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A M E R I C A N C O L L E G E O F
 **C H E S T**
P H Y S I C I A N S

Airway Mucosal Thickening and Bronchial Hyperresponsiveness Induced by Inhaled β_2 -Agonist in Mice*

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Background: Patients with chronic persistent asthma require frequent use of inhaled β_2 -agonist, which may result in aggravation of asthma symptoms. Our recent *in vitro* study has shown that β_2 -agonist stimulates the growth of human airway epithelial cell lines.

Study objective: To determine whether β_2 -agonist likewise affects airway epithelial cell proliferation *in vivo* and, if so, what the mechanism of action is, we examined the effect of salbutamol on the morphology of murine airways.

Methods: Seventy-two BALB/c mice were administered aerosolized salbutamol using "flow-through" nose-only inhalation chambers at daily doses of 0.2 to 20 μg for up to 6 weeks. Morphology of tracheal mucosa, labeling of epithelial cells with 5-bromo-2'-deoxyuridine (BrdU), and bronchial responsiveness were assessed.

Results: Exposure to salbutamol increased the thickness of tracheal epithelial layer and the number of BrdU-positive epithelial cells in a dose- and time-dependent manner: the values in mice receiving 20 μg salbutamol for 6 weeks were 247% and 642%, respectively, of those in control animals receiving saline solution alone. These effects were inhibited by the mitogen-activated protein (MAP) kinase kinase inhibitors PD98059 and U0126. Salbutamol also caused a decrease in the provocative concentration of methacholine to achieve 400% of baseline enhanced pause. Combined treatment with inhaled budesonide attenuated salbutamol-induced airway morphologic changes and bronchial hyperresponsiveness.

Conclusion: β_2 -agonist stimulates proliferation of airway epithelial cells and produces airway wall thickening *in vivo* via MAP kinase-dependent pathway, and these effects are prevented by inhaled corticosteroid. (CHEST 2004; 126:205-212)

Key words: airway epithelium; airway remodeling; bronchial hyperresponsiveness; corticosteroid; salbutamol

Abbreviations: BrdU = 5-bromo-2'-deoxyuridine; DMSO = dimethyl sulfoxide; MAP = mitogen-activated protein; PC₄₀₀ = provocative concentration of methacholine to achieve 400% of baseline enhanced pause; Penh = enhanced pause

Airway remodeling is an important feature of chronic persistent asthma, which includes hypertrophy of airway smooth-muscle cells, metaplasia of goblet cells, collagen deposition to basement membrane, and thickening of airway mucosa.^{1,2} The thickened airway mucosa might be produced by

cytokines and growth factors released from inflammatory cells and airway epithelial cells,^{3,4} and associated with the severity of asthma and bronchial hyperresponsiveness.⁵ The bronchodilator β_2 -adrenergic agonist has been widely used for the treatment of asthma, and because the patients with chronic severe asthma generally require frequent use of inhaled β_2 -agonist, airway mucosa is likely exposed to the drug in high concentrations. It is thus possible that pathologic changes of airway mucosa seen in asthma patients could reflect not only airway inflammation but also the action of β_2 -adrenergic agonist by itself. In fact, we have recently shown that salbutamol, a selective β_2 -adrenergic agonist, enhances the growth of cultured human airway epithelial cells lines through stimulation of mitogen-activated protein (MAP) kinase (ERK1/2) cascade.⁶

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However, the *in vivo* effect of β_2 -agonist on airway remodeling is unknown. Therefore, in the present study, we examined whether exposure to salbutamol aerosols alters morphology of airway mucosa, proliferation of airway epithelial cells, and airway reactivity in mice.

Inhaled glucocorticosteroid is the preferred controller medication for patients with persistent asthma at all levels of severity, and several studies⁷⁻⁹ have demonstrated that treatment with inhaled corticosteroid reduces airway inflammation and bronchial hyperresponsiveness. In addition to its potent anti-inflammatory actions, corticosteroid has recently been shown to inhibit the growth of certain cell types such as airway smooth-muscle cells¹⁰ and lung cancer cells.¹¹ Thus, we also studied whether addition of inhaled budesonide can prevent mucosal morphologic changes and, possibly, bronchial hyperresponsiveness induced by salbutamol.

MATERIALS AND METHODS

Agents

The following drugs and chemicals were used: salbutamol, 5-bromo-2'-deoxyuridine (BrdU) [Sigma-Aldrich; St Louis, MO], budesonide (AstraZeneca; Lund, Sweden), monoclonal anti-BrdU antibody (Boehringer Mannheim; Mannheim, Germany), rabbit anti-mouse IgG (Dako Dimension Labs; Mississauga, ON, Canada), alkaline phosphatase/rabbit anti-alkaline phosphatase conjugate (Dako Labs; Copenhagen, Denmark), PD98059 (New England Biolabs; Beverly, MA), and U0126 (Tocris; Ballwin, MO).

Exposure to Salbutamol Aerosols

The experiments were approved by the Committee on Animal Research, Tokyo Women's Medical University. Female BALB/c mice 6 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME), housed in pathogen-free rooms, and maintained on laboratory chow with free access to food and water. The mice, positioned in restraint cones, were exposed to aerosols of salbutamol or sterile saline solution, 20 min per day, for 6 days per week, for up to 6 weeks using "flow-through" nose-only inhalation chambers (Sasaki Medical; Tokyo, Japan) [Fig 1].¹² Salbutamol was dissolved in sterile saline solution (0.05 μ g/mL, 0.5 μ g/mL, and 5 μ g/mL), and aerosols of the solution were generated with an ultrasonic nebulizer (model 5000D; Shimadzu Medical; Tokyo, Japan) at 0.2 mL/min. Thus, the amounts of salbutamol administered were approximately 0.2 μ g/d, 2 μ g/d, and 20 μ g/d. We chose these doses because 2 μ g salbutamol administered to mice corresponds to approximately 1,000 μ g salbutamol for humans, which is clinically relevant amount used by patients with severe asthma.

To determine whether salbutamol-induced morphologic changes in airway mucosa are associated with the MAP kinase cascade, we examined the effects of two selective MAP kinase (also known as MEK1/2, an upstream activator of the MAP kinase) inhibitors, PD98059¹³ and U0126.¹⁴ Mice were administered for 6 weeks intraperitoneal PD98059 (10 mg/kg) or U0126 (5 mg/kg), dissolved in fresh dimethyl sulfoxide (DMSO) and

finally diluted in saline solution (final concentration DMSO was 0.1%), 60 min before each exposure to 20 μ g salbutamol. The doses of these inhibitors were chosen based on the previous studies,^{15,16} and 0.1% DMSO has been shown to have no effect on MAP kinase cascade in other tissues.¹⁵

We also studied whether inhaled glucocorticoid modifies the effect of salbutamol. Budesonide was dissolved in ethanol and delivered by the jet nebulizer (model AN-100; Murai Medical; Tokyo, Japan). The nebulizer was chilled to 0°C in an ice bath and connected to two serial condensers warmed at 60°C by a heated circulating water bath.¹⁷ The aerosols passed the condensers to ensure complete evaporation of the droplets, and the solvent vapor was removed by dissolution in the stream of water pumped counter currently to the aerosol flow. The concentration of ethanol at the apex of the nose cone was $< 3 \times 10^{-6}\%$ as determined by gas chromatography. Mice received budesonide (20 μ g/kg) 60 min before each exposure to 20 μ g salbutamol for 6 weeks.

Epithelial Layer Thickness and BrdU Labeling

Before and after 2 weeks, 4 weeks, and 6 weeks of exposure to salbutamol, the mice were killed by an overdose of intraperitoneal sodium pentobarbital. The trachea was excised, fixed in periodate-lysine-paraformaldehyde (pH 7.4) for 3 h, and washed in a buffered cacodylate solution with 7% sucrose for 12 h. Each specimen was embedded in paraffin, cut into midsagittal sections of 3- μ m thickness, and stained with hematoxylin-eosin. Sections were coded and blindly examined by a single investigator (Y.E.). The thickness of epithelial layer was assessed on five sections of the same trachea from 5 mm above the carina toward the larynx with 100 μ m apart under light microscope at 1,000 $\times$ magnification (BX60; Olympus; Tokyo, Japan). Using a digitized image projected on a videoscreen (FlexScan L360; Eizo; Tokyo, Japan), the length between the inner limit of basement membrane and the top of airway epithelium was automatically calculated by a computer software program (ImageMeasure; Phoenix Technology; Seattle, WA). Several estimates were made in each section, along with its length at 200- μ m intervals, and the values were averaged.

For the assessment of DNA synthesis, labeling of epithelial cells with BrdU, an analog of thymidine that is incorporated into the nuclear DNA in the S-phase of the cell cycle, was determined. The mice were injected intraperitoneally with BrdU (50 mg/kg body weight) in saline solution 2 h before death. Serial tracheal sections were dewaxed and immunohistochemically stained to identify cells labeled with BrdU according to the method of Bicknell et al.¹⁸ Briefly, after washing with 5% rabbit serum in Tris-buffered saline solution (TBS)/Tween 20 for 15 min, mouse anti-BrdU antibody (10 ng/mL) or the negative control, nonimmune mouse IgG (10 ng/mL) was applied in TBS/Tween 20 plus 1% bovine serum albumin for 1 h. To visualize the bound primary antibody, a second antibody, rabbit anti-mouse IgG (1:20 dilution) was applied for 30 min, followed by a tertiary enzyme-linked immune conjugate: a 1:50 dilution of alkaline phosphatase/rabbit anti-alkaline phosphatase conjugate applied for 30 min. This immune conjugate reacts with the rabbit anti-mouse bridging antibody. Alkaline phosphatase substrate was then applied for 20 min to form the colored reaction products. The BrdU-labeled cells were then counted for basement membrane length of 3 mm in each section, and the values were expressed as the number of BrdU-labeled nuclei of epithelial cells per millimeter of basement membrane.

Bronchial Responsiveness

Bronchial responsiveness was determined in awake, unrestrained mice by using barometric plethysmography (Buxco;

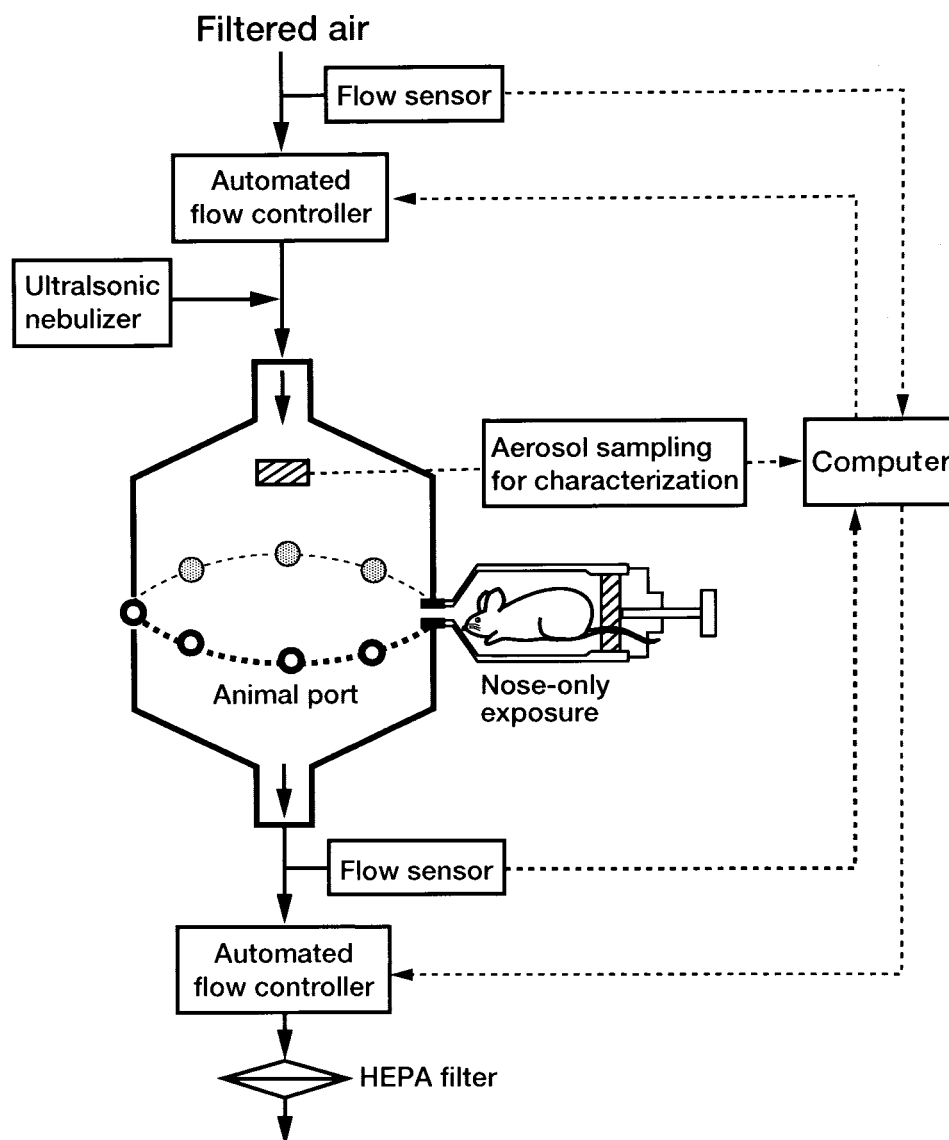


FIGURE 1. Schematic illustration of "flow-through" nose-only exposure system for small animals. HEPA = high-efficiency particulate arresting.

Troy, NY) to measure enhanced pause (Penh), a unitless measure that correlates with the changes in airway resistance during bronchial challenge with methacholine.¹⁹ Following the last exposure to salbutamol at week 6, mice were equilibrated in the plethysmography chamber for 10 min and, after baseline Penh was first established with aerosolized saline solution, dose-response curves to inhaled methacholine were obtained as follows. Aerosols of methacholine dissolved in saline solution increasing in half-log intervals from 1 to 100 mg/mL were delivered to the chamber for 1 min. Because the peak response to methacholine occurred between 3 min and 7 min after the exposure, the average Penh value obtained over these time intervals was used to measure the response to methacholine. Ten minutes were allowed to elapse between aerosol administrations. Data for each mouse were plotted, and the provocative concentrations of methacholine to achieve 400% of baseline Penh (PC₄₀₀) were calculated by using appropriate software (GraphPad Software; San Diego, CA).

Statistics

All values were expressed as means $\pm$ SEM. One-way analysis of variance and Newman-Keuls multiple comparison test were used to determine statistically significant differences between groups, and $p < 0.05$ was considered significant.

RESULTS

Tracheal Epithelial Layer Thickening and DNA Synthesis

As demonstrated in Figure 2, *top, A* and *upper middle, B*, exposure of mice to salbutamol aerosols at a daily dose of 20 μ g for 6 weeks produced an infiltration of mononuclear cells into the tracheal

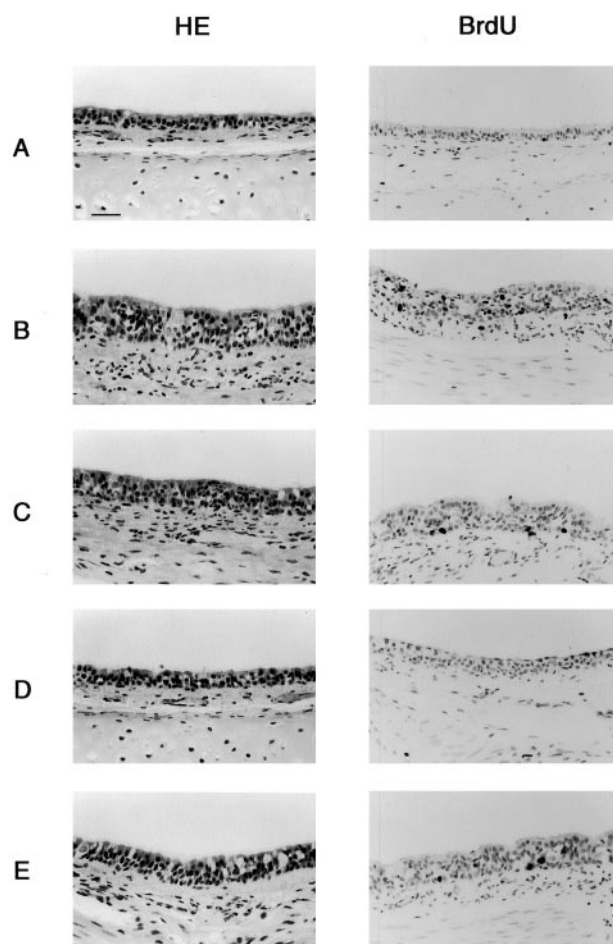


FIGURE 2. Light photomicrographs of mouse tracheal mucosa. Left panels: hematoxylin-eosin (HE) stain. Right panels: immunostaining with antibody against BrdU, which is incorporated into the DNA of replicating cells. The mice received inhaled sterile saline solution (*top*, A), inhaled salbutamol (20 µg) [*upper middle*, B], salbutamol plus intraperitoneal PD98059 (10 mg/kg) [*middle*, C], inhaled budesonide alone (20 µg/kg) [*lower middle*, D], or budesonide plus salbutamol (*bottom*, E) for 6 weeks. Bar indicates 20 µm.

wall and a marked increase in the thickness of epithelial layer, the value being 2.5- to 3-times greater than that of the control mice exposed to sterile saline solution alone. Furthermore, there were much more epithelial cells whose nuclei were stained with BrdU in the salbutamol-treated mice than in the control mice. Thickening of basement membrane or hyperplasia of goblet cells was not observed. The salbutamol-induced increases in the epithelial layer thickness and the number of BrdU-positive epithelial cells were dose- and time-dependent (Fig 3); these measures significantly increased at week 2 and thereafter in the mice receiving salbutamol at daily doses of 2 µg and 20 µg. At week 6, the epithelial layer thickness and the number of BrdU-positive cells in the salbutamol (20 µg)-treated

mice were $247 \pm 24\%$ and $642 \pm 58\%$ of the control mice, respectively ($p < 0.001$, $n = 8$ for each).

To test a possible contribution of MAP kinase cascade to the observed effects of salbutamol, a MAP kinase kinase inhibitor, PD98059 or U0126, was administered before each exposure to 20 µg salbutamol. Treatment with PD98059 reduced the salbutamol-induced increases in epithelial layer thickness and BrdU-positive cell number at any time points by 94 to 115% ($p < 0.01$, $n = 8$ for each) [Figs 2, *middle*, C, 3]. At week 6, treatment with U0126 likewise attenuated the effects of salbutamol on epithelial layer thickness by $86 \pm 7\%$ ($p < 0.01$, $n = 8$) and DNA synthesis by $91 \pm 10\%$ ($p < 0.01$, $n = 8$) [Fig 4]. Inhalation of budesonide (20 µg/kg) for 6 weeks *per se* had no significant effect on tracheal mucosal morphology or DNA synthesis of airway epithelium (Fig 2, *lower middle*, D). However, the salbutamol (20 µg)-induced increases in epithelial layer thickness and the number of BrdU-positive cells were reduced by combined inhalation with budesonide by $72 \pm 11\%$ ($p < 0.01$, $n = 8$) and $80 \pm 9\%$ ($p < 0.01$, $n = 8$), respectively (Figs 2, E, 4).

Bronchial Responsiveness

As shown in Figure 5, exposure of mice to 20 µg salbutamol for 6 weeks induced bronchial hyperresponsiveness to methacholine. The PC_{400} values of the control mice (76.2 ± 10.6 mg/mL) were decreased to 23.0 ± 5.3 mg/mL at the end of salbutamol exposure ($p < 0.05$, $n = 8$). Pretreatment with PD98059 reduced the salbutamol-induced bronchial hyperresponsiveness, the PC_{400} value being 53.1 ± 7.7 mg/mL ($n = 8$). When the mice received inhaled budesonide (20 µg/kg), the PC_{400} value was not significantly changed by the exposure to salbutamol (82.3 ± 11.5 mg/mL and 70.5 ± 10.4 mg/mL, respectively; $n = 8$).

DISCUSSION

Treatment of asthma attack has relied primarily on the reduction of bronchospasm by the use of β_2 -adrenergic agonists, but long-term, regular β_2 -agonist usage may be associated with exacerbations of asthma symptoms and airway hyperresponsiveness.²⁰ In the present study, we found that long-term exposure of mice to aerosols of salbutamol, a specific β_2 -adrenergic agonist, caused a remarkable increase in the thickness of epithelial layer of tracheal mucosa in a time- and dose-dependent manner, and that this effect was accompanied by the increase in the number of BrdU-positive epithelial cells, indicating an up-regulation of DNA synthesis. These results

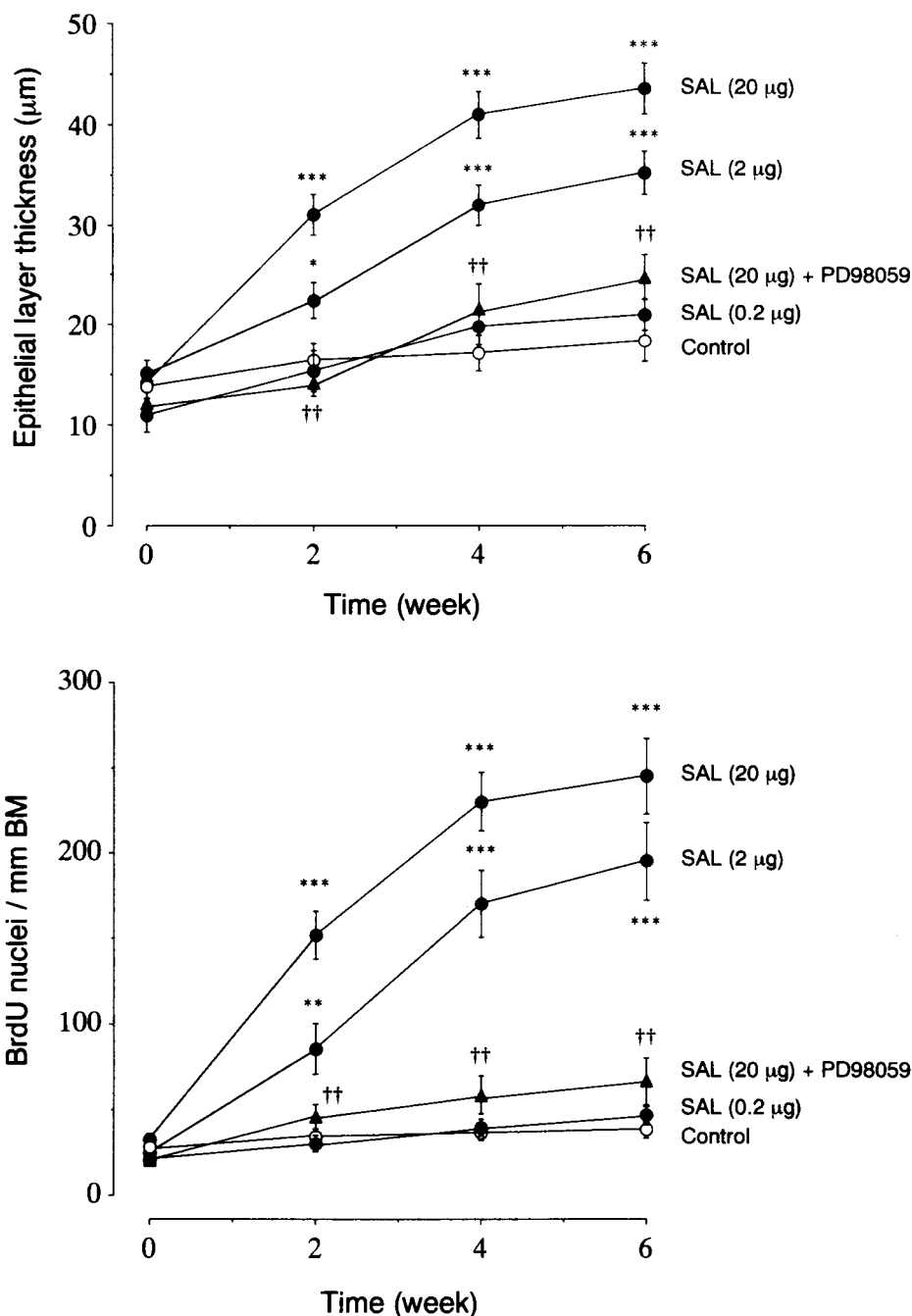


FIGURE 3. Time-dependent increases in the thickness of epithelial layer (*top*) and the number of BrdU-positive epithelial cells per millimeter of basement membrane (BM) [*bottom*] in the mouse trachea during exposure to salbutamol (SAL) at daily doses of 0.2 μg, 2 μg, and 20 μg. For the control experiment, the mice received only sterile saline solution. In some mice, PD98059 (10 mg/kg, intraperitoneal) was administered daily 60 min before exposure to salbutamol. Data are means $\pm$ SEM; n = 8 for each point. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from control values. ††p < 0.01, significantly different from corresponding values for salbutamol (20 μg) alone.

suggest that chronic use of β_2 -agonist produces an enhanced proliferation of airway epithelial cells, thereby producing airway wall thickening, one of the characteristic features of airway remodeling in asthma.^{1,2} In addition, such alterations in airway geom-

etry may result in the development of bronchial hyperresponsiveness,²¹ and we found that inhaled salbutamol for 6 weeks caused hyperresponsiveness to methacholine.

Regarding the effects of long-term administration

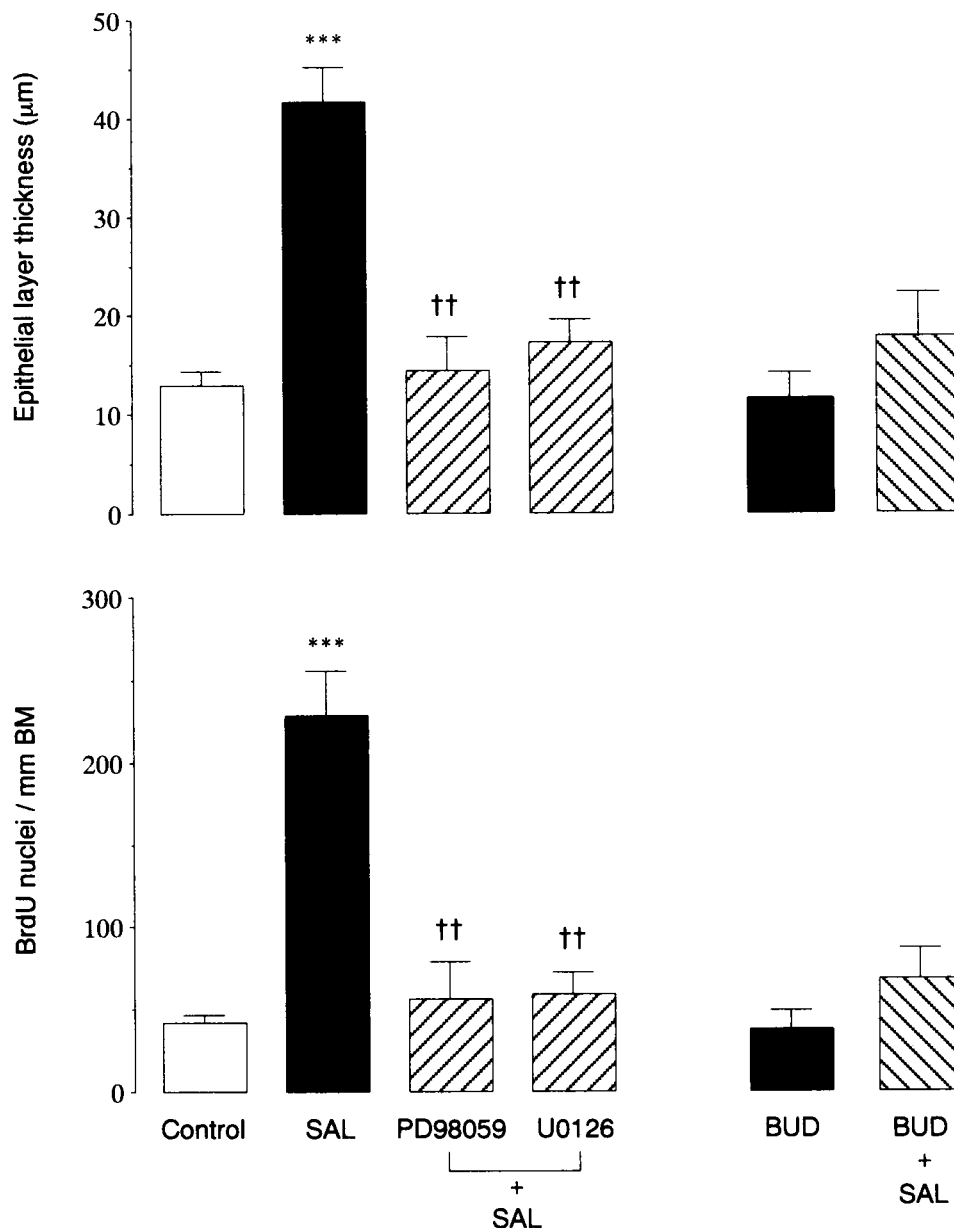


FIGURE 4. Effects of MAP kinase kinase inhibitors and budesonide on salbutamol-induced increases in the thickness of epithelial layer (*top*) and the number of BrdU-positive epithelial cells per millimeter of basement membrane (*bottom*). The mice received sterile saline solution (control) or salbutamol (20 μg) for 6 weeks, and some mice received intraperitoneal PD98059 (10 mg/kg) or U0126 (5 mg/kg), or inhaled budesonide (BUD) [20 μg/kg] 60 min before exposure to salbutamol. Data are means ± SEM; n = 8 for each column. ***p < 0.001, significantly different from control values. ††p < 0.01, significantly different from values for salbutamol alone. See Figure 3 for expansion of abbreviations.

of β_2 -agonist on airway mucosal morphology, Kamauchi and colleagues²² recently reported that salbutamol administered to rats at a daily dose of 0.5 mg/kg by a subcutaneously implanted osmotic pump produced goblet-cell hyperplasia without affecting airway wall thickness. The reason for the difference between their results and ours is uncertain, but could

be due to differences in species, experimental system, and the route and duration of salbutamol administration.

We have shown that incubation of cultured human airway epithelial cell lines, 16-HBE and NCI-H292 cells, with salbutamol stimulated the growth and DNA synthesis through the activation of MAP kinase

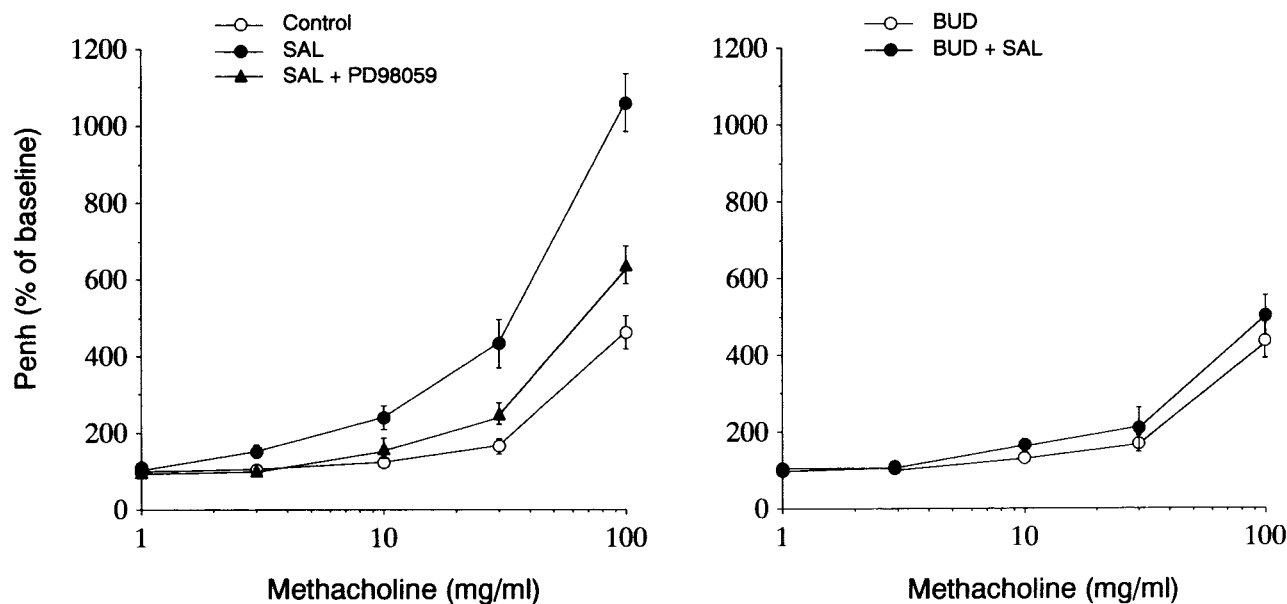


FIGURE 5. Airway responsiveness to methacholine in the mice receiving sterile saline solution (control), salbutamol (20 μ g) alone, salbutamol plus PD98059 (10 mg/kg), budesonide (20 μ g/kg) alone, or budesonide plus salbutamol (20 μ g) for 6 weeks. Values are expressed as percentage of baseline enhanced pause determined with aerosolized saline before methacholine challenge. Data are means $\pm$ SEM; n = 8 for each point. See Figures 3, 4 for expansion of abbreviations.

cascade.⁶ In accord with the *in vitro* findings, the present study confirmed that a β_2 -agonist produces a similar growth-promoting effect on airway epithelial cells *in vivo*. Furthermore, because there is increasing evidence of successful administration and efficacy of MAP kinase kinase inhibitors,^{15,16} we examined the effects of intraperitoneal injection of PD98059 and U0126, which differ only in their affinities for the kinase. Pretreatment of mice with these inhibitors almost completely inhibited the salbutamol-induced increases in tracheal epithelial layer thickness and the number of BrdU-positive epithelial cells, indicating that the growth-promoting effect of salbutamol is mediated by the MAP kinase signaling cascade.

The addition of an inhaled corticosteroid to an inhaled β_2 -agonist gives optimal control of asthma in most patients,²³ and there is evidence that long-term use of corticosteroid may prevent or reverse the thickening of submucosal basement membrane in asthmatic airways.^{24,25} In the present experiment, treatment with budesonide aerosols attenuated the thickening of epithelial layer and the increase in BrdU-positive epithelial cells induced by salbutamol. Although not confirmed, this inhibition of the cell growth may be associated with the suppressive effects of glucocorticosteroid on MAP kinase phosphorylation and/or cyclin D1 that is downstream to the MAP kinase pathway.^{10,11}

Thickening of the airway wall is one of the patho-

logic features of chronic asthma and COPD, and such airway remodeling, probably in association with basement membrane thickening and smooth-muscle hypertrophy/hyperplasia, may contribute to the development of airflow limitation and persistent bronchial hyperresponsiveness.²¹ We thus measured Penh to assess bronchoconstrictor responses to methacholine. In this experiment, the peak response to inhaled methacholine occurred between 3 min and 7 min after the exposure, the time course of which was similar to the previous study by Shore et al.²⁶ In contrast, other studies^{19,27} found the peak response within 3 min, but the mechanism of the discrepancy is unknown. We found that a 6-week exposure of mice to salbutamol increased bronchial hyperresponsiveness to methacholine, and that this effect was reduced by PD98059 and by inhaled budesonide. These changes in bronchial hyperresponsiveness were in parallel with those in airway epithelial layer thickness and DNA synthesis in airway epithelial cells. Therefore, it can be speculated that bronchial hyperresponsiveness induced by the β_2 -agonist is accomplished, at least in part, by MAP kinase-mediated remodeling of airway mucosa. However, corticosteroid has been known to inhibit the decrease in the number of airway β_2 -adrenergic receptors induced by the chronic use of β_2 -agonist²⁸ and, hence, prevent deterioration of bronchial hyperresponsiveness in asthma patients. Further stud-

ies may thus be required to determine whether such mechanism could also be involved in our experimental setting.

In conclusion, we have demonstrated for the first time that long-term exposure of airways to β_2 -agonist causes proliferation of airway epithelial cells and the resultant airway wall thickening and bronchial hyper-responsiveness *in vivo*. These effects may be mediated by the MAP kinase signaling cascade, and prevented by simultaneous exposure to inhaled corticosteroid.

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