pulmonary fibrosis is similarly mediated by suppression of immune cell accumulation and activation in the lung.

Oxidative and nitrosative stress are known to contribute to mustard lung injury and disease pathogenesis (O'Neill et al. 2011; Steinritz et al. 2009). HO-1 is a stress inducible enzyme with both anti-oxidant and antiinflammatory activity (Campbell et al. 2021). Recent studies have suggested that HO-1 may also contribute to the development of profibrotic M2 macrophages in the lung (Campbell et al. 2021; Rossi et al. 2021). In accord with our earlier findings (Malaviya et al. 2015; Sunil et al. 2014), HO-1 was upregulated in lung macrophages following NM exposure. This was mainly evident in enlarged vacuolated macrophages, the typical morphology of foam cells (Poznyak et al. 2021). Further studies are required to determine if HO-1 contributes to the development of profibrotic macrophages in response to NM. Following NM exposure, we also observed increased expression of iNOS in alveolar macrophages and epithelial cells, a response accompanied by increases in BAL levels of nitric oxide oxidation products, which are known to contribute to oxidative stress (Atochina et al. 2004; Kumarathasan et al. 2015; Taylor et al. 2022). Activation of FXR by OCA blunted NM-induced increases in HO-1, iNOS, and nitrates/nitrites in the lung. These findings are consistent with the antioxidant activity of FXR in inflammatory injury in the lung, as well as the liver.
Mechanisms underlying the ability of OCA to reduce oxidative/nitrosative stress in the lung are unknown. Earlier studies demonstrated that FXR agonists including OCA can directly upregulate antioxidants and/or free radical scavengers which may contribute to its activity (Dong et al. 2021; Wang et al. 2020; Zhang et al. 2017). By activating FXR, OCA may also reduce levels of nitric oxide by suppressing NFκB and expression of iNOS (Sarada et al. 2008; So et al. 2020; Tsai et al. 2013). Studies are in progress to investigate these possibilities.

Aberrant regulation of lipid homeostasis appears to be central to the pathogenesis of inflammatory diseases including pulmonary fibrosis and emphysema, atherosclerosis, and hepatic steatosis (Morissette et al. 2015; Schmitz and Grandl 2008; Suryadevara et al. 2020; Xi and Li 2020). The present studies demonstrate that NM causes dysregulated lipid handling; thus, total phospholipids increased in lung lining fluid and lipid-laden macrophages accumulated in the tissue. These findings are in line with earlier studies showing that NM upregulates CD36, a scavenger receptor mediating lipid uptake by macrophages, and downregulates lipid efflux transporters, Abca1 and Abcg1 (Chen and Kolls 2010; Venosa et al. 2019). OCA mitigated the effects of NM on total phospholipids and lipid-laden macrophages. OCA also restored alterations in pulmonary functioning caused by exposure to NM. Thus, treatment of rats with OCA blunted NM-induced emphysematous-like pulmonary dysfunction, as reflected by reduced tissue resistance and air trapping. Impaired pulmonary function has been linked to alterations in lung lipids (Chen and Kolls 2010). The ability of OCA to mitigate NM-induced pulmonary dysfunction is likely due to its capacity to restore lipid homeostasis as a consequence of FXR activation. This is supported by findings that FXR agonists normalize lipid levels in models of atherosclerosis and diabetic neuropathy (Jiang et al. 2007; Mencarelli et al. 2009). Previous
studies have shown that emphysema induced by cigarette smoke exposure is associated with reduced expression of Abca1 and Abcg1 and disrupted cholesterol efflux in the lung (Bodas et al. 2015; Sonett et al. 2018). It remains to be investigated whether decreased emphysematous-like disease and restored pulmonary function following OCA administration are due to FXR-induced upregulation of Abca1 and Abcg1.

Nuclear receptors, such as FXR, are emerging as major regulators of lipid homeostasis in the lung, playing a role in both physiological and pathological processes. Exposure to pulmonary toxicants can dysregulate nuclear receptor function resulting in altered lipid homeostasis and immune cell activation in the lung (Duffney et al. 2020; Francis et al. 2020; Shichino et al. 2019). Elucidating mechanisms underlying impaired activity of FXR and other nuclear receptors following exposure to pulmonary toxicants, including NM as well as SM, may lead to the development of more specific therapeutics to treat diseases associated with aberrant lipid handling and inflammation.

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The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data and available upon request.
Authorship Contributions

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Footnotes

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Figure Legends

Figure 1. Effects of OCA on NM-induced alterations in FXR target genes. Rats were treated with PBS control, NM, OCA, or NM + OCA as described in Materials and Methods. Animals were euthanized on day 28 post PBS or NM administration, alveolar macrophages (AMs) collected and analyzed by real-time PCR for expression of Cyp7a1 and Shp. Data were normalized to GAPDH and presented as a fold change relative to PBS control. Bars, mean ± SE (n = 4 rats/group). *Significantly different (p < 0.05) from PBS; †Significantly different from NM.

Figure 2. Effects of OCA on NM-induced histopathology. Rats were euthanized 28 days after treatment with PBS control, NM, OCA, or NM + OCA as described in Materials and Methods. Lung sections were prepared and stained with Hematoxylin & Eosin. Abbreviations: a, hypercellularity of bronchiolar epithelium; b, alveolar macrophages; c, emphysema; d, edema; f, fibrin deposits. Upper panels, representative sections from 5 rats/group. Original magnification, 20X; scale bar, 100 μm. Lower panels, alveolar epithelial thickness and alveolar space were calculated using ImageJ. Bars, mean ± SE (n = 4-5 rats/group). *Significantly different (p < 0.05) from PBS; †Significantly different from NM.

Figure 3. Effects of OCA on NM-induced fibrosis. Rats were euthanized 28 days after treatment with PBS control, NM, OCA, or NM + OCA as described in Materials and Methods. Lung
sections prepared and stained with Picrosirius Red. Arrows indicate collagen deposition. Upper panel, representative sections from 4-5 rats/group. Original magnification, 20X; scale bar, 100 μm. Lower left panel. Picrosirius Red staining was quantified using ImageJ. Lower right panel.

Frozen lung tissue was assayed for total hydroxyproline content. Data were normalized to total mg tissue for each sample. Bars, mean ± SE (n = 4-5 rats/group). aSignificantly different (p < 0.05) from PBS; bSignificantly different from NM.

Figure 4. OCA blunts markers of NM-induced lung injury. Rats were euthanized 28 days after treatment with PBS control, NM, OCA, or NM + OCA as described in Materials and Methods. BAL was collected, and cell-free supernatants analyzed for total protein, phospholipids, and cells. The percentage neutrophils was determined from Giemsa stained BAL cell cytospins. Bars, mean ± SE (n = 6-8 rats/group). aSignificantly different (p < 0.05) from PBS; bSignificantly different from NM.

Figure 5. OCA reduces inflammatory protein accumulation in the lung following NM exposure. Rats were euthanized 28 days after treatment with PBS control, NM, OCA, or NM + OCA as described in Materials and Methods. BAL was collected and levels of sRAGE and fibrinogen analyzed by western blotting. Left panels. Representative blots from 3-4 rats/group. Right panels. Band density was quantified by ImageJ. Bars, mean ± SE (n = 3 rats/group).

aSignificantly different (p < 0.05) from PBS; bSignificantly different from NM.

Figure 6. OCA blunts NM-induced HO-1 expression. Rats were euthanized 28 days after treatment with PBS control, NM, OCA, or NM + OCA as described in Materials and Methods. Lung sections prepared and immunostained with antibody to HO-1. Binding was visualized using a DAB Vectastain Kit. Representative sections from 4-5 rats/group are shown. Arrows indicate macrophages. Original magnification, 40X; scale bar, 20 μm.
Figure 7. Effects of OCA on iNOS and nitric oxide metabolites. Rats were euthanized 28 days after treatment with PBS control, NM, OCA, or NM + OCA as described in Materials and Methods. **Upper panel.** Lung sections were prepared and immunostained with antibody to iNOS. Binding was visualized using DAB Vectastain Kit. Representative sections from 5 rats/group are shown. Arrows indicate macrophages. Original magnification, 40X; scale bar, 20 μm. **Lower panel.** BAL was collected and analyzed for nitrates and nitrites. Data are presented as the ratio of nitrates/nitrites. Bars, mean ± SE (n = 3-4 rats/group). \(^a\)Significantly different (p < 0.05) from PBS; \(^b\)Significantly different from NM.

Figure 8. OCA reduces foam cell formation. Rats were euthanized 28 days after treatment with PBS control, NM, OCA, or NM + OCA as described in Materials and Methods. **Upper Panels.** Lung sections were prepared and stained with Hematoxylin & Eosin. **Lower panel.** Cytospins prepared from BAL cells were stained with Oil Red O. Representative images from 4-5 rats/group. Original magnification, 20X; scale bar, 50 μm. **Inset.** The total number of positively stained cells was quantified. Data are mean ± SE (n = 4-5 rats/group). \(^a\)Significantly different (p < 0.05) from PBS; \(^b\)Significantly different from NM.

Figure 9. Effects of OCA on NM-induced alterations in hysteresis. Rats were euthanized 28 days after treatment with PBS control, NM, OCA, or NM + OCA as described in the Materials and Methods. Lung hysteresis was assessed using a Scireq flexivent. **Upper panel.** PV-loops were analyzed at PEEPs 3, 6, and 9 cm H₂O. **Lower panel.** PV-loop area (hysteresis) was quantified at each PEEP. Data are mean ± SE (n = 6 rats/group). \(^*\)Significantly different (p < 0.05) from PBS; \(^#\)Significantly different from NM.

Figure 10. Effects of OCA on NM-induced alterations in impedance and tissue resistance. Rats were euthanized 28 days after treatment with PBS control, NM, OCA, or NM + OCA as
described in Materials and Methods. Impedance and tissue resistance were assessed using a Scireq flexivent. Upper panel. Real and imaginary impedance were analyzed at PEEPs 3, 6, and 9. Lower panel. Real impedance was used to calculate resistance; tissue (a/c) and airway (b) components were extrapolated from resistance. Data are mean ± STD (n = 6 rats/group).

*Significantly different (p < 0.05) from PBS; #Significantly different from NM.
Figure 5
Figure 9

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