Effect of Salmeterol on Seasonal Changes in Airway Responsiveness and Exhaled Nitric Oxide in Pollen-Sensitive Asthmatic Subjects*

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**Objective:** Using a model of natural allergen exposure, we examined the effect of regular treatment with salmeterol on allergen-induced changes in airway responsiveness and exhaled nitric oxide (ENO).

**Design:** Double-blind, randomized, parallel-group study.

**Setting:** Specialist allergy unit in a university hospital.

**Patients:** Asthmatic patients sensitized to pollen allergens were randomly allocated to monotherapy with salmeterol (n = 14) or placebo (n = 13).

**Interventions:** Salmeterol, 25 μg, and placebo inhalers, two puffs bid, for 6 weeks.

**Measurements:** Spirometry, the level of a provocative concentration of a substance (methacholine) causing a 20% fall in FEV₁ (PC₂₀), the PC₂₀ level for adenosine 5′-monophosphate (AMP), and ENO were measured before the pollen season and were repeated at the height of the pollen season after 6 weeks of treatment with salmeterol or placebo.

**Results:** The decrease in FEV₁ during the pollen season was significantly larger in the placebo group than in the salmeterol group, the mean difference in the change between the groups being 0.20 L (95% confidence interval, 0.03 to 0.35; p = 0.047). Changes in PC₂₀ for methacholine, PC₂₀ for AMP, and ENO levels were not significantly different between treatment groups. However, a mean (± SEM) decrease in the PC₂₀ for methacholine of −1.0 ± 0.4 doubling concentrations was observed within the placebo group (p = 0.03), whereas no significant changes were observed within the salmeterol group. A significant decrease in PC₂₀ for AMP (doubling concentrations) was observed within the placebo group (−2.1 ± 0.6; p = 0.003) and the salmeterol group (−1.5 ± 0.4; p = 0.003). ENO concentrations increased significantly among the placebo and the salmeterol groups during natural pollen exposure.

**Conclusion:** These observations indicate that natural pollen exposure and the regular use of salmeterol are not associated with a greater increase in ENO and airway responsiveness than allergen exposure alone.

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**Key words:** adenosine 5′-monophosphate; airway responsiveness; allergens; allergic asthma; methacholine; nitric oxide; salmeterol

**Abbreviations:** AMP = adenosine 5′-monophosphate; CI = confidence interval; ENO = exhaled nitric oxide; MDI = metered-dose inhaler; NO = nitric oxide; PC₂₀ = provocative concentration of a substance causing a 20% fall in FEV₁; ppb = parts per billion

Inhaled β₂-adrenergic agonists are the most effective of the available bronchodilator drugs used to treat acute asthma symptoms.¹ However, there is a concern that treatment with short-acting β₂-agonists may be causally associated with the increase in asthma morbidity.² Partly as a result of these concerns, the International Asthma Guidelines³ recommend that inhaled β₂-agonists be used “as required” for the relief of symptoms rather than as part of a regularly scheduled regimen.

Salmeterol is a very effective β₂-agonist, with...
long-lasting bronchodilator activity up to 12 h. It is recommended in combination with anti-inflammatory agents (such as inhaled corticosteroids) in patients with chronic asthma, particularly moderate and severe persistent disease. Concern that the adverse effects of regular short-acting inhaled β2-agonists on asthma morbidity also might occur with salmeterol has been a major factor in many clinical trials. Salmeterol studies have demonstrated no increase in asthma exacerbations compared with placebo even when used without anti-inflammatory drugs. However, several studies have demonstrated that the regular use of inhaled salmeterol produces tolerance to their bronchoprotective effect against bronchospasm induced by exercise, allergens, and methacholine. The loss of β-agonist protection against bronchoconstriction is more pronounced in relation to the effect of inhaled adenosine 5’-monophosphate (AMP), which induces bronchospasm indirectly through the activation of mast cells, than to the effect of inhaled methacholine, which causes bronchoconstriction mainly by the direct stimulation of cholinergic receptors on airway smooth muscle.

Some studies have demonstrated an increased responsiveness to methacholine after the regular use of salmeterol, although others have not. Furthermore, regular treatment with salmeterol enhances allergen-induced early bronchoconstrictor responses. Subsequent clinical studies also have suggested that, compared to placebo, 1 week of regular therapy with salmeterol may lead to an increase in airway inflammation 24 h after allergen challenge. Consequently, it was hypothesized that, especially in allergic asthmatic patients, there might be an interactive effect on inflammation between the regular use of β2-agonists and the exposure to allergens, resulting in an increased airway responsiveness. It remains, however, unclear how relevant the observations made under conditions of laboratory challenge with large quantities of allergens are to the natural course of asthma.

This study was designed to assess the interaction of inhaled salmeterol and natural allergen exposure with airway responsiveness to direct and indirect bronchoconstrictor agents and with airway inflammation in subjects with allergic asthma. To this end, we selected subjects with pollen-induced asthma to measure the effect of 6 weeks of treatment with salmeterol and placebo on allergen-induced airway responsiveness to methacholine and AMP and on allergen-induced changes in exhaled nitric oxide (ENO) concentrations during natural pollen exposure. ENO is currently considered to be a marker of airway inflammation.

Materials and Methods

Subjects

Thirty asthmatic subjects aged 17 to 55 years, from our outpatient Allergy Clinic, were studied, having met the following inclusion criteria: a history of mild seasonal asthma for at least 2 years; an FEV1 > 80% of the predicted value; a skin-prick test result (wheat diameter, ≥ 3 mm) that was positive for pollen allergens (eg, grass pollen and/or Parietaria judaica and/or Olea europaea); and no skin sensitization for other perennial allergens that were tested, namely, house dust mites, Alternaria, Aspergillus, Penicillium, Cladosporium, and cat and dog dander (Abelló SA; Madrid, Spain). Outside of the pollen season, these subjects had no asthma symptoms, and none had required asthma therapy, including β2-agonists, for at least 4 months before the first evaluation. All 30 subjects were life-long nonsmokers, and none had a history of chronic bronchitis, emphysema, or respiratory tract infections during the 4 weeks before the study began. Current or ex-smokers, pregnant women, and patients with significant renal, hepatic, or cardiovascular disease were specifically excluded. The study protocol was approved by the Medical Ethics Committee of the Hospital Universitario Dr. Peset and the National Regulatory Agency (case No. 00-0545). Written informed consent was obtained from each subject prior to participation.

Study Design

This was a double-blind, randomized, placebo-controlled, parallel-group study. Subjects were first evaluated between mid-January and the end of February, before the pollen season began. Subjects visited the laboratory twice, at the same time of day (± 2 h). On the first day, spirometry and methacholine challenge tests were performed. The subjects returned 5 to 7 days after the initial evaluation, and measurements were made in the following order: ENO; spirometry; and AMP challenge. Subjects then were randomized to receive 50 µg (2 puffs) salmeterol xinafoate (Serevent; GlaxoSmithKline; Madrid, Spain) twice per day by metered-dose inhaler (MDI) or 2 puffs twice per day by MDI of placebo, which they commenced taking in April. Salmeterol and placebo were administered using a large volume spacer device (Volumatic; GlaxoSmithKline). Albuterol MDI and antihistamines (ie, cetirizine and loratadine) were used on an “as-needed” basis to control pulmonary or nasal symptoms, respectively. No other medications were allowed to be used during the study. Subjects were asked not to take albuterol for at least 8 h and antihistamines for at least 72 h before each challenge.

Subjects returned to the laboratory at the height of the pollen season (seasonal assessment) in May-June (after 6 weeks of treatment), having taken their last dose of trial medication 12 to 15 h earlier. Spirometry and methacholine challenge were performed the first day, whereas the determination of ENO and AMP challenge were performed after 5 to 7 days. Therapy with the test medication was resumed after the methacholine challenge and continued until 12 to 15 h before the AMP challenge. Used canisters were collected and were weighed before and after use in order to check compliance. Treatment compliance was reported as the actual canister weight loss as a percentage of the expected weight loss. The latter was estimated from the known weight loss per dose and the number of doses prescribed.

Pulmonary Function

Spirometry was performed with a calibrated dry rolling seal spirometer (model 2130; SensorMedics; Yorba Linda, CA) ac-
Inhalation Challenge Tests

Inhalation provocation tests were performed using the method described by Chai et al\textsuperscript{23} with some modifications.\textsuperscript{22} Methacholine and AMP (Sigma Chemical Co; St. Louis, MO) were dissolved freshly in 0.9% saline solution to produce a doubling concentration range of 0.095 to 25 mg/mL for methacholine and from 0.39 to 400 mg/mL for AMP. Each solution was administered from a jet nebulizer attached to a breath-activated dosimeter (model MB3; Mefar, Brescia, Italy) at a nebulization time of 1 s with a pause time of 6 s. The nebulizer delivers particles with an aerodynamic mass median diameter of 3.5 to 4.0 pm at an output of 10 μL per breath. Subjects inhaled the aerosolized methacholine and AMP solutions in five inhalations from functional residual capacity to total lung capacity through a mouth-piece with the subject’s nose clipped. Normal saline solution was inhaled initially, followed by five breaths of doubling concentrations of methacholine or AMP at 2 to 3-min intervals. Due to the effect of a deep inspiration on subsequent airway tone,\textsuperscript{23} single administrations of methacholine or AMP at 2 to 3-min intervals. Due to the effect of a deep inspiration on subsequent airway tone,\textsuperscript{23} single measurements of FEV\textsubscript{1} were made 60 to 90 s after the inhalation. The highest value was recorded or when the highest concentration of AMP given (400 mg/mL). A PC\textsubscript{20} value for AMP calculated as \( \log \text{PC}_{20}/\log 2 \). A PC\textsubscript{20} value for AMP calculated as \( \log \text{PC}_{20}/\log 2 \).

Data Analysis

The primary outcome of the study was the PC\textsubscript{20} for AMP level. Secondary outcomes of the study were PC\textsubscript{20} for methacholine, FEV\textsubscript{1}, and ENO levels. The power of the study was calculated on the basis of an estimate of within-subject variability from a previous study\textsuperscript{25} in which PC\textsubscript{20} for AMP values were measured in subjects with mild asthma. The within-subject SD was 0.45 (log value). With a α value set at 5% and a power of 80%, 26 patients (13 in each group) were required to detect a 1.5 doubling concentration difference in the PC\textsubscript{20} for AMP level. A PC\textsubscript{20} for methacholine value of 25 mg/mL was assigned to eight subjects (four in each group) in the pre-pollen season assessment, and to one subject (salmeterol group) in the pollen season assessment, in whom FEV\textsubscript{1} dropped < 20% even when the highest concentration of methacholine was used. Further, a PC\textsubscript{20} value for AMP could not be calculated in nine subjects (salmeterol group, four subjects; placebo group, five subjects) during the pre-pollen season assessment and in two subjects (one in each group) during the pollen season. On these occasions, the PC\textsubscript{20} value was censored to the highest concentration of AMP given (400 mg/mL).

All PC\textsubscript{20} and ENO values were log-transformed before analysis and were presented as geometric means. Changes in PC\textsubscript{20} were expressed in terms of doubling concentrations of methacholine or AMP calculated as \( \Delta \log \text{PC}_{20} / \log 2 \).

Comparisons of the baseline characteristics of the two groups were performed by unpaired Student \( t \)-tests for continuous data and by Fisher exact tests for categoric data. Changes in FEV\textsubscript{1}, ENO, and PC\textsubscript{20} were assessed by paired \( t \)-tests within each randomized group and by an unpaired \( t \)-test to compare differences between groups. Correlations were explored using the Spearman rank correlation test. \( p \) Values are two-sided, and values < 0.05 were considered to be statistically significant. Data were analyzed with a standard statistical software package (SPSS, version 6.01 for Windows; SPSS Inc; Chicago, IL).

Results

Of the 30 subjects initially recruited, 3 were withdrawn from the study before the completion of the first month of treatment. One patient (placebo group) declined the previous acceptance of participation, while two patients (one in each group) failed to complete the study due to worsening of asthma and the need to use oral steroids. Only the data for the remaining 27 subjects who completed the study period were included in the final analysis. Of those who completed the study, 14 were randomized to receive salmeterol 13 and 13 to receive placebo. One subject (salmeterol group) dropped out of the study after the methacholine challenge was performed during natural pollen exposure. However, the remainder of the study was completed. Thus, in the salmeterol group, there were evaluable data on 13 subjects for FEV\textsubscript{1}, methacholine PC\textsubscript{20}, and ENO, and there were evaluable data on 13 subjects for the AMP PC\textsubscript{20}. The two groups (Table 1) did not differ with respect to age, sex, time since diagnosis of asthma, pulmonary function, or ENO. PC\textsubscript{20} values for methacholine and AMP were higher in the placebo group, although the differences were not significant. The mean treatment compliance rate was 83% in the salmeterol group and 90% in the placebo group (\( p = 0.34 \)).
Lung Function

In the salmeterol group, the FEV₁ increased from a mean (± SEM) baseline value of 3.18 ± 0.19 L during the pre-pollen season period to 3.21 ± 0.20 L during the pollen season (p = 0.59). In the placebo group, the mean FEV₁ values decreased from 3.21 ± 0.27 L during the pre-pollen season period to 3.03 ± 0.22 L during the pollen season (p = 0.07). The decrease in FEV₁ was significantly greater in the placebo group than in the salmeterol group, the mean difference in the change between the groups being 0.20 L (95% confidence interval [CI], 0.03 to 0.35; p = 0.047).

Methacholine PC₂₀

Values of PC₂₀ before and during the pollen season for the two treatment groups are shown in Figure 1. The geometric mean methacholine PC₂₀ values decreased in the salmeterol group from 5.4 mg/mL (range, 0.6 to 25.0 mg/mL) during the pre-pollen season assessment to 4.8 mg/mL (range, 0.5 to 25.0 mg/mL; p = 0.69) during the pollen season. The mean (± SEM) reduction was −0.2 ± 0.5 doubling concentrations. In the placebo group, there was a decrease from 7.6 mg/mL (range, 0.8 to 25.0 mg/mL) in the pre-pollen season assessment to 3.7 mg/mL (range, 0.4 to 22.5 mg/mL; p = 0.03) during the pollen season. The mean (± SEM) reduction was −1.0 ± 0.4 doubling concentrations. Changes in methacholine PC₂₀ values were not significantly different between the salmeterol and placebo groups, the mean difference being 0.8 doubling concentrations (95% CI, −0.5 to 2.2 doubling concentrations; p = 0.20).

AMP PC₂₀

The values for PC₂₀ before and during the pollen season for the two treatment groups are shown in Figure 2. The geometric mean AMP PC₂₀ value decreased in the salmeterol group from 134.9 mg/mL (range, 10.7 to 400.0 mg/mL) during the pre-pollen season assessment to 47.9 mg/mL (range, 4.0 to 400.0 mg/mL; p = 0.003) during the pollen season. The mean (± SEM) reduction was −1.5 ± 0.4 doubling concentrations. In the placebo group, there was a decrease from 223.9 mg/mL.

Table 1—Baseline Characteristics of the Study Patients*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Salmeterol Group (n = 14)</th>
<th>Placebo Group (n = 13)</th>
<th>p Value</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>38.3 ± 2.4</td>
<td>41.8 ± 3.1</td>
<td>0.37</td>
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<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
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<td>3</td>
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<tr>
<td>Female</td>
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<tr>
<td>Duration of asthma, yr</td>
<td>10.1 ± 1.4</td>
<td>10.1 ± 2.1</td>
<td>0.99</td>
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<tr>
<td>FEV₁, % predicted</td>
<td>106.1 ± 2.9</td>
<td>109.0 ± 4.9</td>
<td>0.62</td>
</tr>
<tr>
<td>FEV₁/FVC, %</td>
<td>79.8 ± 1.2</td>
<td>77.8 ± 2.2</td>
<td>0.42</td>
</tr>
<tr>
<td>PC₂₀, mg/mL</td>
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<td></td>
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<tr>
<td>Methacholine</td>
<td>5.4 (0.6–25.0)</td>
<td>7.6 (0.8–25.0)</td>
<td>0.52</td>
</tr>
<tr>
<td>AMP</td>
<td>147.9 (10.7–400.0)</td>
<td>223.9 (12.6–400.0)</td>
<td>0.35</td>
</tr>
<tr>
<td>ENO, ppb</td>
<td>26.9 (6.6–87.2)</td>
<td>27.5 (13.4–120.0)</td>
<td>0.95</td>
</tr>
</tbody>
</table>

*Values are given as the mean ± SEM or the geometric mean (range), unless otherwise indicated.

Figure 1. Individual PC₂₀ values within the two groups, before and during the pollen season are shown. Geometric means are represented by horizontal lines. Allergen-induced changes in PC₂₀ were not significantly different between the placebo and salmeterol groups (p = 0.20).

Figure 2. Individual values for AMP PC₂₀ within the two groups, before and during the pollen season are shown. Geometric means are represented by horizontal lines. Allergen-induced changes in PC₂₀ were not significantly different between the placebo and salmeterol groups (p = 0.36).
(range, 12.6 to 400.0 mg/mL) in the pre-pollen season assessment to 50.1 mg/mL (range, 2.3 to 400.0 mg/mL; p = 0.003) during the pollen season. The mean (± SEM) reduction was −2.1 ± 0.6 doubling concentrations. Changes in AMP PC20 values were not significantly different between the salmeterol and placebo groups, the mean difference being 0.6 doubling concentrations (95% CI, −0.8 to 2.1 doubling concentrations; p = 0.36).

ENO

The values of ENO before and during the pollen season for the two treatment groups are shown in Figure 3. The geometric mean ENO values increased in the salmeterol group from 26.9 ppb (range, 6.6 to 87.2 ppb) during the pre-pollen season assessment to 61.7 ppb (range, 24.0 to 137.0 ppb; p < 0.001) during the pollen season. In the placebo group, there was an increase from 27.5 ppb (range, 13.4 to 120.0 ppb) in the pre-pollen season assessment to 70.8 ppb (range, 27.8 to 142.3 ppb; p < 0.001) during the pollen season. Changes in ENO were not significantly different between the salmeterol and placebo groups, the mean difference being 8.0 ppb (95% CI, −15.6 to 31.7 ppb; p = 0.49).

Correlations

There were no significant correlations between allergen-induced changes in FEV1 and changes in either methacholine PC20 values (placebo group: ρ = 0.17; p = 0.59; salmeterol group: ρ = 0.27; p = 0.34) or AMP PC20 values (placebo group: ρ = 0.15; p = 0.62; salmeterol group: ρ = 0.27; p = 0.37). Furthermore, there were no significant correlations between allergen-induced changes in ENO levels and changes in either methacholine PC20 values (placebo group: ρ = 0.18; p = 0.55; salmeterol group: ρ = −0.47; p = 0.09) or AMP PC20 values (placebo group: ρ = 0.27; p = 0.37; salmeterol group: ρ = 0.12; p = 0.69). Allergen-induced changes in methacholine and AMP PC20 values were not significantly related (placebo group: ρ = 0.20; p = 0.51; salmeterol group: ρ = 0.11; p = 0.72).

FIGURE 3. Individual values for NO concentrations within the two groups, before and during the pollen season are shown. Geometric means are represented by horizontal lines. Allergen-induced changes in exhaled NO were not significantly different between the placebo and salmeterol groups (p = 0.49).

Discussion

The results of the present study have confirmed that natural allergen exposure of subjects with pollen-induced asthma is associated with increased airway responsiveness and ENO concentrations. However, we were unable to detect an effect of regular treatment with salmeterol on the allergen-induced rise in ENO levels and airway responsiveness to either the indirect bronchoconstrictor AMP or the direct bronchoconstrictor methacholine.

Cockcroft and colleagues13 found that a 6-day course of salmeterol therapy produced a significant increase in airway responsiveness to allergens. Furthermore, in another study26 from the same group it was demonstrated that the regular use of albuterol increased the late asthmatic response to allergens and the associated allergen-induced increase in airway responsiveness. If these findings in the laboratory translate into similar occurrences during natural allergen exposure, then patients with allergic asthma who are exposed to a natural allergen might be more likely to experience a β2-agonist-induced increase in airway responsiveness.20 Consequently, it was hypothesized that the regular use of β2-agonists in conjunction with allergen exposure might result in increased airway responsiveness.15,20 In contrast to this hypothesis, we showed no enhancement of the seasonal increase in airway responsiveness in pollen-sensitive asthmatic subjects receiving regular salmeterol therapy compared with those receiving placebo. On the contrary, the results of our study demonstrate that salmeterol significantly inhibits the fall in FEV1 values during the pollen season compared with placebo. Indeed, salmeterol abolishes the effects of natural allergen exposure on airway responsiveness to methacholine. One possible explanation for this discrepancy is the difference between natural and experimental allergen exposure in the dose of allergen and the time course of delivery. Under conditions of natural exposure during a pollen season, the duration of exposure is longer. Therefore, a single
inhalation of an allergen in the laboratory can cause a mild increase in airway responsiveness, whereas repeated allergen exposures, such as exposures that occur during the pollen season, can cause greater increases in airway responsiveness. Alternatively, the time of measurement after the last doses of study medication might have influenced our data. We chose to measure airway responsiveness 12 h after the last dose of the study drug was administered, which is the normal dose interval during maintenance treatment with salmeterol and, therefore, is clinically relevant. No correlation was found between the changes in PC_{20} and in FEV_{1} values, and, therefore, the effect of salmeterol on airway responsiveness could not be explained by an improvement in airway caliber. However, it has been suggested in the literature that salmeterol has effects for beyond 12 h, and, thus, it might be that salmeterol was still partly active at the time of measurement and that subjects were still in a partly protected stage. Finally, another possible explanation for differences between studies of the effect of long-acting 2-agonists is the prevalence of different 2-adrenoceptor polymorphisms. In the present study, 2-adrenoceptor polymorphisms were not determined, and so the relevance of this factor in our patients cannot be ascertained.

The study was adequately powered at 80% to detect a 1.5 doubling concentration difference in AMP PC_{20} at the 5% level, but we found a difference of only 0.6 doubling concentrations. In addition, the allergen-induced change in AMP PC_{20} was smaller in the salmeterol group (1.5 doubling concentrations) than in the placebo group (2.1 doubling concentrations). Therefore, we are confident that this cannot be attributed to insufficient subject numbers (type II error). In addition, we included in the analysis censored data on the eight patients in whom we were unable to measure methacholine PC_{20} and on the nine patients in whom we were unable to measure AMP PC_{20} during the pre-pollen season assessment. However, because the number of censored values was similar in each group, the effect of this was to underestimate the allergen-induced changes within both groups.

We deliberately studied patients who did not use inhaled corticosteroids, as we wanted to investigate the isolated effects of the regular use of salmeterol. One could argue that this study is mostly theoretical, as regular monotherapy with long-acting 2-agonists is not generally advocated in guidelines nowadays. However, in the presence of inhaled corticosteroids, any effect of salmeterol on ENO may have been attenuated, and, equally, any effect of this drug on airway responsiveness may have been masked. Furthermore, although there is convincing evidence that, in pollen-sensitive asthmatic subjects, therapy with inhaled steroids protect against the seasonal increase in airway responsiveness, a significant reduction in glucocorticosteroid receptor-binding affinity has been observed in ragweed-allergic patients with asthma during the pollen season compared with pre-pollen season measurements. These findings could suggest that high allergen exposure in sensitized subjects with asthma might contribute to poor asthma control by reducing the effectiveness of steroid treatment. On the other hand, long-acting 2-agonists, including salmeterol, have been shown to prime corticosteroid receptors for increased corticosteroid binding and nuclear translocation. This provides a potential mechanism for complementary anti-inflammatory cellular effects of inhaled corticosteroids and salmeterol. Thus, it could be argued that the effects of salmeterol on the allergen-induced increases in airway responsiveness might be different in subjects treated concomitantly with inhaled steroids, and it would be of interest to repeat this type of study but adding therapy with an inhaled corticosteroid to that with salmeterol.

In a previous study performed in patients with house dust mite-induced asthma, no interactive effects were detected between allergen exposure and the regular use of formoterol during a period of 12 weeks. To the best of our knowledge, there are no studies that have investigated the effects of treatment with inhaled salmeterol on the increase in airway responsiveness during the pollen season in pollen-sensitive asthmatic subjects. The results of our study demonstrate that, in this group of subjects, inhaled salmeterol abolishes the effects of natural allergen exposure on methacholine responsiveness, but no protection was observed on allergen-induced changes in AMP responsiveness. The difference in the effects of salmeterol on allergen-induced increases in airway responsiveness to methacholine and AMP suggests that different mechanisms may be involved. Inhaled AMP causes bronchoconstriction by releasing mediators from mast cells, which then act on smooth muscle, while methacholine causes bronchoconstriction by direct action on the smooth muscle. When compared to the effect on direct-acting stimuli, short-acting inhaled 2-agonists provide greater protection against indirect stimuli, and their regular use results in greater tolerance; this may be due to a 2-agonist mast cell-stabilizing effect that is more susceptible to down-regulation. Thus, the differential effect of salmeterol on the allergen-induced increases in airway responsiveness to direct and indirect bronchoconstrictors may relate to the differential susceptibility of mast cell receptors and smooth muscle 2-receptors. The situation, however, is complicated further by the re-
sults of a study that suggested that, in contrast to albuterol, salmeterol does not have an additional protective effect against AMP challenge compared with histamine challenge, indicating that this partial agonist does not have a mast cell-stabilizing effect in vivo. Whatever the mechanism, what is clear from our study is the definite absence of deterioration in airway responsiveness during natural allergen exposure with the administration of salmeterol, which was one of the central questions being addressed and had been widely feared to be the case.\textsuperscript{15,26}

In keeping with previous reports,\textsuperscript{34} we have detected that, in subjects with pollen-induced asthma, natural exposure to an allergen is associated with increased concentrations of ENO. However, when applying a validated methodology of ENO determination,\textsuperscript{17} we were unable to detect an effect of salmeterol on the allergen-induced rise in ENO levels. Since it has been postulated that the measurement of ENO may be a noninvasive method of assessing airway inflammation,\textsuperscript{16} our results suggest that salmeterol intake is not associated with an increase in allergen-induced airway inflammation. These data are consistent with those from a variety of other studies\textsuperscript{35,36} that have suggested that salmeterol neither abates nor amplifies airway inflammation induced by allergen challenge.

Contrary to our findings, there are clinical studies both for and against a potential anti-inflammatory action of salmeterol. In subjects with atopic asthma, salmeterol intake has been associated with a decrease in the serum concentration of eosinophil cationic protein during natural pollen exposure.\textsuperscript{37} It also inhibits the increase in the percentage of eosinophils in induced sputum after allergen challenge.\textsuperscript{38} In contrast, Boulet et al\textsuperscript{14} reported that there was an increase in mast cell and total leukocyte counts in bronchial biopsies of asthmatic subjects taking salmeterol regularly for 1 week 24 h after an allergen challenge. The variability of salmeterol effects observed in these studies, compared to those in our study, may be due to the methods used to determine allergen-induced airway inflammation. First, we used natural allergen exposure, rather than allergen challenge. The variability of salmeterol effects observed in these studies, compared to those in our study, may be due to the methods used to determine allergen-induced airway inflammation. First, we used natural allergen exposure, rather than allergen challenge, which is a more persistent stimulus for inflammation. Therefore, the inflammatory signal may have been too persistent for down-regulation by salmeterol. Second, we determined the level of allergen-induced inflammation in the lower airways by changes in ENO concentrations. Although ENO has been proposed as a marker of airway inflammation in asthma patients,\textsuperscript{16} the correlations between ENO and direct measures of airway inflammation have been of relatively small magnitude.\textsuperscript{39} In addition, the precise mechanism that links NO with airway inflammation remains to be elucidated. It has been suggested that atopic status may be the most important determinant of enhanced NO production and that the inhalation of allergens in sensitized subjects may have a direct effect on NO production, regardless of the presence of inflammation in the airways.\textsuperscript{40} A second explanation for our results might be the absence of an inhibitory effect of salmeterol on the mechanism that regulates the increased NO production during natural allergen exposure.

In conclusion, using a model of natural allergen exposure, we were unable to detect any potentiating effect of regular treatment with salmeterol on the allergen-induced increases in ENO levels and airway responsiveness to either direct or indirect bronchoconstrictor agents. There is no convincing evidence, however, that long-acting $\beta_2$-agonists have anti-inflammatory properties, and patients should be instructed to use these drugs only in combination with anti-inflammatory medication.

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