Simvastatin Inhibits Smoke-Induced Airway Epithelial Injury: Implications for COPD Therapy

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Introduction

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the United States, and current treatments do not significantly alter the progression of the disease \(^1\). Approximately 20 million men and women in the United States have COPD with a mortality of 20 out of 100,000 \(^2\). The most common cause of COPD in the general population is cigarette smoking \(^2;3\). Persistent inflammation is a key component of COPD pathogenesis. Thickened bronchiolar walls accompanied by an influx of inflammatory cells including neutrophils, macrophages, and lymphocytes are characteristic of COPD \(^4;8\). Besides lowering cholesterol, the statin drugs (‘statins’) possess pleiotropic anti-inflammatory, immunomodulatory, anti-oxidant, and anti-proliferative properties, with potential clinical applications beyond cardiovascular disease \(^9;10\). Due to these pleiotropic effects, the statins have been proposed as a potentially novel treatment for inflammatory respiratory diseases, including bronchial asthma and COPD \(^11;12\). Compelling reductions in COPD morbidity and mortality, and improvements in lung function have been attributed to statin use \(^11;13-18\). Additionally, statins have been found to reduce neutrophil transendothelial migration in cell culture \(^19;20\) and neutrophilic inflammation and influx into lung tissues of animals \(^19;21-24\).

The anti-inflammatory effects of statins are mediated partly via inhibition of the mevalonate pathway. Specifically, statins inhibit the enzyme 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (HMGR), the rate-limiting step in cholesterol biosynthesis. The immediate product of HMG-CoA reductase is
mevalonate (MA), which is metabolized into the non-sterol isoprenoids farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), and into cholesterol. These metabolites are necessary for the post-translational isoprenylation and subsequent activation of intracellular monomeric small G-proteins (Ras, Rho, Rac, Cdc42, etc.). These G-proteins (or GTPases) control many important biological functions, including immune cell function, cytoskeletal dynamics, transmigration, and cell proliferation \(^9;10;25\), and are thought to mediate at least some of the beneficial effects of statins.

Others have published the beneficial effects of simvastatin in attenuating smoke-induced emphysema, pulmonary hypertension, and small airway remodeling \(^26;27\). However, no studies have evaluated whether systemic treatment with statins inhibits smoke-induced large airway or bronchial epithelial damage. This question is highly relevant to human smoke-related lung diseases, such as COPD. Therefore, in this study we hypothesized that systemic treatment with simvastatin can (1) attenuate acute tobacco smoke-induced lung inflammation, and (2) inhibit airway epithelial injury. We evaluated whether the mevalonate pathway and lung Ras and Rho GTPase activation were affected by treatment with simvastatin. We utilized the spontaneously hypertensive (SH) rat model of COPD because of its robust neutrophilic inflammatory response to tobacco smoke exposure \(^28-34\). This model produces COPD like pathologies, including mucin hypersecretion, airway epithelial squamation and thickening, airspace enlargement and pulmonary function changes with four weeks exposure to tobacco smoke. We chose to test simvastatin's effect with an acute tobacco
smoke exposure that induces lung inflammation to isolate simvastatin's preventative effects on leukocyte recruitment and airway epithelial injury from possible effects of promoting recovery. In addition, this choice of tobacco smoke exposure allowed us to separate simvastatin's preventative effects on leukocyte recruitment and airway epithelial injury from prevention of mucus accumulation due to mucin hypersecretion and the loss of ciliated epithelial cells, which occurs with a longer tobacco smoke exposure and could cause further inflammation.
Materials and Methods

Animals

Twelve-week-old male SH rats were purchased from Charles River Laboratories (Portage, MI). Upon arrival, all animals were housed in polycarbonate cages under a 12-hour light-dark pattern with continuous access to food and water. Animals were acclimated to the new housing environment for one week before tobacco smoke exposure began. All animals were handled according to the U.S. Animal Welfare Acts, and all procedures were performed under the supervision of the University Animal Care and Use Committee (University of California, Davis).

Tobacco Smoke Exposure

Groups of six SH rats each were exposed to filtered air (FA) or to tobacco smoke (approximately 80-90 mg/m$^3$ total suspended particulates (TSP)) for 6 hours/day for 3 days. Whole body exposure to cigarette smoke was done using a TE10 smoke exposure system $^{35}$ that combusts 3R4F research cigarettes (Tobacco and Health Research Institute, University of Kentucky, KY) with a 35 ml puff volume of 2 seconds duration once each minute (Federal Trade Commission smoking standard). In addition to TSP, exposure conditions were monitored daily for both nicotine and carbon monoxide concentrations.

Simvastatin Delivery

Simvastatin was purchased from Sigma (St. Louis, MO). Simvastatin was made as a 4-mg/ml stock solution that was dissolved in 10% ethanol as the drug
vehicle. The drug vehicle was made with 100 µl of ethanol, 150 µl of 0.1 N NaOH, and 750 µl of phosphate-buffered saline (PBS) to make 1 ml of 10% ethanol. Hydrochloric acid (HCl, 0.1 N) was added as necessary to obtain a pH solution of 7.0.

Rats were given 20mg/kg simvastatin via intra-peritoneal (i.p.) injection daily for either (1) seven days prior to smoke exposure and 30 minutes prior to daily tobacco smoke exposures, or (2) only 30 minutes prior to daily tobacco smoke exposures.

We chose this dose based on previously published work by us and others that have shown this to be an effective non-toxic dose. It is difficult to extrapolate what the equivalent dose in humans would be. In rats the half-life of simvastatin metabolism in the blood is four minutes when compared to humans, which is seven hours. However, simvastatin is likely to remain at therapeutic levels in the tissue much longer.

**Pulmonary function test**

Rats were deeply anesthetized with Ketamine and Xylazine 18-20 hours following the final day of tobacco smoke exposure. A midline incision was made over the cervical trachea. Once the rats were tracheo-cannulated, the cannula was then connected to the Scireq Flexivent (Montreal, Canada) positive pressure ventilator, pulmonary mechanics measurement and data acquisition system. Animals were paralyzed by succinylcholine. The lung volume and pressure were measured twice while a standard respiratory cycle was simulated—once with the
catheter open to room air and once with it closed. The rats were ventilated at a frequency of 90 breaths/min with a tidal volume of 10 ml/kg. Lung mechanics were evaluated using a forced oscillation technique. Measures of respiratory system input impedance were obtained that allows for the unique distinction between central and peripheral lung mechanics. A "snapshot perturbation" maneuver was imposed to measure resistance (R), compliance (C), and elastance (E) of the whole respiratory system (airways, lung, and chest wall).

**Tissue Preparation**

Following the pulmonary function test, rats were given an overdose of sodium pentobarbital. The trachea was cannulated, the left lung bronchus tied, and the right lung lavaged with Ca$^{2+}$/Mg$^{2+}$-free Hank’s buffered salt solution (HBSS). Bronchoalveolar lavage (BAL) was performed using a three-in/three-out pattern of intratracheal instillation and removal with the same HBSS aliquot in order to enrich total cell and protein recovery. BAL fluid (BALF) was collected in tubes and kept on ice prior to processing. The lavaged lung lobes were frozen in liquid nitrogen and stored at -80°C until use. For histology, the suture on the left lung bronchus was released, and the lung was inflated with 4% paraformaldehyde at 30 cm water pressure for 1 hour, followed by storage of the inflation-fixed lung immersed in fixative.

**Histology**

Histology was performed using cross-sectional lung tissue slices containing the first and second intrapulmonary airway generations from rats.
exposed to filtered air or tobacco smoke. Five-micron thick sections were cut from paraffin-embedded tissue blocks using a microtome. Sections were placed on glass slides and baked overnight at 37°C. Sections were subsequently deparaffinized in toluene and hydrated through a graded series of alcohol solutions. For hematoxylin and eosin (H&E) staining, sections were stained with the following American Master Tech Scientific materials: Harris Hematoxylin, Differentiating Solution, Bluing Solution, and Eosin Y Stain. Sections were then dehydrated in ethanol and mounted with Clear Mount (American Tech Master Scientific, Inc., Lodi, CA).

**BALF Analysis**

The BALF was centrifuged at 250 xg for 10 minutes at 4°C to separate cells from the supernatant fluid. After centrifugation, the cell pellet was resuspended in Ca^{2+}/Mg^{2+}-free HBSS. The cell suspension was assayed for cell viability as determined by trypan blue exclusion. Total cell number was determined using a standard hemocytometer. Cytospin slides (Shandon, Pittsburgh, PA) were prepared using aliquots of cell suspension that were then stained with Hema 3 (Fisher Scientific, Pittsburgh, PA). Cell differentials in BALF were assessed by counting the number of macrophages, neutrophils, lymphocytes, and eosinophils on the cytocentrifuge slides using light microscopy (500 cells counted per sample). The proportion of each cell type was multiplied by the total cell number per ml to determine total neutrophils, macrophages, lymphocytes and eosinophils per ml. Eosinophils are not reported since they
made up less than 0.01% of the total cells and were too few for statistical analysis.

**Airway epithelial injury**

Airway epithelial injury was evaluated by measuring the percentage of sloughed airway epithelium. Using Image J software (NIH Freeware) the length of sloughed airway epithelium was quantitatively measured and divided by the total airway epithelium in H&E stained lung sections at interpulmonary generation two. P value was determined by Wilcoxin rank sum test.

**Western Blot analysis of tissue**

Western blot methods are described in Bratt et al. 38 and were done with the following modifications. SDS-PAGE electrophoresis was performed using both cytosolic and membrane fractions (at 30 ug total protein) under reducing conditions and transferred to a polyvinylidene difluoride membrane. Membranes were probed using 0.001 mg/mL rabbit anti-mouse Ras (Cell Signaling Technology, Inc., Danvers, MA); 0.001 mg/mL monoclonal anti-Rho (EMD Millipore, Billerica, MA); 0.1 ug/mL monoclonal anti-E-cadherin (BD Bioscience, San Jose, CA); or rabbit anti-mouse α-Actinin IgG primary antibodies in 0.05% Tween in PBS (TBST) followed by incubation in 40 ng/mL HRP-conjugated goat anti-rabbit IgG (Pierce Biotechnology, Rockford, Ill) or HRP-conjugated goat anti-mouse IgG (R&D Systems, Minneapolis, MN) secondary antibodies in 5% dry milk in TBST. Protein bands were then visualized using Western Lightning Plus-ECL substrate kit (PerkinElmer, Shelton, CT) and Image Reader LAS-4000 V2.1
(Fuji Photo Film Co, Cypress, CA). Individual band intensity was calculated using ImageJ (NIH Freeware).

**Cholesterol Assay**

Rat lungs were homogenized in a homogenization buffer by hand using a dounce homogenizer as described above. Lung homogenate was centrifuged at 5000 xg for 5 minutes to remove large tissue debris. The supernatant was collected and aliquoted into volumes used to measure protein concentration and cholesterol. Lipids were isolated by adding a large volume of chloroform:methanol (2:1) solution, mixing (by vortex and sonication), and further addition of 1M NaCl solution, which allows the separation of organic (chloroform) phase from the aqueous phase by centrifugation (3000 xg for 5 min). The bottom (chloroform) phase was collected and dried using speed vacuuming. Lipid pellets were resuspended in phosphate buffer (0.1 M potassium phosphate pH 7.4, 50 mM NaCl, 5 mM deoxycholic acid, 0.1% Triton X-100) and sonicated for at least 1 minute to mix. Cholesterol was then measured via a fluorometric assay, the Amplex® Red Cholesterol Assay Kit (Invitrogen, Grand Island, NY), according to the manufacturer's instructions.

**Rho and Ras GTPase Analysis**

Rat lung homogenates were prepared using isolation buffer (250mM sucrose; 20mM HEPES, pH 7.4; 2mM EDTA; and 3mM NaN₃) containing protease inhibitors (Protease Inhibitor (Sigma, 1:100), and 1mM PMSF) and phosphatase inhibitors (Sigma, 1:100, 1mM Na₂VO₄, and 1mM NaF). Lung tissue
homogenization was performed by hand using a dounce homogenizer. Crude homogenates were initially centrifuged at 800 xg for 5 min at 4°C. The supernatant was then removed and saved as total lung homogenate. A 500 μL aliquot of this total homogenate was centrifuged at 31,000 xg at 4°C for 30 minutes to separate the cytosolic (soluble) and membrane (insoluble) components. The supernatant from this step was saved as the cytosolic fraction, and the remaining pellet was resuspended in 500 μL of isolation buffer (same volume of the cytosolic fraction). The total protein concentration of the cytosolic fraction from each sample was measured using the Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) and was used to determine protein loading volumes for each sample.

We performed Western blots on our samples to assure that the cytosolic fractions were not contaminated by the membrane fractions and vice versa. We used E-cadherin as an indicator of membrane fractions since this protein is only expressed in cell membranes. None of our cytosolic fractions had evidence of membrane components, and our membrane fractions positively confirmed E-cadherin expression. Previously, we also measured GAPDH to confirm cytosolic fractions as further confirmation of our method, but this data are not included.

**Statistical Analysis**

To increase the validity of tests of statistical significance, log-transformation was applied to outcome variables with skewed distributions before performing a one-way ANOVA with Bonferroni corrections for multiple comparisons where appropriate. The Wilcoxin rank sum test was used to analyze
airway epithelial injury measurement and HO-1 densitometric analysis due to grossly non-normal distributions. A p-value of less than 0.05 was used to indicate statistical significance.
Results

Simvastatin requires a pre-treatment period to reduce leukocyte recruitment to the lung.

To determine whether simvastatin can prevent or reduce tobacco smoke-induced lung inflammation, we measured leukocytes recovered in the BALF. Three days of cigarette smoke exposure significantly increased total leukocytes, macrophages and neutrophils (Figure 1) compared with filtered air or filtered air/simvastatin exposure. Lymphocytes made up less than 3% of the total cells and were not increased by tobacco smoke exposure. SH rats treated with simvastatin for one week prior to smoke exposure and during the exposure course had reduced tobacco smoke-induced total leukocytes, macrophages, and neutrophils recruited to the lung compared to SH rats receiving a smoke-only treatment. On the other hand, treatment with simvastatin concurrently with smoke exposure days had no effect, indicating that the anti-inflammatory effect of simvastatin is not immediate, or a pre-exposure treatment period is required for the greatest benefit.

Simvastatin prevents airway inflammation and epithelial injury.

To determine whether simvastatin can prevent or reduce tobacco smoke-induced damage to the lung, we evaluated hematoxylin and eosin stained lung sections. Airway epithelial injury was quantified by measuring the percentage of sloughed airway epithelium (Figure 2). Rats exposed to tobacco smoke only had extensive damage to the airways, including cellular infiltration into the subepithelial space and marked denudation of the epithelial layer. Numerous
neutrophils and macrophages were also present in the airways. The lung histology of rats treated with simvastatin on smoke exposure days appeared identical to the histology from the tobacco smoke only group. However, rats pretreated with simvastatin for 1 week prior to smoke exposure (and that continued through the smoke exposure period) prevented tobacco smoke induced large airway epithelial injury ($p = 0.02$) (Figure 2). Four of the six rats had virtually no damage or signs of inflammation, and were indistinguishable from filtered air controls. One rat had a small amount of cellular infiltration in the epithelium and a few inflammatory cells in the airway, and the other rat had clear damage and inflammation in the lung but to a lesser extent than all but one of the rats in the tobacco smoke only group.

**Simvastatin alters pulmonary function.**

Three days of tobacco smoke exposure did not significantly alter airway resistance, respiratory system compliance, or elastance (Figure 3). However, simvastatin pretreatment reduced lung compliance (Figure 3B) and increased lung elastance (Figure 3C) compared to the smoke only treatment. Simvastatin given on exposure days only did not alter pulmonary function.

**Simvastatin causes weight loss.**

Rats exposed to tobacco smoke only or tobacco smoke plus simvastatin, regardless of when simvastatin treatment began, lost significant weight compared to corresponding filtered air exposed animals (Table 1). Pre-treatment with Simvastatin was associated with weight loss, whether the animals were
exposed to filtered air or tobacco smoke. In fact, animals exposed to tobacco smoke and pretreated with simvastatin lost more weight than any other group.

**Simvastatin did not alter lung Rho or Ras GTPase membrane localization.**

Part of statins’ anti-inflammatory effects have been proposed to occur by inhibition of GTPase membrane localization \(^{10;19;24;39;40}\). Therefore, we tested whether simvastatin attenuated or inhibited tobacco smoke-induced membrane translocation of Ras and Rho. However, we did not detect smoke- or simvastatin-induced changes in Ras or Rho translocation from the cytosol to the membrane in whole lung homogenate (Figure 4).

**Simvastatin did not alter whole lung cholesterol levels.**

The mevalonate pathway branches downstream into the isoprenoids farnesylpyrophosphate and geranylgeranylpyrophosphate, and ultimately cholesterol. Since the statins deplete serum cholesterol as their main effect and given that cholesterol has been associated with increased lung inflammation in some animal models \(^{41}\), we evaluated whole lung total cholesterol levels. We did not observe any smoke- or simvastatin-induced changes in lung total cholesterol in any of our experiments (Figure 5).

**Simvastatin reduces oxidative stress in smoke exposed rats.**

We investigated simvastatin's effects on oxidative stress as a possible mechanism of simvastatin's prevention of airway injury. Heme oxidase 1 (HO-1) expression is increased with oxidative stress and was assayed as a marker of
oxidative stress. In smoke exposed rats treatment with simvastatin for one week prior to smoke exposure and during the exposure course significantly decreased HO-1 expression by western blot (Figure 6). However, smoke induced increases compared to filtered air controls did not reach significance. HO-1 can have antioxidant properties as well as being a marker for oxidative stress. Simvastatin has been reported to increase HO-1\textsuperscript{42-47}. Simvastatin did not increase HO-1 expression in filtered air exposed rats indicating that this effect did not occur in this study.
Discussion

This study demonstrates a causal relationship between statin use and the prevention of tobacco smoke-induced inflammatory cell recruitment to the lung and acute bronchial epithelial damage. We show three major novel findings regarding statins’ protective anti-inflammatory effects in smoke-induced lung damage: 1) Pre-treatment with simvastatin 1-week prior to tobacco smoke exposure not only reduces acute inflammatory cell recruitment to the lung, but also completely prevents smoke-induced acute airway epithelial injury and denudation; 2) The anti-inflammatory effect of simvastatin requires a 1-week pre-treatment period, whereas there was no benefit when simvastatin was given concurrently with smoke exposure, indicating that the underlying mechanism of simvastatin’s anti-inflammatory effect is not immediate and must precede smoke exposure; and 3) These results were independent of simvastatin’s effect on recovery, mucus accumulation and airway remodeling, changes which only occur with longer tobacco smoke exposures in our SH rat model.

The above findings suggest that statins may be appropriate for patients with a wide variety of bronchial diseases, such as the airways disease component of COPD (i.e. chronic bronchitis and not only emphysema). It is unclear why simvastatin protected the SH rats from acute airway epithelial damage. Systemic treatment with simvastatin may prevent smoke-induced damage via direct epithelial effects or prevent the secondary damage incurred by the epithelium from the influx of activated leukocytes. Other possibilities include statin anti-oxidative properties, which in the case of smoke-induced injury may
play a major role \textsuperscript{48-51}. We investigated whether treatment with simvastatin for one week prior to smoke exposure and during the exposure reduced HO-1 a marker of oxidative stress. Simvastatin significantly reduce HO-1 expression in smoke exposed rats supporting the hypothesis that simvastatin prevents airway injury by reducing oxidative stress. However, care should be taken not to over interpret these results because smoke induced increases in HO-1 expression compared to filtered air control did not reach significance. Thus, it is not clear how much oxidative stress contributed to airway injury in this study. Additionally, we do not know whether any oxidative stress is a direct result of smoke exposure or secondary to neutrophil activation and subsequent respiratory burst.

Statin 'pre-conditioning' appears to be a critical process for simvastatin's anti-inflammatory effect, which depended on the 1-week pre-treatment period. We speculate that pre-conditioning could be due to the a priori modulation of immune cell responses \textsuperscript{52,53}, decreased proinflammatory cytokine/chemokine gene expression at the level of the airway epithelium \textsuperscript{54,55} or mesenchyme, or reduced vascular recruitment of leukocytes into lung tissue \textsuperscript{56-58}, amongst other mechanisms. Statins have been shown to inhibit LFA1 by binding to it and preventing high affinity binding of leukocytes to vascular endothelium to facilitate transmigration into inflamed tissues. We speculate that simvastatin is not preventing leukocyte recruitment through inhibition of LFA1 because it would not require pretreatment.

Given the central role of the airway epithelium in both innate and adaptive immune responses \textsuperscript{59,60}, where it is the central mediator of smoke-induced pro-
inflammatory responses, additional studies are required to elucidate the protective effect of statins on bronchial epithelium for statin dosing and administration. Future work is needed to determine whether simvastatin is acting on the lung tissue or directly on leukocytes or both to prevent leukocyte recruitment.

By demonstrating that simvastatin reduces both inflammatory cell recruitment to the lung and airway injury before the development of mucus accumulation and airway remodeling, we show that simvastatin prevents inflammatory cell recruitment and acute airway injury independently of these other important factors. Our results suggest that the short-term statin benefits in smoke-induced lung injury, which may be an early event in some phenotypes of COPD, do not depend on reducing mucus accumulation and airway remodeling. Within 10 days of treatment, there is clear protection from statins in preventing further airway epithelial injury and leukocyte recruitment. The latter is a key component of acute COPD exacerbations and may, at least in part, explain why simvastatin-use in large human epidemiologic studies has been associated with a reduced risk of COPD exacerbations and improved lung function\textsuperscript{11,18}.

Prior studies show that simvastatin can prevent or reverse smoke-induced emphysema and reverse pulmonary hypertension\textsuperscript{29,30}, and when given chronically over 16 weeks with tobacco smoke, simvastatin attenuates smoke-induced small airway remodeling\textsuperscript{31}. To our knowledge, this study is the first to report that systemic pre-treatment with simvastatin inhibits smoke-induced
bronchial epithelial injury and denudation. The beneficial effects of simvastatin in our model occurred within ten days, which is a relatively short time period. Other animal studies have shown statin anti-inflammation in the lung; however, the statin was administered during the animals’ lung development and for a month (or longer). 21;29;31.

The anti-inflammatory effects of statins are thought to be mediated partly via inhibition of isoprenoid synthesis. Therefore, we investigated if simvastatin reduced the pool of available FPP and GGPP, thereby decreasing the proportion of isoprenylated/membrane-bound Ras and Rho, respectively. We expected to see smoke-induced cell membrane translocation (or activation) of Ras and/or Rho in our homogenized whole rat lungs. However, we did not detect smoke-induced or simvastatin-induced translocalization of these GTPases (between cytosolic and membrane fractions), indicating that any smoke-induced changes may be transient, subtle or restricted to certain cells and/or compartments within the lung, and not detectable in whole lung homogenate. Given these possibilities, testing whether co-treatment with geranylgeranyl pyrophosphate and farnesyl pyrophosphate, the isoprenoid molecules upstream of Rho and Ras activation, respectively, reverse the effects of simvastatin may be the best way to determine whether Rho and Ras activation modulate the protective effects of simvastatin.

Rats pre-treated with simvastatin and exposed to tobacco smoke had reduced lung compliance (and increased lung elastance) (Figure 3). In humans, COPD typically manifests with abnormally high lung compliance due to emphysema and the resultant loss of lung elastic recoil. Thus, normalizing or
lowering lung compliance is usually desirable in COPD. However, the acute smoke exposure does not mimic this human pathophysiology. We speculate that the decrease in lung compliance may be due to a reduction in alveolar surfactant production\(^{28}\) rather than due to the anti-inflammatory effect of simvastatin because 3 days of smoke exposure did not alter compliance as compared to filtered-air controls. Also, any alterations in local cholesterol concentrations in resident alveolar cells due to simvastatin were not detectible by our assay; thus, we cannot relate this to cholesterol per se.

We were surprised to see a weight loss effect in the “pre-treatment” experimental arm at the 20mg/kg simvastatin dose because both the literature and our previous work did not reveal any evidence of toxicity or gross weight loss in both rats and mice at this dose or higher doses\(^{24;36}\). There is no analog in humans. Statin use does not cause weight loss in humans as far as is known from clinical reports covering the past 30 years of continued statin use.

This work highlights simvastatin as a potential novel therapy for tobacco smoke-induced inflammatory lung disease, such as COPD. We demonstrate that simvastatin attenuates tobacco acute smoke-induced leukocyte recruitment to the lung and further show that simvastatin pre-treatment for 1-week almost completely prevents smoke-induced acute bronchial epithelial injury. The protective statin effect may be due to direct effects on the lung or mucosal surface during leukocyte-airway epithelial cell interactions \(^{36;61}\). We envision simvastatin (and possibly other statins) as having the potential to become important adjunctive therapies in COPD, added to the current standard inhaler
regimens. Additional animal studies are needed to investigate the underlying mechanisms of action. Also, ongoing clinical trials in both asthma and COPD will be of high interest.
Table 1. Weight change over 10 days of the study. Tobacco smoke or treatment with simvastatin (Sim) for one week prior to smoke exposure and continued with tobacco smoke exposure caused weight loss, while treatment with simvastatin on the tobacco smoke exposure days only had no effect. All groups were analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons.

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Figure Legends
Figure 1. Pre-treatment with simvastatin (Sim) one week prior to tobacco smoke exposure and continued through the exposure time frame reduced tobacco smoke-induced leukocyte recruitment to the lung while treatment on the smoke exposure days only had no effect. A) Total leukocytes recovered in the BALF. B) Macrophage recovered in the BALF. C) Neutrophils recovered in the BALF. All groups were analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons.

Figure 2. Epithelial and inflammatory changes to the bronchial wall of spontaneous hypertensive rats exposed to filtered air or tobacco smoke and treated with simvastatin. The tobacco smoke only exposure was associated with significant airway epithelial injury.
and sloughing. Pretreatment with simvastatin provided almost complete epithelial protection to tobacco smoke if given one week prior to and during exposure to tobacco smoke, but not if treatment only began concurrently with tobacco smoke exposure. Top: Hematoxilin and Eosin staining of bronchial airways at interpulmonary generation 2. Bottom: Quantitative analysis of the percentage of airway epithelial sloughing. were analyzed by the Wilcoxin rank sum test.
**Figure 3.** Treatment with simvastatin (Sim) for one week prior to smoke exposure and continued during the smoke exposure period reduced lung compliance and increased lung elastance, while Sim treatment on exposure days only had no effect. A) Resistance, B) Compliance, C) Elastance. All groups were analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons.

**Figure 4.** Treatment with simvastatin (Sim) for one week prior to smoke exposure and continued during the exposure did not change localization of Ras and Rho (between cytosolic and membrane fractions). A) Western blots of active Ras (top) and membrane
to cytosol ratio of western blot densitometry (bottom). B) Western blots of active Rho (top) and membrane to cytosol ratio of western blot densitometry (bottom). All groups were analyzed by one-way ANOVA.
Figure 5: Treatment with simvastatin (Sim) for one week prior to smoke exposure and continued during the exposure had no effect on total cholesterol in rat whole lung.
homogenate. Total cholesterol in whole lung homogenate lysates was measured and normalized to total protein, as described in Material and Methods. All groups were analyzed by one-way ANOVA.

Figure 6. HO-1 protein expression was measured by western blot as a marker for oxidative stress. Top: western blot of HO-1 and αActinin (as a control) from 100 µg of total proteins of whole lung homogenates. Bottom: densitometric analysis of HO-1 expression normalized to αActinin control. (Mean +/- SEM). p value determined by Wilcoxin Rank sum test.
Reference List


