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Use of a Chemical Chaperone to Attenuate Allergic Airway Inflammation

Thesis submitted to the University of Vermont College of Arts and Sciences for The College Honors Program

By

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**Abstract**

**Rationale:** Asthma is a chronic inflammatory disease characterized by increased airway inflammation and fibrosis. So far, endoplasmic reticulum (ER) stress has been shown to play a role in several inflammatory diseases, however, its involvement in the pathogenesis of allergic asthma has not been clearly understood. It is also known that ER stress inhibitor chemical chaperone tauroursodeoxycholic acid (TUDCA) has been shown to attenuate inflammation in obese and diabetic conditions, but its therapeutic potential in allergic asthma is currently unknown.

**Objective:** The current study was designed to investigate the role of ER stress in house dust mite (HDM)-induced allergic asthma, and the therapeutic efficacy of TUDCA in attenuating the hallmarks of allergic asthma (airway inflammation, mucus metaplasia and fibrosis), using a mouse model of HDM-induced allergic airways disease.

**Methods:** Inflammation was measured by inflammatory cell counts (cell differentials) and cytokine analyses (ELISA). ER stress was examined by the expression levels of ER stress markers ATF6, ERp57, GRP78, GRP94, and CHOP. Further, changes in the airways to indicate fibrosis was determined by quantifying alpha-smooth muscle actin (α-SMA) cell expression and hydroxyproline content in the lungs. HDM and TUDCA were administered using a HDM-induced allergic airways disease murine model for preventive and therapeutic regimens.

**Results:** TUDCA administered during the HDM-challenge phase (as a prophylactic), significantly decreased inflammatory cells and cytokines, ER stress markers, peri-bronchial collagen content and α-SMA cell expression. Moreover, TUDCA administered after the HDM-challenge phase (as a therapeutic), markedly decreased HDM-induced airway inflammation, ER stress makers, but not airway remodeling.

**Conclusion:** These results suggest that the inhibition of ER stress by a chemical chaperone, TUDCA, could be helpful in the treatment of asthmatic patients.
Introduction

Allergic asthma is an inflammatory disorder caused by repeated exposure to allergens resulting in chronic airway inflammation, airway remodeling with the presence of increased α-SMA expression and mucus metaplasia, and airways hyperresponsiveness (1, 3, 27). Environmental and genetic factors are known to play a role in disease pathogenesis (4); however, the causes of asthma are not completely understood. Allergic airway inflammation results from initial exposure (inhaled or cutaneous), thus causing sensitization to the subsequent allergen contact (5). This event is characterized by inflammatory cells and cytokines, which respond in a coordinated, albeit dysfunctional manner (17).

House dust mite (HDM) is a multifaceted allergen to which 50-80% of asthmatics are allergic (6). Nelson et al., demonstrated evidence of significantly increased Immunoglobulin E levels in response to HDM exposure as compared to other allergens such as cat, white oak, Bahia grass, and short ragweed in patients with acute asthma (7). Further, house dust mite contributes to the pathogenesis of allergic asthma in patients that are genetically pre-disposed to asthma (8). HDM exposure has been demonstrated to elicit the inflammatory response, with increases in the recruitment of eosinophils, neutrophils, macrophages, and lymphocytes (16). Thus, the HDM-induced allergic airways disease model is widely used to investigate the progression and attenuation of allergic airway inflammation and fibrosis.

The endoplasmic reticulum (ER) is a network of membranes in the cell with two regions—rough and smooth. The smooth ER serves as a storage for lipid storage and secretion. In contrast, the rough ER has bound ribosomes and is responsible for synthesis and folding of proteins. In addition, it plays a key role in allergic airway inflammation (9, 13) and other inflammatory disease conditions (15, 22). Protein synthesis and folding is normally regulated in the ER under physiological conditions. However, the synthesis of proteins is augmented during pathological
conditions, which leads to increased demand for protein folding. These events collectively create an imbalance in protein synthesis and capacity to fold, resulting in increased accumulation of misfolded/unfolded proteins in the ER lumen, the condition referred to as ER stress. Subsequently, ER stress activates the signaling pathways to reestablish the ER homeostasis, otherwise known as the Unfolded Protein Response (UPR) (10, 22, 23). The abundance of misfolded proteins is sensed by three ER transmembrane proteins: protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1 α (IRE1 α), and activating transcription factor 6 (ATF6) (11). Sustained UPR can cause CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP)-induced cell death (10), and the accumulation of collagen in the lung (12, 13). Another key

Molecular structure of TUDCA. [http://pubs.rsc.org/en/content/articlehtml/2015/ra/c5ra18985c]

Chemical chaperone tauroursodeoxycholic acid (TUDCA) (molecule above), the taurine conjugate form of ursodeoxycholic acid (UDCA), has been shown to inhibit ER stress (14, 20). Exogenous administration of TUDCA has also been found to alleviate ER stress in liver and adipose tissues of diabetic and obese mice (2). In addition, TUDCA has exhibited anti-apoptotic properties in decreasing the expression of ER stress-associated proteins (20). Previously, Hoffman et al., demonstrated that the targeted knockdown of ER stress transducers ATF6 and endoplasmic reticulum resident protein 57 (ERp57) attenuated ER stress-induced airway inflammation and fibrosis (13). Moreover, there are no effective treatments for chronic allergen-induced airway inflammation and remodeling. For that reason, the ER stress inhibitor chemical chaperone TUDCA
might be utilized as a potential therapeutic, particularly in patients with atopic asthma and abnormal lung function.

The main goal of this investigation was to determine the effect of ER stress inhibitor TUDCA on airway inflammation and remodeling in a murine model of allergic airways disease. Within this context, my primary objective was to establish a suitable method to quantitate immunohistochemical (IHC) staining of α-SMA, ERp57, and mucus metaplasia, along with the biochemical evaluation of collagen in the lungs as measurements of the pathophysiology of allergic asthma. Our results demonstrate that HDM-induced ER stress is a substantial contributor to the airways inflammation and remodeling, which can be attenuated by TUDCA.

Methods

Human Samples

Lung tissues from patients with physician-diagnosed asthma and non-asthmatic subjects were obtained from the Department of Medicine of the University of California, San Francisco (UCSF), and the Department of Pathobiology of the Cleveland Clinic. The Institutional Review Boards of UCSF and the Cleveland Clinic approved provision of de-identified materials for research at the University of Vermont. All subjects were nonsmokers defined as never smokers or former smokers with no smoking for at least 1 year before enrollment and total pack-years of ≤15. All asthmatic patients refrained from inhaled corticosteroids for 6 weeks before enrollment in the study. Lung biopsy specimens from 6 non-asthmatic subjects and 6 asthmatic patients were from the UCSF airway tissue bank, and lung biopsy specimens from 3 non-asthmatic subjects and 3 asthmatic patients were from Cleveland Clinic.

Animals

Bitransgenic and triple transgenic mice carrying the rat club cell secretory protein (CCSP) promoter 5’ to the open reading frame for the reverse tetracycline transactivator (CCSP-rtTA; line
1, which in adult lungs expresses in bronchiolar and type II epithelial cells) plus 7 tetracycline operon 5’ to the open reading frame for Cre recombinase (TetOP-Cre) mice were provided by Dr. Whitsett (Cincinnati Children’s Hospital). In the Anathy laboratory CCSP-rtTA1, TetO-Cre1 mice were bred with mice carrying the ERp57\textsuperscript{loxP/loxP} alleles. Mice expressing CCSP-rtTA/TetO-Cre/ERp57\textsuperscript{loxP/loxP} were used to ablate ERp57 from lung epithelial cells (denoted as ΔEpi-ERp57) by feeding doxycycline-containing chow (6 g/kg; Purina Diet Tech, St Louis, MO) 5 days before exposure to HDM. Mice were maintained on doxycycline-containing food until completion of the experiment. Double-transgenic littermates containing either CCSP-rtTA/TetO-Cre or CCSP-rtTA/ERp57\textsuperscript{loxP/loxP} (Ctr mice) and fed doxycycline-containing food were used as controls in the experiments. Age-matched male and female mice were used for all experiments.

For TUDCA treatment studies, twelve week-old female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Guidelines for the experiments approving the use of biological samples and vertebrate animals were set forth by the Institutional Animal Care and Use Committee and the Institutional Review Board of the University of Vermont.

**HDM-Induced Allergic Airways Disease Mouse Model**

Mice were first anesthetized using isoflurane and challenged with 25 μg of HDM (GREER, Lenoir, NC) extract suspended in PBS for the sensitization phase, or the primary exposure to the allergen, through an intranasal administration on day 1 and again as a booster on day 8. The challenges were repeated consecutively on days 15 to 19 (Figs. 3 and 5). The control groups were administered sterile PBS as vehicle controls at all time points.

**TUDCA Treatment**

For the preventative regimen experiments, mice were administered TUDCA (0.5, 1.0, and 5.0 mg/kg body weight; dissolved in PBS) via the nasopharynx on days 14, 17, and 20 (Fig. 3) during the HDM challenge phase. Mice were euthanized 24 hours after the last TUDCA administration.
In the therapeutic regimen experiments, TUDCA (0.25, 0.75, and 5.0 mg/kg body weight) was administered on days 20, 22, 24, 26, and 28. Mice were euthanized 24 hours after the final treatment (Fig. 5). PBS served as the vehicle control in both experiments.

**Bronchoalveolar lavage fluid (BALF) Collection and Processing**

Mice were euthanized by injecting 0.1 mL of pentobarbital sodium (50 mg/mL; intraperitoneal), and BALF was collected by lavaging lungs with 1.0 ml of sterile PBS. Samples were centrifuged at 1200g for 5 min at 4 °C. Supernatants were transferred to another set of tubes and stored at -80°C for further analysis. Cell pellets were re-suspended in 5% Bovine Serum Albumin (BSA) in PBS for total and differential cell counts (eosinophils, neutrophils, macrophages, and lymphocytes). Total white blood cell counts were monitored using an Advia 120 Automated Hematology Analyzer System (18).

For differential cell counts, the remaining re-suspended cells were pipetted into cytopsin cartridges overlaid on super frost slides and filters. An appropriate amount of 5% BSA in PBS was added to the cartridges, and samples were centrifuged at 600rpm at room temperature for 10 min. Further, slides were air dried, fixed in 100% methanol for 5 min, and stained using Hema3 stain reagents (Fisher Scientific). The slides were dipped in each reagent 10 times followed by rinsing with water. Differentials were conducted on a minimum of 300 cells per sample.

**Immunoblot Analysis**

Following dissection, right lung lobes were flash frozen for protein analysis. Lungs were pulverized and lysed in buffer containing 137 mM Tris-HCl (pH 8.0), 130 mM NaCl, and 1% NP-40. The protein samples from cell lysates were prepared in the same buffer and insoluble proteins were pelleted by centrifugation at 10000g for 10 min at 4°C. Further, concentration of protein in each samples were measured by Bradford Assay (DC Protein Assay, BioRad Laboratories, Hercules, CA). Following protein quantitation of the supernatant, lung lysates (25 μg protein) were
resuspended in loading buffer with dithiothreitol (DTT), boiled at 95°C for 5 min, and cooled on ice. Further, the protein samples were loaded onto 12% SDS-PAGE gels and electrophoresis was carried out under reducing conditions at constant voltage of 150 V for 3 hrs.

After the run, proteins were transferred to PVDF using a wet transfer protocol. Briefly, two thick Whatman filter papers and one PVDF membrane were cut to the dimensions of the gel. The PVDF membrane was activated by dipping it in 100% methanol for 15-20 sec. Activated PVDF membrane, sponges, and filter papers were soaked in transfer buffer. A gel sandwich structure was prepared by stacking a sponge on top of a sheet of Whatman filter paper in a transfer cassette along with the PVDF membrane and the gel followed by another sponge and filter paper on to the cassette. The cassette was closed firmly before placing it in the electric module. In this procedure, wetness was maintained throughout by dipping the sponges, filter papers and membrane in transfer buffer. The transfer apparatus was connected to the electric module and the wet transfer was carried out at 100 V for 1h. After the wet transfer, blots were probed using the standard immunoblotting protocol.

The primary antibodies used were: Activating transcription factor 6 (ATF6) (1:1000, anti-rabbit) and CCAAT/enhancer-binding protein homologous protein (CHOP) (1:2000, anti-mouse) both from Abcam, Cambridge, MA, and endoplasmic reticulum resident protein 57 (ERp57) (1:1000, anti-rabbit) and glucose-regulated protein 78 (GRP78) (1:1000, anti-rat) from Stressgen, San Diego, CA. β-actin was from Sigma, St. Louis, MO. The blots were incubated with ECL (Enhanced Chemiluminescence) western blotting substrate (Promega, Madison, WI) for 2 min, and the blots were exposed to X-ray films under dark conditions. The X-ray films were developed and the protein bands were analyzed.
**Alpha-Smooth Muscle Actin and ERp57 Immunohistochemistry**

For murine lung tissue samples, the left lung of each sample was inflated by injecting 10% neutral buffered formalin through the trachea as well as being fixed prior to paraffin embedding. Paraffin blocks were cut into 5-μm sections and mounted to slides by the Surgical Pathology Department in the UVM Medical Center. The paraffin-embedded lung sections were stained by the Masson’s trichrome method by the Histology Department to measure the peri-bronchoalveolar collagen content of the airways.

Alternatively, the standard immunohistochemistry protocol (Abcam) was performed to measure the α-SMA content in both human and murine lung tissue samples using reagents from the Vectastain ABC kit (Vector Laboratories). The paraffin embedded tissues were dewaxed in xylene, 100%, 95%, 70% and 50% ethanol, and rinsed with water. Slides were placed in 95°C sodium citrate buffer for 20 min, to expose and retrieve the antigens. Blocking serum in PBS (phosphate buffered saline) was added to minimize any non-specific background binding. Samples were incubated overnight with the specific primary, α-SMA antibody (1:8000, anti-mouse, Sigma) or ERp57 antibody (1:250, anti-rabbit, Enzo Life Sciences) at 4 °C. After washing slides in TBSt (Tris-Buffer Saline with 0.05% tween), slides were incubated for 1h with biotinylated universal antibody. The last incubations involved the avidin DH/biotinylated enzyme complex with alkaline phosphatase reagents (ABC-AP) and the Vector red alkaline phosphatase substrate kit. Finally, slides were rehydrated with changes of xylene and alcohols and cover slips were placed using Permaslip mounting medium. Images were captured at a magnification of 20X using the Olympus BX50 light microscope (UVM Microscopy Imaging Center). Samples were analyzed for quantifying α-SMA and ERp57 in human and murine samples through scoring tissue samples. Scores were conducted by three individuals in which tissue samples were blindly evaluated on a scale from 0-3, 0 denoting little to no indication of remodeling and mucus metaplasia and 3
denoting substantial positivity. In relation to the TUDCA treatment, murine samples were analyzed for quantifying protein such as α-SMA and ERp57 positive area of airways using MetaMorph software.

For each murine sample in the TUDCA experiment, three airways were randomly imaged from the top, middle, and bottom regions of lung sections. Using the software, the pixel distances were first calibrated (1 pixel=0.368 μm) with the loaded lens of the Olympus BX50 imaging objective (20X magnification) for all images. For all of the measurements, the color intensity range corresponding to the α-SMA staining was chosen using the settings on three image scales; Hue (180/255), Saturation (0/240), and Intensity (0/104). To measure the color intensity within an airway, the outer and inner layers of airways (labeled Area 1 and 2, respectively) were traced. The inner area was traced to eliminate the lumen of the airway from the calculation. The measurements were exported into a spreadsheet with which total area and color intensities were calculated. The total area was calculated by subtracting the inner outline (Area 2) from the outer outline (Area 1) of the airway. The color intensity was calculated in a similar manner. The percentage of positive staining was calculated by dividing the threshold by the total area of the airway and multiplying by 100. Averages of each sample were clustered into specific groups and graphs were plotted.

**Assessment of Mucus Metaplasia**

Paraffin blocks of lung tissues were cut into 5-μm sections and mounted to slides by the Surgical Pathology Department in the UVM Medical Center. Mucus metaplasia was assessed by the periodic acid Schiff staining (PAS). The images were captured using an Olympus BX50 light microscope, and mucus metaplasia was measured by quantifying positively stained area of airways for PAS staining, using MetaMorph software, as described in the previous section.
**Hydroxyproline Assay**

Increases in sub-epithelial collagen and fibrosis in the lung were analyzed by determining the hydroxyproline content (19). The lungs were crushed into a fine powder and hydrolyzed in sealed glass tubes containing 2 mL of 6N HCl at 110 °C overnight (18 hrs). The samples were neutralized using 10N and 1N sodium hydroxide, and incubated with chloramine T solution at room temperature for 20 min. Further, perchloric acid solution (1.0 ml) was added, samples were vortexed, and incubated at room temperature for 5 min. To this solution, Ehrlich’s reagent solution (DMBA) was added and incubated at 60°C for 20 min. Samples (200µL) were pipetted into each well of a 96-well plate and the optical density was measured at 561 nm. All the reagents used in this assay were obtained from Sigma.

**Statistics**

Data were statistically analyzed by one-way analysis of variance (ANOVA) and Tukey’s multiple comparison analysis. Histological scores were analyzed by a Kruskal-Wallis and Dunn’s multiple comparison post hoc test. Data from multiple experiments were averaged and expressed as mean values ± SEM and results at P<0.05 were considered statistically significant.

**Results**

**Increased ERp57 levels are associated with asthma pathogenesis in human subjects**

To evaluate the possible role of ER stress transducer ERp57 in the pathogenesis of asthma, paraffin-embedded human lung tissue samples were stained for ERp57 by immunohistochemistry. Immunostaining analysis revealed significant increases in ERp57-positive staining in the lung samples from asthmatic human subjects versus the non-asthmatics. Furthermore, airway epithelium of the asthmatic patients exhibited predominantly increased ERp57 levels, as revealed by semi-quantitative scoring (p<0.002; Figs. 1A and 1B).
Fig. 1. ERp57 levels are increased in the lung tissue samples from asthmatic patients.
A. Immunohistochemical (IHC) staining for ERp57 (Red) in paraffin-embedded human lung tissue samples obtained from asthmatic human subjects. B. Lung tissue samples stained with secondary antibody alone. Scale bars=50μm. C. Quantitation by histological scores for ERp57 (p<0.002) by ANOVA.

**Lung epithelium–specific ablation of ERp57 decreases allergen-induced airways fibrosis**

To examine the role of ERp57 in airway fibrosis, the presence of α-SMA cells in the airways of murine lung tissue samples was evaluated by α-SMA immunostaining in control and lung epithelial cell-specific ERp57 deficient (ΔEpi-ERp57) mice after HDM exposure. The HDM challenge increased α-SMA in the peri-bronchoalveolar region of lung tissue sections from HDM-challenged control mice as compared to HDM-challenged ΔEpi-ERp57 mice (Fig. 2A). Semi-quantitative scoring for these sections revealed significant decreases in α-SMA staining in HDM-challenged ΔEpi-ERp57 mice when compared to control mice (Fig. 2B). Collectively these results
contribute to the indication that ER stress mediators may play a critical role in allergen-induced airways fibrosis.

Fig. 2. Ablation of ERp57 in lung epithelial cells decreases smooth muscle hypertrophy.
A. IHC staining for α-SMA in PBS- and HDM-challenged lungs from Ctl and Epi-ERp57 mice. B. Histologic scores for α-SMA. *P<0.05 indicates significant differences compared with PBS groups. #P<0.05 indicates significant differences compared with HDM groups. Scale bars= 50 μm.

Preventative regimen of TUDCA attenuates HDM-induced ER Stress

To evaluate the potential of ER stress inhibitor chemical chaperone TUDCA as a preventative treatment for allergic asthma, mice were instilled with 0.5mg/kg and 1.0 mg/kg body weight dose of TUDCA via the nasopharynx during the HDM-challenge phase (Fig. 3A). Analysis of immunoblots for the expression of ER stress markers demonstrated decreases in ATF6\(^{50}\), ERp57, and CHOP protein expression in TUDCA-treated HDM-challenged mice as compared to the vehicle-treated HDM-challenged mice (Fig. 3B). β-actin served as a loading control. Further, analysis of the inflammatory profiles in BAL fluid revealed a significant dose-dependent decrease in eosinophils and lymphocytes in TUDCA-treated HDM-challenged mice as compared to vehicle-
treated HDM-challenged mice (Fig. 3C). Our analysis also showed that, although not dose-dependent, there was a significant decrease in macrophages in TUDCA-treated mice (p<0.05; Fig. 3C). For the PAS staining, the doses of TUDCA at 1 mg/kg significantly decreased mucus metaplasia as seen in the reduction in intensity of the PAS-stained epithelial cells in the airway of the lung sections of TUDCA-treated HDM-challenged mice as compared to vehicle-treated or untreated HDM-challenged mice (Fig. 3D).
Fig. 3. Preventive regimen of TUDCA attenuates HDM-induced airways inflammation and ER stress. A. Schematic representing the time points of HDM or PBS instillation and TUDCA treatment. HDM (25 μg/mouse) was instilled intranasally while TUDCA (0.5 and 1 mg/kg body weight) was administered via nasopharynx during the HDM-challenge phase. B. Western blot analysis for ER stress markers in whole lung lysates of vehicle-treated and TUDCA-treated HDM-challenged mice. β-actin was used as a loading control. C. Analysis of inflammatory and immune cells in the bronchoalveolar lavage fluid (BALF). Data are mean ± SEM of 6-8 mice/group. *P < 0.05 as compared to vehicle-treated group. #P < 0.05 indicates as compared to vehicle untreated HDM challenged mice. ‡P < 0.05 as compared to vehicle treated HDM challenged mice. §P < 0.05 as compared to mice treated with 0.5 mg/kg body weight dose of TUDCA. D. Representative images of PAS-stained lung tissue sections of vehicle-treated and TUDCA-treated HDM-challenged mice (×20 magnification) and quantification of percentage of area positively stained for PAS staining. *P < 0.05 as compared to their respective PBS controls. #P < 0.05 as compared to vehicle untreated HDM challenged mice. ‡P < 0.05 as compared to vehicle-treated HDM-challenged mice.

Preventative regimen of TUDCA decreases HDM-induced airways fibrosis

Alpha-Smooth Muscle Actin (α-SMA) cell expression is a factor in airway remodeling as well as collagen deposition (27). Therefore, analysis of α-SMA and collagen levels were measured. Immunostaining was conducted for cell expression of α-SMA to compare intensity of staining
between the vehicle-treated or untreated HDM-challenged mice. There were significant decreases in α-SMA levels in the peribronchiolar region of lung sections from TUDCA-treated HDM-challenged mice as compared to vehicle-treated or untreated HDM-challenged mice (p<0.05; Fig. 4A). In addition, measurement of collagen by levels of hydroxyproline indicated a significant decrease in collagen in the lungs of TUDCA-treated HDM-challenged mice as compared to vehicle-treated HDM-challenged mice (p<0.05; Fig. 4B). Collectively, these experiments strongly support the impact of HDM-induced ER stress in airways inflammation and sub-epithelial airway remodeling and demonstrate the potential of TUDCA to alleviate ER stress, airways inflammation, and remodeling.
Fig. 4. Preventive regimen of TUDCA decreases HDM-induced airways fibrosis. A. Representative images of α-SMA-stained lung tissue sections of vehicle-treated and TUDCA-treated HDM-challenged mice (×20 magnification) and the quantification of percentage of area that positively stained for α-SMA. *P< 0.05 as compared to their respective PBS controls. #P< 0.05 as compared to HDM-challenged mice. B. Measurement of collagen by hydroxyproline assay. Data are mean ± SEM of 6-8 mice/group. *P < 0.05 as compared to PBS control. #P < 0.05 as compared to HDM challenged vehicle untreated group.

**TUDCA in therapeutic regimen decreases airways inflammation**

Based on the preventative effect of TUDCA on the development of allergic airways disease, we next investigated the therapeutic potential of TUDCA administration after the establishment of allergic airways disease. To address this, we administered different doses of TUDCA via nasopharynx after the HDM-challenge phase (Fig. 5A). Analysis of inflammatory cells in BAL fluid indicated that all three doses of TUDCA significantly reduced eosinophils and neutrophils (Fig. 5B). Interestingly, only one dose of TUDCA (0.75 mg/kg body weight) markedly decreased total cell counts, lymphocytes, and macrophages in HDM-challenged mice (Fig. 5B). These results indicate that TUDCA-inhibited pro-inflammatory cytokines decreases the airways inflammation in the lung.
Fig. 5. Therapeutic regimen of TUDCA decreases HDM-induced airways inflammation.

A. Schematic representing the time points of HDM or PBS instillation and TUDCA treatment. HDM (25 μg/mouse) was instilled intranasally while TUDCA (0.25, 0.75 and 5 mg/kg body weight) was administered via nasopharynx after the HDM-challenge phase. B. Analysis of leukocytes in the bronchoalveolar lavage fluid (BALF). Data are mean ± SEM of 6-8 mice/group. *P < 0.05 indicates as compared to their respective PBS controls. #P < 0.05 indicates as compared to HDM-challenged mice. †P < 0.05 indicates comparison between mice treated with 0.25 mg/kg body weight dose of TUDCA and 0.75 mg/kg body weight dose of TUDCA. ♦P < 0.05 indicates comparison between mice treated with 0.75 mg/kg body weight dose of TUDCA and 5 mg/kg body weight dose of TUDCA.

**Therapeutic treatment of TUDCA inhibits ER stress**

ER stress was measured by analyzing the expression of ER stress marker proteins in lung lysates. The expression of GRP94 and GRP78 was substantially decreased in TUDCA-treated HDM-challenged mice when compared to vehicle-treated HDM-challenged mice (Fig. 6). However, there was a trend towards decrease in ERp57 and CHOP expressions in TUDCA-treated mice when compared to vehicle-treated mice (Fig. 6). β-actin served as a loading control.
Fig. 6. Therapeutic regimen of TUDCA attenuates HDM-induced ER stress. Western blot analysis for ER stress markers in whole lung lysates of vehicle-treated and TUDCA-treated HDM-challenged mice for n=3/group. β-actin was used as a loading control.

**Therapeutic regimen of TUDCA does not protect from HDM-induced airways fibrosis**

To examine the therapeutic efficacy of TUDCA in reversing allergen-induced airway remodeling, we assessed airways smooth muscle and collagen content in TUDCA-treated and vehicle-treated HDM-challenged mice as well as the extent of mucus metaplasia. The therapeutic regimen of TUDCA did not alleviate airways fibrosis, as indicated by the absence of changes in α-SMA cell expression levels in TUDCA-treated HDM-challenged mice compared to vehicle-treated HDM-challenged mice (Fig. 7A). Analysis of hydroxyproline content did not display a significant change in collagen levels in TUDCA-treated HDM-challenged mice versus vehicle-treated HDM-challenged mice (Fig. 7B). However, as revealed in the quantification of the PAS staining, the intensity of PAS-stained epithelial cells was significantly decreased in the airways of TUDCA-treated HDM-challenged mice compared to the vehicle-treated HDM-challenged mice (Fig. 7C). Collectively, these results indicate that the therapeutic treatment of TUDCA does not diminish HDM-induced airways fibrosis.
Fig. 7. Therapeutic regimen of TUDCA does not decrease HDM-induced airways fibrosis. A: Representative images of α-SMA-stained lung tissue sections of vehicle-treated and TUDCA-treated HDM-challenged mice (×20 magnification) and the quantification of percentage of area that positively stained for α-SMA. Differences were not significant between the groups. B: Measurement of collagen by hydroxyproline assay. Data are mean ± SEM of 6-8 mice/group. *P < 0.05 as compared to their respective PBS controls. Statistical differences were not significant between vehicle-treated and TUDCA-treated HDM-challenged mice. C. Representative images of PAS-stained lung tissue sections of vehicle-treated and TUDCA-treated HDM-challenged mice (×20 magnification) and quantification of percentage of area positively stained for PAS. *P < 0.05 as compared to their respective PBS controls. #P < 0.05 as compared to HDM-challenged mice. ‡P < 0.05 as compared between mice treated with 0.25 mg/kg body weight dose of TUDCA and 0.75 mg/kg body weight dose of TUDCA. §P < 0.05 as compared between mice treated with 0.75 mg/kg body weight dose of TUDCA and 5 mg/kg body weight dose of TUDCA.
Discussion

The present investigation was carried out to determine the role of ER transducer ERp57 in allergic asthma, using lung tissue sections from asthmatic human subjects and a mouse model of HDM-induced allergic asthma. Furthermore, we evaluated the efficacy of ER stress inhibitor TUDCA in alleviating allergic asthma, using a mouse model of HDM-induced allergic airways disease both during (preventive regimen) and after the HDM-challenge phase (therapeutic regimen). Our results show that asthmatic patients exhibit a marked increase in ERp57 levels as compared to non-asthmatic subjects, predominantly in the lung epithelium (Fig. 1). In addition, epithelium-specific downregulation of ERp57 significantly decreased allergen-induced inflammation and airways fibrosis in a murine model of HDM-induced allergic asthma. Interestingly, both preventive and therapeutic regimens of TUDCA efficiently attenuated HDM-induced ER stress and airways inflammation. In addition, the preventive regimen of TUDCA significantly reduced airways fibrosis in HDM-challenged mice, whereas the therapeutic regimen did not.

As stated previously, ER stress is a condition caused by an imbalance in synthesis and folding of proteins that leads to the accumulation of unfolded proteins in ER lumen (10, 22, 23). ER stress has been shown to play a key role in inflammatory diseases including atherosclerosis, diabetes, obesity, and cardiovascular diseases (2, 15, 22). Furthermore, Hoffman et al. have demonstrated that the targeted knock-down of ER stress transducers ATF6α and ERp57 attenuated HDM-induced allergic airways disease pathologies, including airways inflammation and fibrosis (13). In support of these findings, immunohistochemistry performed in this study demonstrated increased levels of ERp57 in airways epithelial regions of lung tissue sections from asthmatics as compared to non-asthmatics (Fig. 1). In addition, the airways epithelial-specific deletion of ERp57
in mice exhibited significant decreases in HDM-induced airways fibrosis when compared to wild type mice (Fig. 2A). Collectively, these results indicate that ER stress plays a significant role in the pathogenesis of allergic asthma.

Recently, Makhija et al. demonstrated that the chemical chaperones glycerol, trehalose, and trimethylamine-N-oxide (TMAO) significantly decreased UPR markers, airways inflammation, and remodeling in a mouse model of ovalbumin-induced allergic airway inflammation (25). Furthermore, the chemical chaperone, TUDCA, has been observed to inhibit ER stress responses in liver and adipose tissues of diabetic and obese mice (14, 2). Since ER stress contributes to the pathogenesis of inflammatory diseases (22), and the inhibition of ER stress might be advantageous as a therapeutic strategy, TUDCA was tested to determine its efficiency in attenuation of airways inflammation and fibrosis using mouse model of HDM-induced allergic airways disease.

House dust mite (HDM), the most common complex airborne allergen, contains multiple components, which regulate the development of pathophysiology associated with allergic asthma, and approximately 50-80% of asthmatics are allergic to HDM (6). Therefore, HDM is a useful model allergen with which to study the pathogenesis of allergic airways diseases and efficacy of therapeutics to alleviate allergic asthma. Several studies have shown that high levels of inflammatory cells such as eosinophils, lymphocytes, and macrophages, significantly contribute to the pathogenesis of asthma (7, 8). Therefore, the inflammatory cell counts were analyzed by cell differentials, and interestingly, both the preventive and therapeutic regimens of TUDCA significantly decreased HDM-induced inflammatory cells, including eosinophils and neutrophils, the major cell types associated with the pathogenesis of asthma. In addition, TUDCA markedly decreased lymphocytes and macrophages in HDM-challenged mice (Figs. 3 and 5). These results
indicate that TUDCA may play a critical role in recruitment and/or activation of inflammatory cells that are crucial in immune responses.

Furthermore, the expression of HDM-induced ER stress markers, including ATF6\textsuperscript{30}, ERp57, and CHOP, was markedly reduced in lung lysates of mice that received TUDCA during the HDM-challenge phase, whereas the therapeutic regimen only significantly reduced the expression of GRP78 and GRP94. Also, we observed a trend towards decreases in the expression of ERp57 and CHOP in these mice. These results indicate that the effect of TUDCA on ER stress proteins could be variable, depending upon the dose and treatment regimen. Moreover, the decreased expression of ER stress markers corresponds with the reduction in inflammatory cells, indicating that TUDCA-mediated attenuation of ER stress might play a critical role in bringing down airway inflammation.

Airways fibrosis is characterized by structural changes within the lungs that causes bronchoconstriction of the airways, thus leading to decreased lung function (1, 24). Increased levels of α-SMA cell expression and collagen in the airway play a causal role in the development of airways fibrosis (21, 27). In this study, TUDCA instilled during the HDM-challenge phase significantly decreased levels of α-SMA cell expression, total collagen/hydroxyproline content, and peri-bronchiolar fibrosis in the lungs of HDM-challenged mice (Fig. 4). However, TUDCA administered after the development of disease did not improve airway fibrotic remodeling, as measured by α-SMA and collagen/hydroxyproline content (Fig. 7). Although, there was a trend towards a decrease in α-SMA and hydroxyproline content in these mice, based on these results we speculate that TUDCA alone may not be sufficient for the resolution of airway fibrotic remodeling. Nevertheless, TUDCA in combination with anti-fibrotic agents (26) or other chemical chaperones (25) could be helpful in attenuating the pathologies of allergic airways disease.
In conclusion, the present investigation demonstrated the role of ER transducer ERp57 in pathogenesis of allergic asthma. In addition, our results showed the efficacy of both preventive and therapeutic regimens of TUDCA in alleviating allergen-induced airways inflammation and ER stress. Interestingly, preventive but not therapeutic regimens of TUDCA significantly reduced airways fibrosis in HDM-challenged mice. Taken together, our results suggest that the inhibition of ER stress by chemical chaperones could be an important strategy in the management of allergic airways diseases.

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