Environmental tobacco smoke exposure does not prevent corticosteroids reducing inflammation, remodeling, and airway hyperreactivity in mice exposed to allergen

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Environmental tobacco smoke exposure does not prevent corticosteroids reducing inflammation, remodeling, and airway hyperreactivity in mice exposed to allergen. Am J Physiol Lung Cell Mol Physiol 297: L380–L387, 2009. First published June 12, 2009; doi:10.1152/ajplung.90588.2008.—The ability of corticosteroids to reduce airway inflammation and improve lung function is significantly reduced in asthmatics who are tobacco smokers compared with asthmatics who are nonsmokers. As not only high levels of tobacco smoke exposure in active smokers, but also significantly lower levels of tobacco smoke exposure from passive environmental tobacco smoke (ETS) exposure in nonsmokers can increase both asthma symptoms and the frequency of asthma exacerbations, we utilized a mouse model to determine whether corticosteroids can reduce levels of airway inflammation, airway remodeling, and airway hyperreactivity in mice exposed to the combination of chronic ETS and ovalbumin (OVA) allergen. Chronic ETS exposure alone did not induce increases in eosinophilic airway inflammation, airway remodeling, or airway hyperreactivity. Mice exposed to chronic OVA allergen had significantly increased levels of peribronchial fibrosis, increased thickening of the smooth muscle layer, increased mucus, and increased airway hyperreactivity, which was significantly enhanced by coexposure to the combination of chronic ETS and chronic OVA allergen. Administration of corticosteroids to mice exposed to chronic ETS and OVA allergen significantly reduced levels of eosinophilic airway inflammation, mucus production, peribronchial smooth muscle thickness, airway hyperreactivity, and the number of peribronchial TGF-β1+ cells. Overall, this study demonstrates that corticosteroids can significantly reduce levels of eosinophilic inflammation, mucus expression, airway remodeling, and airway hyperreactivity in chronic ETS-exposed mice challenged with allergen.

asthma is a disease associated with airway inflammation that is responsive to corticosteroid therapy in most individuals as demonstrated in studies using bronchial biopsies (9). In conjunction with reducing airway inflammation in asthma, corticosteroids improve clinical outcomes (symptoms, frequency of rescue β2-adrenergic agonist inhaler use) (9, 14) as well as prevent asthma exacerbations (27) and deaths from asthma (33). Corticosteroids are also effective in improving pulmonary function (14), inhibiting the late-phase lower airway response to allergen challenge (2), and reducing airway hyperresponsiveness (AHR) (14).

In contrast to the efficacy of corticosteroids in nonsmoking asthmatics, the efficacy of inhaled corticosteroids (4, 17, 35), as well as oral corticosteroids (5) in asthmatics who smoke tobacco, is significantly reduced. For example, in a double blind placebo-controlled study of inhaled corticosteroids (1,000 μg fluticasone daily for 3 wk) in mild asthmatics (forced expiratory volume in 1 s, FEV1 87% of predicted), the asthmatics who did not smoke had significant improvements in pulmonary function (FEV1), airway responsiveness (provocative concentration of methacholine causing a 20% fall in FEV1, MCh PC20), and sputum eosinophils, whereas the asthmatics who did smoke did not have any of these improvements with inhaled corticosteroid therapy (4). In a second double blind placebo-controlled study of asthmatics, administration of inhaled beclomethasone (320 μg HFA-BDP daily for 8 wk) in mild asthmatics (FEV1 80% of predicted) who did not smoke resulted in significant improvements in pulmonary function (FEV1), airway responsiveness (MCh PC20), and sputum eosinophils, whereas in asthmatics who smoked, statistically significant improvements were not noted on a.m. peak flow rates (PEFR) and sputum eosinophils, but not FEV1 or airway responsiveness (17). A third double blind study compared low-dose vs. high-dose inhaled corticosteroids (400 or 2,000 μg beclomethasone daily for 12 wk) in mild asthmatics (FEV1 85% of predicted) who smoked or did not smoke (35). The asthmatics who were not smokers and received the low dose of inhaled corticosteroid demonstrated significant improvements in PEFR after 3 mo of therapy, whereas the asthmatics who smoked did not demonstrate improvements in PEFR (35). In addition, the smoking asthmatics who received the low dose of inhaled corticosteroid had significantly more asthma exacerbations compared with the asthmatics on low-dose inhaled corticosteroids who did not smoke (35). In contrast to the difference in response between smokers and nonsmokers to the low dose of inhaled corticosteroid (400 μg beclomethasone daily), the asthmatics who received the high dose of inhaled corticosteroid (2,000 μg beclomethasone daily) demonstrated improvements in PEFR in both nonsmokers (mean improvement 18 l/min) as well as smokers (mean improvement 11 l/min) (35). Thus, this study suggests that smokers with asthma who receive low-dose inhaled corticosteroid do not have improvements in lung function, but that high doses of inhaled corticosteroids in smokers with asthma can improve lung function but not to the same degree as in asthmatics who do not smoke. Overall, the several studies of inhaled corticosteroids in asthma (4, 17, 35) suggest that asthmatics who smoke have a significantly reduced therapeutic response to corticosteroids compared with asthmatics who do not smoke and that the difference in response may be influenced by corticosteroid dose, but is not due to baseline differences in either bronchodilator
response, levels of airway responsiveness, or sputum eosinophils (4, 17, 34, 35).

Studies have also compared the therapeutic efficacy of oral corticosteroids in asthmatics who smoke compared with asthmatics who do not smoke (5). In a placebo-controlled, cross-over study with prednisolone (40 mg daily for 2 wk) in asthmatics with stable symptoms (FEV1 70% predicted), there was a significant improvement after oral prednisolone in pulmonary function (FEV1, morning PEF) and asthma control score in never-smokers with asthma, but no change in smokers with asthma (5). Thus, smoking also impairs the efficacy of short-term oral corticosteroid treatment in chronic asthma.

While there are now several studies that demonstrate that asthmatics with high levels of tobacco smoke exposure (i.e., 7-25-yr pack year history of smoking in the various studies) (4, 5, 17, 35) have an impaired response to corticosteroids, as well as higher rates of adverse asthma outcomes compared with non-smokers with asthma (16, 30), there is no current information as to whether significantly lower levels of tobacco smoke exposure from second-hand exposure to environmental tobacco smoke (ETS) might also impair the corticosteroid response in asthmatics who do not smoke but are exposed to ETS. The importance of even such low levels of tobacco smoke exposure in asthmatics is suggested from epidemiological studies of ETS exposure and adverse asthma outcomes (6, 10, 15) as well as gene association studies demonstrating a link between a region on chromosome 17q21 with ETS and asthma (3). Exposure to ETS has been linked to several adverse asthma outcomes including increased prevalence of asthma, increased severity of asthma symptoms, increased frequency of asthma medication use, and increased emergency room visits by asthmatic children (6, 10, 15). In addition to these epidemiological studies of ETS exposure and asthma, experimental ETS challenge studies in humans indicate that passive smoke exposure has adverse effects on airflow and/or airway responsiveness in asthma (22, 32). Therefore, as no studies in humans have reported on the effect of chronic ETS exposure on corticosteroid responsiveness in asthma, we have used a well-defined mouse model of chronic ETS exposure (24, 28, 29) to investigate whether exposure of mice to chronic low levels of tobacco smoke in ETS would limit the ability of corticosteroids to reduce airway inflammation, mucus expression, airway remodeling, and AHR in mice exposed to chronic allergen.

METHODS

Therapeutic intervention with corticosteroids in ETS-exposed mice. We have previously demonstrated that in mice, chronic ETS exposure increases levels of chronic OVA allergen-induced eosinophilic inflammation, airway remodeling, and AHR compared with mice exposed to chronic ovalbumin (OVA) allergen with no ETS exposure (24). These studies (24) demonstrate that ETS has a significant biological effect in this mouse model of asthma and allow us to determine whether ETS exposure will impair the ability of corticosteroids to inhibit inflammation, remodeling, and AHR in ETS-exposed mice. Thus, to determine whether corticosteroids could inhibit the development of airway inflammation, remodeling, and AHR in mice exposed to low levels of second-hand tobacco smoke, in this study mice were chronically exposed to ETS as well as either no OVA, OVA, or OVA + corticosteroid. We also included as controls mice not exposed to ETS (no OVA and OVA) as we have previously demonstrated that chronic ETS exposure increases levels of chronic OVA allergen-induced airway remodeling (24). The group of ETS-exposed mice that received the OVA + corticosteroid was administered dexamethasone (1 mg/kg ip in 100 µl of sterile, endotoxin-free PBS), a dose of corticosteroid that we have previously demonstrated reduces airway inflammation, remodeling, and AHR in mice not exposed to tobacco smoke (8, 23). The first dose of dexamethasone was administered 6 h before the first intranasal OVA challenge, and the therapeutic intervention was continued daily for the duration of the 1-mo period of ETS exposure and twice-weekly OVA challenges.

Mouse model of chronic OVA and ETS-induced airway remodeling. Five different groups of 8-10-wk-old female BALB/c mice (12 mice/group, The Jackson Laboratory, Bar Harbor, ME) were exposed to either chronic ETS (no OVA, OVA, OVA + corticosteroid) or as a control no ETS (no OVA, OVA) as previously described in this laboratory (8, 23). In these studies, mice were immunized subcutaneously on days 0, 7, 14, and 21 with 25 µg of OVA (grade V, Sigma) adsorbed to 1 mg of alum (Aldrich) in 200 µl of normal saline as previously described (8, 23). OVA-challenged mice received intranasal OVA challenges on days 27, 29, and 31 under isoflurane (Vedco, St. Joseph, MO) anesthesia, which were then repeated twice a week for 1 mo. The no-OVA age- and sex-matched control mice were sensitized but not challenged with OVA during the 1-mo study. The groups of mice exposed to ETS had their first ETS exposure on day 33 after the mice had been sensitized with OVA subcutaneously and received intranasal OVA challenges on days 27, 29, and 31 as previously described in this laboratory (24). Chronic ETS was continued daily for the subsequent duration of the 1-mo period of twice-weekly intranasal OVA challenges.

Three groups of mice (no OVA, OVA, OVA + corticosteroid) were subjected to chronic ETS (side-stream smoke from 6 cigarettes/day each administered over ~5 min with a 15-min break between cigarettes, 5 days/wk) generated by burning 2R4F reference cigarettes (2.45 mg of nicotine/cigarette; purchased from Tobacco Research Institute, Univ. of Kentucky, Lexington, KY) using a smoking machine (McChesney-Jaeger CSM-SSM Single Cigarette Machine; CH Technologies, Westwood, NJ) regulated by programmable controls provided with JASPER Windows 9×2/000 software over RS-232 communication ports (CH Technologies) as previously described in this laboratory (24). Each smoldering cigarette is puffed for ~2 s, once every 25 s, for a total of 12 puffs/cigarette, at a flow rate of 5 l/min. The outflow from the smoking machine was adjusted to mimic an exposure to ETS by producing a mixture of room air (98%) and mainstream smoke (2%). The mice were exposed to the ETS in a 12-port nose-only directed flow inhalation exposure system (Jaeger-NYU 12 port). Nose ports were monitored for total suspended particulates, which we have previously reported to be 173 ± 5.3 µg/m³ using a gravimetric method (24). All animal experimental protocols were approved by the University of California San Diego Animal Subjects Committee.

Mice were killed 24 h after the final chronic OVA and/or ETS challenge, and bronchoalveolar lavage (BAL) fluid and lungs were analyzed. Lungs in the different groups of mice were equivalently inflated with an intratracheal injection of a similar volume of 4% paraformaldehyde solution (Sigma Chemicals, St. Louis, MO) to preserve the pulmonary architecture. Lungs from the different experimental groups were processed as a batch for either histological staining or immunostaining under identical conditions. Stained and immunostained slides were all quantified under identical light microscope conditions, including magnification (×20), gain, camera position, and background illumination. The quantitative histological and image analysis of all coded slides was performed by research associates blinded to the coding of all the slides.

Effect of ETS on the ability of corticosteroids to reduce eosinophilic airway inflammation. To determine if exposure to ETS interfered with the ability of corticosteroids to reduce levels of eosinophilic airway inflammation, we quantitated total BAL eosinophil counts and the number of peribronchial major basic protein positive (MBP+) cells as previously described (8, 23). In brief, lung sections were processed for MBP immunohistochemistry using an anti-mouse MBP antibody (kindly provided by Dr. James Lee, Mayo Clinic,
Scottsdale, AZ). The number of individual cells staining positive for MBP in the peribronchial space was counted using a light microscope. Results are expressed as the number of peribronchial cells staining positive for MBP per bronchiole with 150–200 μm of internal diameter. At least 10 bronchioles were counted in each slide.

**Effect of ETS on the ability of corticosteroids to reduce airway mucus.** The number of periodic acid-Schiff (PAS)-positive and PAS-negative airway epithelial cells in individual bronchioles were counted as previously described (8, 23). At least 10 bronchioles were counted in each slide. Results are expressed as the percentage of PAS-positive cells per bronchiole, which is calculated from the number of PAS-positive epithelial cells per bronchus divided by the total number of epithelial cells of each bronchiole.

**Effect of ETS on the ability of corticosteroids to reduce peribronchial fibrosis.** The area of peribronchial trichrome staining in paraffin-embedded lung was outlined and quantified using a light microscope (Leica DMLS, Leica Microsystems) attached to an image analysis system (Image-Pro Plus, Media Cybernetics) as previously described (8, 23). Results are expressed as the area of trichrome staining per micrometer length of basement membrane of bronchioles 150–200 μm of internal diameter.

**Effect of ETS on the ability of corticosteroids to reduce peribronchial TGF-β1 + cells.** The number of peribronchial cells expressing TGF-β1 was assessed in lung sections processed for immunohistochemistry using an anti-TGF-β1 primary antibody (Santa Cruz, CA), the immunoperoxidase method, and image analysis quantitation as previously described (8, 23). Results are expressed as the number of TGF-β1-positive cells/bronchus (8, 23).

**Effect of ETS on the ability of corticosteroids to reduce the thickness of the peribronchial smooth muscle layer.** The thickness of the airway smooth muscle layer was measured using an image analysis system, and the peribronchial area that stained with an antibody to α-smooth muscle actin quantitated as previously described (8, 23). In brief, the thickness of the smooth muscle layer (the transverse diameter) was measured from the innermost aspect to the outermost aspect of the smooth muscle layer. The smooth muscle layer thickness in at least 10 bronchioles of similar size (150–200 μm) was counted on each slide.

Lung sections were also immunostained with an anti-α-smooth muscle actin primary antibody (Sigma-Aldrich). The area of α-smooth muscle actin staining was outlined and quantified using a light microscope attached to an image analysis system as previously described (8, 23). Results are expressed as the area of α-smooth muscle actin staining per micrometer length of basement membrane of bronchioles 150–200 μm of internal diameter.

**Effect of ETS on the ability of corticosteroids to reduce airway hyperreactivity.** AHR to MCh was assessed 24 h after the final chronic OVA and/or chronic ETS challenge (after 1 mo of repetitive OVA ± ETS challenges) in intubated and ventilated mice (flexiVent ventilator; Scireq, Montreal, PQ) as previously described in this laboratory (20). The frequency-independent airway resistance was determined in mice exposed to nebulized PBS and MCh (3, 24, 48 mg/ml) (20).

Percentage reduction in inflammation and remodeling in response to corticosteroid therapy. To calculate the % reduction in response to corticosteroid therapy in individual indices of airway inflammation and remodeling, the absolute increase in each of the individual indices of airway inflammation and remodeling in response to OVA + ETS were calculated according to the formula (OVA + ETS) – (no OVA + ETS) or OVA + ETS). This value is the maximum increase induced by OVA + ETS above baseline values. The reduction of this value induced by corticosteroid therapy was expressed as a %.

Statistical analysis. Results in the different groups of mice were compared by ANOVA using the nonparametric Kruskal-Wallis test followed by posttesting using Dunn’s multiple comparison of means. All results are presented as means ± SD. A statistical software package (Graph Pad Prism, San Diego, CA) was used for the analysis. P values of < 0.05 were considered statistically significant.

**RESULTS**

**Effect of ETS on the ability of corticosteroids to reduce eosinophilic airway inflammation.** Exposure of mice to chronic ETS alone did not induce an increase in BAL eosinophils (Fig. 1A) or MBP+ peribronchial eosinophils (Fig. 1B) compared with non-ETS-exposed mice [ETS + no OVA vs. no ETS + no OVA, P = not significant (ns)]. In contrast, exposure of mice to chronic OVA challenge alone induced a significant increase in the number of BAL eosinophils compared with non-OVA-challenged mice (8.83 ± 2.98 × 10^4 vs. 0.20 ± 0.01 × 10^4 BAL eosinophils; OVA + no ETS vs. no OVA + no ETS, P < 0.0005) (Fig. 1A). A similar significant increase in the number of MBP+ peribronchial eosinophils was noted in mice exposed to chronic OVA challenge alone compared with non-OVA-
challenged mice (31.1 ± 5.8 vs. 0.7 ± 0.3 peribronchial MBP+ eosinophils; OVA + no ETS vs. no OVA + no ETS, \( P < 0.0005 \)) (Fig. 1B).

The combination of chronic ETS and chronic OVA allergen exposure induced significantly increased levels of BAL eosinophils compared with chronic ETS alone (ETS + OVA vs. ETS + no OVA, \( P < 0.0005 \)) (Fig. 1A) or compared with chronic OVA allergen alone (ETS + OVA vs. no ETS + OVA, \( P < 0.05 \)) (Fig. 1A). Similarly, the combination of chronic ETS and chronic OVA allergen exposure induced significantly increased levels of peribronchial MBP+ eosinophils compared with chronic ETS alone (ETS + OVA vs. ETS + no OVA, \( P < 0.0005 \)) (Fig. 1A). Similarly, administration of corticosteroids to mice exposed to chronic ETS and OVA allergen significantly reduced levels of peribronchial eosinophils by \( \approx 95\% \) compared with chronic ETS and OVA allergen-challenged mice that did not receive corticosteroids (14.19 ± 4.07 × 10^3 vs. 0.83 ± 0.27 × 10^4 BAL eosinophils; ETS + OVA vs. ETS + OVA + corticosteroids, \( P < 0.0005 \)) (Fig. 1A). Similarly, administration of corticosteroids to mice exposed to chronic ETS and OVA allergen significantly reduced levels of peribronchial eosinophils by \( \approx 94\% \) compared with chronic ETS and OVA allergen-challenged mice that did not receive corticosteroids (48.4 ± 16.9 vs. 2.9 ± 1.3 peribronchial MBP+ eosinophils; ETS + OVA vs. ETS + OVA + corticosteroids, \( P < 0.0005 \)) (Fig. 1B).

**Effect of ETS on the ability of corticosteroids to reduce airway mucus.** Exposure of mice to chronic ETS alone did not induce a change in the % of airway epithelium that stained positive with PAS compared with non-ETS-exposed mice (ETS + no OVA vs. no ETS + no OVA) (\( P = \text{ns} \)) (Fig. 2). In contrast, exposure of mice to chronic OVA challenge alone induced a significant increase in the % of airway epithelium that stained positive with PAS compared with non-OVA-challenged mice (32.5 ± 1.9 vs. 0.0 ± 0.0% PAS-positive cells/bronchus) (OVA + no ETS vs. no OVA + no ETS) (\( P < 0.0001 \)) (Fig. 2). The combination of chronic ETS and chronic OVA allergen exposure induced a significant increase in the % of airway epithelium that stained positive with PAS compared with chronic ETS alone (ETS + OVA vs. ETS + no OVA) (\( P < 0.0001 \)) or compared with chronic OVA alone (\( P < 0.0001 \)) (Fig. 2).

Administration of corticosteroids to mice exposed to chronic ETS and OVA allergen significantly reduced levels of mucus expression by \( \sim 81\% \) compared with chronic ETS and OVA allergen-challenged mice that did not receive corticosteroids (51.3 ± 11.2 vs. 9.6 ± 12.1% PAS-positive cells/bronchus) (ETS + OVA vs. ETS + OVA + corticosteroids) (\( P < 0.0001 \)) (Fig. 2).

**Effect of ETS on the ability of corticosteroids to reduce peribronchial fibrosis.** Exposure of mice to chronic ETS alone did not increase levels of peribronchial fibrosis as assessed by image analysis quantitation of the area of peribronchial trichrome staining (ETS + no OVA vs. no ETS + no OVA) (\( P = \text{ns} \)) (Fig. 3). A significant increase in the area of peribronchial trichrome staining was noted in mice following exposure to chronic OVA alone compared with non-OVA-exposed mice (0.27 ± 0.16 vs. 0.71 ± 0.33 \( \mu \)m^2/\( \mu \)m circumference of bronchiole) (no ETS + no OVA vs. no ETS + OVA) (\( P < 0.0001 \)) (Fig. 3). The combination of chronic ETS and chronic OVA allergen exposure induced a significantly greater increase in levels of peribronchial trichrome staining compared with chronic ETS alone (ETS + OVA vs. ETS + no OVA) (\( P < 0.0001 \)) (Fig. 3) or compared with chronic OVA allergen alone (ETS + OVA vs. no ETS + OVA) (\( P < 0.05 \)) (Fig. 3).

Administration of corticosteroids to mice exposed to chronic ETS and OVA allergen significantly reduced levels of peribronchial trichrome staining by \( \approx 47\% \) compared with chronic ETS and OVA allergen-challenged mice that did not receive corticosteroids (0.89 ± 0.47 vs. 0.61 ± 0.41 \( \mu \)m^2/\( \mu \)m circumference of bronchiole) (ETS + OVA vs. ETS + OVA + corticosteroids) (\( P < 0.0001 \)) (Fig. 3).

**Effect of ETS on the ability of corticosteroids to reduce peribronchial TGF-\( \beta \)-positive cells.** Exposure of mice to chronic ETS alone did not induce an increase in the number of peribronchial TGF-\( \beta \)-positive cells (Fig. 4) compared with non-ETS-exposed mice (ETS + no OVA vs. no ETS + no OVA) (\( P = \text{ns} \)). In contrast, exposure of mice to chronic OVA challenge alone induced a significant increase in the number of peribronchial TGF-\( \beta \)-positive cells compared with non-OVA-challenged mice (50.4 ± 14.7 vs. 2.0 ± 1.6 TGF-\( \beta \)-positive cells/bronchus) (OVA + no ETS vs. no OVA + no ETS) (\( P < 0.0001 \)) (Fig. 4). The combination of chronic ETS and chronic OVA allergen exposure induced a significant increase in the number of peribronchial TGF-\( \beta \)-positive cells compared with chronic ETS alone (ETS + OVA vs. ETS + no OVA) (\( P < 0.0001 \)) (Fig. 4) or compared with chronic OVA allergen alone (ETS + OVA vs. no ETS + OVA) (\( P < 0.0001 \)) (Fig. 4).

Administration of corticosteroids to mice exposed to chronic ETS and OVA allergen significantly reduced the number of peribronchial TGF-\( \beta \)-positive cells by \( \sim 72\% \) compared with chronic ETS and OVA allergen-challenged mice that did not
receive corticosteroids (65.1 ± 17.4 vs. 19.5 ± 8.2 TGF-β1-positive cells/bronchus) (ETS + OVA vs. ETS + OVA + corticosteroids) (P = 0.0001) (Fig. 4).

**Effect of ETS on the ability of corticosteroids to reduce the thickness of the peribronchial smooth muscle layer.** Exposure of mice to chronic ETS alone did not induce an increase in thickness of the peribronchial smooth muscle layer compared with non-ETS-exposed mice (ETS + no OVA vs. no ETS + no OVA) (P = ns) (Fig. 5A). In contrast, exposure of mice to chronic OVA alone induced an increase in thickness of the peribronchial smooth muscle layer compared with non-ETS-exposed mice (8.7 ± 2.1 vs. 3.4 ± 1.2 μm) (no ETS + OVA vs. no ETS + no OVA) (P < 0.0001) (Fig. 5A). The combination of chronic ETS and chronic OVA allergen exposure induced significantly increased levels of thickness of the peribronchial smooth muscle layer compared with chronic ETS alone (ETS + OVA vs. ETS + no OVA) (P < 0.0001) (Fig. 5A) or compared with chronic OVA allergen alone (ETS + OVA vs. no ETS + OVA) (P < 0.01) (Fig. 5A).

**Fig. 3.** Mouse lungs were stained with trichrome, and the area of peribronchial trichrome staining quantitated in μm²/μm length of bronchus by image analysis in both non-ETS-exposed mice (no OVA, OVA) and chronic ETS-exposed mice (no OVA, OVA, OVA + corticosteroids). In ETS-exposed mice challenged with OVA, corticosteroids significantly reduced the area of peribronchial trichrome staining (P < 0.0001; ETS + OVA + corticosteroids vs. ETS + OVA).

**Fig. 4.** Mouse lungs were immunostained with an anti-TGF-β1 antibody, and the number of peribronchial cells immunostaining positive for TGF-β1 quantitated by image analysis in both non-ETS-exposed mice (no OVA, OVA) and chronic ETS-exposed mice (no OVA, OVA, OVA + corticosteroids). In ETS-exposed mice challenged with OVA, corticosteroids significantly reduced the number of peribronchial cells immunostaining positive for TGF-β1 (P < 0.0001; ETS + OVA + corticosteroids vs. ETS + OVA).

**Fig. 5.** Mouse lungs were processed for immunohistology to measure both the thickness of the peribronchial smooth muscle layer in micrometers as assessed by image analysis (A) as well as the area of peribronchial region immunostaining positive with an α-smooth muscle actin antibody (quantitated in μm²/μm length of the basement membrane of the bronchus by image analysis) (B). In ETS-exposed mice challenged with OVA, corticosteroids significantly reduced the thickness of the peribronchial smooth muscle layer (P < 0.0001; ETS + OVA + corticosteroids vs. ETS + OVA) (A) as well as the area of the peribronchial region immunostaining positive with an α-smooth muscle actin antibody (P < 0.0001; ETS + OVA + corticosteroids vs. ETS + OVA) (B).
Administration of corticosteroids to mice exposed to chronic ETS and OVA allergen significantly reduced the thickness of the peribronchial smooth muscle layer by ~70% compared with chronic ETS and OVA allergen-challenged mice that did not receive corticosteroids (9.3 ± 2.4 vs. 5.6 ± 1.8 μm) (ETS + OVA vs. ETS + OVA + corticosteroids) (P < 0.0001) (Fig. 5A).

In addition to measuring the thickness of the smooth muscle layer, we also determined the area of peribronchial α-smooth muscle actin immunostaining. Exposure of mice to chronic ETS alone did not increase the area of peribronchial α-smooth muscle actin immunostaining compared with non-ETS-exposed mice (ETS + no OVA vs. no ETS + no OVA) (P = ns) (Fig. 5B). In contrast, exposure of mice to chronic OVA challenge alone induced a significant increase in the area of peribronchial α-smooth muscle actin immunostaining compared with either chronic ETS alone (ETS + OVA vs. ETS + no OVA) (P < 0.0001) (Fig. 5B). The combination of chronic ETS and chronic OVA allergen exposure induced significantly increased levels of peribronchial α-smooth muscle actin immunostaining compared with either chronic ETS alone (ETS + OVA vs. ETS + no OVA) or compared with chronic OVA allergen alone (ETS + OVA vs. no ETS + OVA) (P < 0.005) (Fig. 5B).

Administration of corticosteroids to mice exposed to chronic ETS and OVA allergen significantly reduced the area of peribronchial α-smooth muscle actin immunostaining by ~58% compared with chronic ETS and OVA allergen-challenged mice that did not receive corticosteroids (1.38 ± 0.61 vs. 0.89 ± 0.44 μm²/μm circumference of bronchiole) (ETS + OVA vs. ETS + OVA + corticosteroids) (P < 0.0001) (Fig. 5B).

Effect of ETS on the ability of corticosteroids to reduce airway hyperreactivity. To determine whether the increased thickness of the smooth muscle layer associated with exposure of mice to chronic ETS and chronic OVA was associated with increased responsiveness of airway smooth muscle, we determined levels of airway responsiveness to MCh in mice exposed to chronic ETS and/or chronic OVA allergen. Exposure of mice to chronic ETS alone did not induce a change in AHR to MCh compared with non-ETS-exposed mice (no OVA + ETS vs. no OVA + no ETS) (P = ns) (Fig. 6). In contrast, exposure of mice to chronic OVA challenge alone induced a significant increase in AHR compared with non-OVA-challenged mice (OVA + no ETS vs. no-OVA + no ETS) (48 mg/ml MCh, P < 0.0001) (Fig. 6). The combination of chronic ETS and chronic OVA allergen exposure induced a significantly greater increase in AHR compared with chronic ETS alone (OVA + ETS vs. no OVA + ETS) (48 mg/ml MCh, P < 0.0001) (Fig. 6) or compared with chronic OVA allergen alone (OVA + ETS vs. OVA + no ETS) (48 mg/ml MCh, P < 0.005) (Fig. 6).

Administration of corticosteroids to mice exposed to chronic ETS and OVA allergen significantly reduced AHR compared with chronic ETS and OVA allergen-challenged mice that did not receive corticosteroids (OVA + ETS vs. OVA + ETS + corticosteroids) (P < 0.005) (Fig. 6).

**DISCUSSION**

This study demonstrates that exposure of mice to low levels of tobacco smoke contained in ETS does not impair the ability of corticosteroids to inhibit airway inflammation, mucus expression, airway remodeling, and AHR in a mouse model of chronic allergen-induced asthma. Indeed, chronic exposure to ETS did not prevent corticosteroids from nearly completely inhibiting eosinophilic inflammation (~95% reduction) and mucus expression (~81% reduction). These levels of inhibition of airway inflammation in ETS-exposed mice are similar to levels of inhibition of airway inflammation achieved by corticosteroids in non-ETS-exposed mice in previous studies from our laboratory (8, 23). In addition, ETS exposure did not inhibit the ability of corticosteroids to reduce levels of airway remodeling and AHR. Although the levels of tobacco smoke exposure in ETS are significantly lower than levels of tobacco smoke exposure in active smokers, ETS induced significant proinflammatory effects in this mouse model as evidenced by increased levels of airway inflammation, mucus, remodeling, and AHR in ETS- and OVA-exposed mice compared with either ETS alone, or to OVA alone, exposed mice. Thus, the inability of ETS to impair the function of corticosteroids cannot be ascribed to a lack of biological activity of the ETS exposure in the airway in the mice studied.

In allergen- and ETS-exposed mice, the increased levels of peribronchial eosinophils and cells expressing TGF-β1 may contribute to airway remodeling as studies in mice depleted of eosinophils (7, 19) as well as studies in asthmatics depleted of eosinophils with anti-IL-5 (13) demonstrate reduced levels of airway remodeling. In addition, inhibiting TGF-β1 signaling in mice inhibits airway remodeling in several (1, 18, 21), but not all, studies (12). Thus, the ability of corticosteroids to significantly reduce levels of peribronchial eosinophils and cells expressing TGF-β1 in ETS- and allergen-exposed mice may contribute to a reduction in levels of airway remodeling and airway responsiveness. Interestingly, recent studies investigating the effect of inhaled corticosteroids (400 μg budesonide daily for 3 years) on reducing the decline in lung function in newly diagnosed
asthma (25) demonstrated that budesonide reduced the decline in lung function in nonsmokers (−2.45% placebo vs. −1.53% budesonide) as well as in smokers (−4.36% placebo vs. −2.84% budesonide), suggesting that inhaled corticosteroids may have an effect on reducing airway remodeling in smokers similar to what we have noted with the effect of corticosteroids reducing airway remodeling in ETS-exposed mice.

AHR is a cardinal feature of asthma. Levels of AHR to direct stimuli such as MCh are associated with asthma severity and the minimum treatment needed to control symptoms of asthma (26). Interestingly, a study investigating a treatment strategy aimed at reducing MCh AHR demonstrated a lower rate of mild asthma exacerbations, a greater improvement in the FEV1, and a significant reduction in the thickness of the subepithelial reticular layer in airway biopsies in the AHR strategy group (31). Thus, our demonstration that corticosteroids reduce AHR in the presence of exposure to ETS in a mouse model would be of importance if similar results were shown in human asthmatics.

The potential additional importance of the ability of corticosteroids to reduce airway inflammation, mucus expression, and AHR in ETS-exposed asthmatics is suggested from the large number of children, as well as adults, with asthma who are exposed to ETS. In a cross-sectional study of nonsmoking urban children aged 8–14 years, ~68% had evidence of ETS exposure as assessed by salivary cotinine levels (15). In a prospective cohort of nonsmoking adult asthmatics aged 18–50 years in California, ~29% reported some regular ETS exposure (defined as most days or nights) during an 18-mo study period (11). In cross-sectional analyses comparing ETS-exposed and non-ETS-exposed asthmatics, the ETS-exposed asthmatics had increased asthma severity, increased health care utilization for asthma (emergency department visits, urgent physician visits, hospitalizations), and worse asthma-specific quality of life (11). The asthmatics who reported cessation of ETS exposure over the 18-mo study experienced a reduction in asthma severity and decreased health care utilization consistent with improved asthma (11). Conversely, asthmatics with newly initiated significant exposure to ETS over the follow-up period had worsening of asthma severity and asthma-specific quality of life measures (11). Overall, these studies suggest an important role for ETS in asthma severity and quality of life.

We are not aware of any current human studies that have evaluated whether ETS limits the effectiveness of corticosteroids in asthma, as has been demonstrated in studies of corticosteroids and smoking asthmatics (4, 5, 17, 34, 35). Our study using a mouse model has the advantage of being able to accurately provide a well-defined level of exposure to ETS to determine whether corticosteroids are effective in the presence of ETS. The limitation of this study, as with all studies in mouse models, is that it is unknown how the results in the mouse model will translate to the effect of corticosteroids in humans with asthma exposed to ETS.

In summary, studies in asthmatics have not yet addressed whether exposure to low levels of tobacco smoke present in ETS results in impairment of response to corticosteroids, as has been reported in asthmatics who are exposed to high levels of tobacco smoke exposure (4, 5, 17, 34, 35). Using a mouse model, our studies suggest that exposure to low levels of tobacco smoke in ETS does not impair the ability of corticosteroids to reduce airway inflammation, mucus expression, airway remodeling, and AHR. However, further human studies are needed to determine whether similar results would be observed in asthmatics exposed to ETS who are treated with low or high doses of inhaled corticosteroids. Results from such studies may be of particular importance to children with the 17q21 gene variant who are at increased risk of development of asthma on exposure to ETS in early childhood (3).

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