Salmeterol, an inhaled \(\beta_2\)-agonist with a prolonged duration of action, has been used extensively in Europe and now in the United States for the treatment of asthma.\(^1\)\(^-\)\(^4\) Because of its long duration of action, it also has the potential to treat nocturnal worsening of asthma, referred to as nocturnal asthma (NA).\(^5\)\(^,\)\(^6\) In moderately severe NA, it is unknown if salmeterol alone can improve this condition. Several studies have shown efficacy of salmeterol in NA, but mainly in combination with inhaled corticosteroids.\(^3\)\(^,\)\(^5\)\(^-\)\(^8\) The ability of salmeterol to improve asthma symptoms is thought to be due to smooth muscle relaxation, and possibly by exerting anti-inflammatory effects, although the latter mechanism is somewhat controversial. Gardiner and colleagues\(^9\) evaluated BAL fluid from subjects with asthma and found no change in total BAL cell count and differential count after salmeterol use. However, Butchers and colleagues\(^10\) showed that salmeterol reduced the release of inflammatory mediators such as histamine, leukotriene C\(_4\), and leukotriene D\(_4\) from human lung tissue in vitro. Given the paucity of information on the solitary efficacy of salmeterol in NA and the conflicting literature on its anti-inflammatory properties, the goal of this study was to determine if salmeterol independently improves symptoms and lung function while decreasing airway inflammation in patients with moderate to severe NA.

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Materials and Methods

Subjects

Ten asthmatics were recruited from the general Denver community. They met diagnostic criteria for asthma.11 All 10 experienced NA, defined as a documented fall in overnight peak expiratory flow rate (PEFR) of >20% on at least four of seven nights of testing at home during the run-in period. Exclusion criteria included use of oral or inhaled steroids, methotrexate, cromolyn, or antibiotics within 6 weeks, astemizole within 12 weeks, any investigational drug within 30 days, upper respiratory tract infection within 6 weeks, variable shift schedule, any history of significant nonasthmatic pulmonary disease, significant medical illness as determined by the principal investigator, or pregnancy. In addition, subjects could have no history of tobacco use over the past 1 year and <5 total pack-years of use. Informed consent was obtained for this Institutional Review Board-approved study. Subject characteristics are shown in Table 1.

Protocol

The study was a randomized double-blind placebo-controlled crossover study. The study consisted of two treatment periods, each 6 weeks in length, with a 1-week washout period. Prior to randomization, subjects underwent screening, including a history and physical examination, chest radiograph, ECG, and analysis of CBC count and chemistry. Subjects were also instructed on the use of a peak flowmeter and keeping their peak flows during the run-in period (7 to 14 days) to document a nocturnal decrement in lung function prior to randomization. They were then randomized to treatment with salmeterol, 100 μg/d (two puffs at 7 AM and 7 PM) or placebo during first treatment period, followed by the alternate therapy during the second period after a 1-week washout phase. Subjects kept diary cards, in which they recorded their PEFRs prior to bedtime and in the morning, and rated their symptoms of chest tightness, wheezing, shortness of breath, and cough from 0 to 4. The washout phase consisted of treatment with a short-acting inhaled β2-agonist as needed only. Each treatment period included a 4 AM methacholine challenge followed by bronchoscopy with BAL at the end of weeks 1 and 6.

Methacholine Challenge

Methacholine challenge was performed as previously described.12 Methacholine was administered as an aerosol in increasing concentrations (0.0175 to 25.0 mg/dL) at 5-min intervals via a nebulizer (De Vilbiss 646; Somerset, Pa) powered by pressurized air (20 psi) delivered through a dosimeter (Rosenthal-French: Baltimore) that was triggered by a solenoid valve set to remain open 0.6 s. Subjects performed five inspiratory capacity inhalations at each concentration of methacholine, followed by spirometry (Moose; Cybermedic; Louisville, Colo) 3 min later. The challenge was concluded after reaching the concentration of methacholine that provoked a 20% or greater reduction in FEV1 from prechallenge baseline (PC20).

Bronchoscopy With BAL

Bronchoscopy with BAL was performed within 30 min of methacholine challenge using identical procedures previously described.12 Prior to the procedure and after methacholine challenge, subjects received 0.18 mg albuterol from a metered dose inhaler, 60 mg codeine and 0.6 mg atropine IM. In addition, 4% lidocaine (Xylocaine) was used to anesthetize the upper airway and 1% lidocaine (Xylocaine) was applied to the laryngeal area, trachea, and orifice of the right middle lobe or lingula via the bronchoscope. Subjects were randomized to undergo bronchoscopy with BAL of the right middle lobe or lingula the first week with the alternate lobe lavaged during the second bronchoscopy at week 6 of the treatment period. A similar randomization was performed for the bronchoscopy site during the second treatment period. BAL was performed using five 60-mL aliquots of sterile normal saline solution at 37°C. Lavage fluid was obtained by immediate gentle hand suction applied to each instilling syringe. Nasal oxygen at 3 to 4 L/min and pulse oximetry were used throughout the procedure. Subjects were monitored postbronchoscopy via heart rate, BP, and pulse oximetry until hospital discharge 4 to 6 h later.

BAL Fluid Analysis

Cell Count and Differential Count: The lavage fluid was immediately put on ice, and the aliquots were combined and centrifuged for 10 min at 1,200 rpm at 4°C to separate cells from fluid. Differential cell counts were done from a known volume of lavage with a Giemsa-type stain (Diff-Quick; Dade Diagnostics, Inc; Aguada, PR). Cell counts were done with fresh lavage fluid and at least 500 cells were counted to obtain the differential cell count. Results are expressed as cells per milliliter of BAL fluid.

Eosinophil Studies—Charcot-Leyden Crystal Protein and Major Basic Protein: Charcot-Leyden crystal protein was measured as previously described.13,14 One hundred microliter BAL fluid aliquots were placed in a “double-sandwich” radioimmunoassay. Results are expressed in nanograms per milliliter. Major basic protein was also measured as previously described via radioimmunoassay. Results are expressed in nanograms per milliliter.14

Macrophage Studies—Leukotriene B4 and Thromboxane B2: The BAL fluid macrophages were resuspended at a concentration of 1×106 macrophages per milliliter in Dulbecco’s modified eagle medium (DMEM) with 10% fetal bovine serum and 100 μg/mL penicillin/100 μg/mL streptomycin. These cells were then plated in a 24- or 48-well plastic culture plate (depending on yield) and allowed to adhere for 2 h at 37°C/10% CO2. After this 2-h adherence, the cells were washed three times with cold phosphate-buffered saline solution and new media (DMEM/0.1% bovine serum albumin) containing calcium ionophore (10 μmol/L A23187) or a vehicle control (0.001% DMSO) was then added. The supernatants were then harvested and analyzed for the eicosanoids leukotriene B4 (LTB4) and thromboxane B2 (TXB2). The plated cells were washed again three times (phosphate-buffered saline solution Ca2+ and Mg2+), lysed (0.2N NaOH), and protein content per well was determined. LTB4 and TXB2 were quantified in the supernatant of these adherent cells by a modified enzyme immunoassay, and they were indexed to BAL protein levels. All eicosanoids were reported as picogram per microgram protein with and without the addition of A23187.

Table 1—Subject Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>29.9±1.2</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>8/2</td>
</tr>
<tr>
<td>Nocturnal awakenings</td>
<td>&gt;4/wk</td>
</tr>
<tr>
<td>Medications Inhaled short-acting</td>
<td>β2 agonists only</td>
</tr>
<tr>
<td>﹪% pred FEV1, 4 PM</td>
<td>76.3±2.1%</td>
</tr>
<tr>
<td>﹪% pred FEV1, 4 AM</td>
<td>57.7±3.9%</td>
</tr>
<tr>
<td>Overnight fall PEFR, %</td>
<td>24.3±3.4%</td>
</tr>
<tr>
<td>measured bedtime to AM</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Years with asthma</td>
<td></td>
</tr>
</tbody>
</table>
Eicosanoid production was determined by enzyme immunoassay,\textsuperscript{15} and cellular lysate protein was determined by using a modified Lowry assay.\textsuperscript{16} LTB\textsubscript{4} was measured as a representative of the 5-lipoxygenase pathway and TXB\textsubscript{2} was measured as a representative of the cyclo-oxygenase pathway. The LTB\textsubscript{4} antibody was purchased (from Advanced Magnetics Inc; Cambridge, Mass), while the TXB\textsubscript{2} antibody was a generous gift (of Dr. Frank Fitzpatrick; UCHSC). The sensitivity of the LTB\textsubscript{4} assay was routinely 30 pg/mL and TXB\textsubscript{2} was 15 pg/mL. Protein values averaged 89.9 μg/1×10\textsuperscript{6} cells.

Statistical Analysis

Analysis of physiologic and lavage variables listed above were performed via a two-period crossover analysis of variance model.\textsuperscript{17} Carryover effects were tested for significance at the 10% alpha level and period/treatment effects were tested for significance at the 5% alpha level. Results are expressed as mean±SEM.

RESULTS

Subjects

The subject characteristics are shown in Table 1. The 10 subjects consisted of eight men and two women with an average age of 29.9±1.9 years. All patients were maintained on a regimen of β\textsubscript{2}-agonists only. All patients had a history of asthma of >10 years and all exhibited nocturnal symptoms of asthma more than four times a week. No subject had a history of cigarette use.

Symptoms

The mean number of nocturnal awakenings decreased while the subjects took salmeterol and this decrease trended toward significance (0.9±0.1 awakenings per night with placebo vs 0.4±0.1 awakenings per night with salmeterol; p=0.08) (Fig 1). In addition, the percentage of nights with awakenings decreased with salmeterol (69.8±8.7%) vs 30.6±10.8% for placebo and salmeterol, respectively; p=0.02) (Fig 1). The percent of days (24-h period) with the use of supplemental inhaled β\textsubscript{2}-agonists also significantly decreased with salmeterol (85.9±9.4% vs 70.4±10.1% for placebo and salmeterol; respectively; p=0.04). Symptoms of wheezing, shortness of

![Figure 1. The number of awakenings per night due to symptoms of asthma (chest tightness, wheezing, and/or coughing), the percentage of nights with awakening, and the percentage of days (24-h periods) of supplemental albuterol use while subjects used placebo (P) and salmeterol (S).](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21747/)
breath, and cough did not change significantly with salmeterol. There were no adverse events directly related to salmeterol use.

Pulmonary Function

The mean percent predicted 4 am FEV₁ improved, while patients received salmeterol as compared with placebo, but only during week 1 was there a trend toward significance (57.7±3.9% with placebo vs 69.0±3.9% with salmeterol; p=0.06). After week 6, the percent predicted FEV₁ at 4 am was not significantly different between the two groups, as the placebo group improved, and the improvement with salmeterol was maintained (62.3±4.13% vs 68.7±4.72% for placebo and salmeterol, respectively; p=0.28). The FVC at 4 am did not change significantly with salmeterol during weeks 1 or 6 (81.7±4.2% with placebo vs 88.5±4.2% with salmeterol after week 1, p=0.27; 86.3±3.6% with placebo vs 85.6±3.9% with salmeterol after week 6, p=0.69). Furthermore, the percent overnight fall in FEV₁, measured from bedtime to 4 am, did not significantly improve with salmeterol during weeks 1 or 6 (20.9±3.2% with placebo vs 16.5±5.9% with salmeterol after week 1, p=0.56; 23.8±5.0% with placebo vs 18.0±5.9% with salmeterol after week 6, p=0.28). The PC₂₀ for methacholine was higher after week 1 with salmeterol (0.04±0.04 mg/mL with placebo vs 0.18±0.06 mg/mL with salmeterol; p=0.05). After week 6, the PC₂₀ results were not significantly different between salmeterol and placebo as the placebo group improved, and the PC₂₀ with salmeterol did not change significantly (0.13±0.07 mg/mL with placebo vs 0.13±0.07 mg/mL with salmeterol; p=0.84).

BAL Fluid Analysis

Three subjects were unable to undergo bronchoscopy at all four time points due to significantly reduced FEV₁. Of these three subjects, two subjects underwent two of four bronchoscopies and the third subject underwent three of four bronchoscopies. Analysis of the BAL fluid cell count and differential count showed a trend toward a significant improvement in the number of eosinophils when subjects used salmeterol, but only after week 6 (3.2±0.6×10⁴/mL with placebo vs 13.2±0.6×10⁴/mL with salmeterol after week 6, p=0.01; 2.6±0.1×10⁴/mL with placebo vs 1.3±0.1×10⁴/mL with salmeterol after week 6, p=0.08). Within the salmeterol group, LTB₄ and TXB₂ production after stimulation of BAL macrophages with A23187 decreased from week 1 to week 6, and approached statistical significance (LTB₄: 20.9±87 pg/μg protein after week 1 to 623±418 pg/μg protein after week 6, p=0.07; TXB₂: 44.4±19.4 pg/μg protein after week 1 to 9.9±3.8 pg/μg protein after week 6, p=0.12). There were no significant differences between the absolute number and percentage of macrophages, lymphocytes, or neutrophils, eosinophil cationic protein (ECP), and Charcot-Leyden crystal protein after either week 1 or week 6. BAL fluid results are summarized in Table 2.

DISCUSSION

Our study reveals that salmeterol does improve symptoms of NA by decreasing the percentage of nights with awakenings. However, the objective mea-

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Table 2—BAL Variables*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Week 1</th>
<th>Week 6</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Salmeterol</td>
</tr>
<tr>
<td>Cell count and differential†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total WBC, ×10⁹/mL</td>
<td>10.1±1.5</td>
<td>15.4±2.2</td>
</tr>
<tr>
<td>%eos</td>
<td>3.2±0.7</td>
<td>7.3±2.7</td>
</tr>
<tr>
<td>%macs</td>
<td>84.4±2.6</td>
<td>79.0±5.3</td>
</tr>
<tr>
<td>%lymphs</td>
<td>10.8±2.7</td>
<td>10.0±2.6</td>
</tr>
<tr>
<td>%pmonos</td>
<td>1.4±0.4</td>
<td>3.9±1.7</td>
</tr>
<tr>
<td>Eosinophil products†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLC, pg/mL</td>
<td>8.5±2.6</td>
<td>20.0±10.4</td>
</tr>
<tr>
<td>MBP, μg/mL</td>
<td>6.6±1.6</td>
<td>6.4±0.8</td>
</tr>
<tr>
<td>Macrophage products†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTB₄, pg/μg protein, control</td>
<td>46.0±28.3</td>
<td>77.8±71.8</td>
</tr>
<tr>
<td>LTB₄, pg/μg protein, stim.†</td>
<td>1,341±601</td>
<td>2,092±878</td>
</tr>
<tr>
<td>TXB₂, pg/μg protein, control</td>
<td>46.9±22.9</td>
<td>77.8±38.3</td>
</tr>
<tr>
<td>TXB₂, pg/μg protein, stim.†</td>
<td>22.0±9.0</td>
<td>44.2±19.4</td>
</tr>
</tbody>
</table>

* eon=eosinophils; macs=macrophages; lymphs=lymphocytes; pmonos=neutrophils; CLC=Charcot-Leyden crystal protein; MBP=macrophage basic protein.
† No statistically significant difference between placebo and salmeterol.
‡ Stim=Macrophages stimulated with A23187.
measurements of 4 AM FEV₁ and overnight fall in lung function were not significantly improved. Except for a trend toward a decrease in BAL eosinophils and LTB₄ at week 6, there were no significant anti-inflammatory effects. However, salmeterol did not decrease airway inflammation or worsen lung function and bronchial reactivity over the 6-week trial.

Several studies have shown efficacy of salmeterol in NA. Three studies by Greening et al., Fitzpatrick et al. and Brambilla et al. showed improvement of early morning PEFRs, number of nocturnal awakenings, and overall circadian variation of lung function with salmeterol. Britton et al. and Lundbeck et al. also noted similar findings. In addition, Fitzpatrick and colleagues demonstrated that subjects experienced increased stage 4 sleep when taking the 50-μg salmeterol dose, inferring an improvement in sleep quality. Importantly, most or all of the subjects in these three studies were using oral and/or inhaled corticosteroids and many subjects were also using theophylline preparations, ipratropium bromide, and/or oral β₂-agonists. This is an important difference from our study in that our subjects were not taking any anti-inflammatory or other types of medications. This difference may also explain why we did not find any objective measurement of improvement in overnight lung function with salmeterol. Therefore, salmeterol may be a useful adjunct to treatment of NA asthma if anti-inflammatory medications are already in use, particularly in patients with moderate to severe asthma. We believe that this is an important point and, in fact, it is supported by Faurschou et al. and Greening et al. They evaluated the effect of salmeterol in patients with poorly controlled NA who were using other agents, including corticosteroids. In these studies, adding salmeterol significantly improved nocturnal lung function compared with placebo.

This study was conducted as a pilot study to evaluate the effect of salmeterol on both lung function and BAL inflammatory parameters, and hence the sample size used was small (10 subjects in a crossover design). As in many small studies, our power to detect statistically significant differences in lung function for the salmeterol group compared with the placebo group is also small. Power calculations were performed at the study’s completion and showed that we would need to study 90 subjects to have 80% power to detect the observed difference between active and placebo treatment in mean 4 AM FEV₁ at 6 weeks of 6.4% predicted (SD=18.11). However, more interesting to us than the limited power was the small effect size we observed. The difference in 4 AM FEV₁ (6.4% predicted) between placebo and salmeterol at week 6 that we observed was smaller than what is usually considered a clinically relevant difference (10 to 12%). This finding suggests that the efficacy of salmeterol in a group of patients with moderately severe asthma may not be of a clinically significant magnitude when salmeterol is used alone, although 6 weeks may not have been a sufficiently long treatment period to demonstrate anti-inflammatory effects. A larger sample size in this study would increase our power to detect a statistically significant difference in means between the salmeterol and placebo groups, but it is unlikely that an increased sample size will change the magnitude of the effect being observed. Perhaps the addition of an anti-inflammatory medication is needed to achieve significant improvement in lung function and inflammatory parameters in patients with moderately severe asthma.

The anti-inflammatory effect of long-acting inhaled β₂-agonists is controversial, with studies both supporting and refuting this mechanism of action. Pedersen and coworkers illustrated that salmeterol inhibited the early and late asthmatic responses to allergen, with a concomitant decrease in serum ECP as compared with placebo. This work in part supports the findings by Wong and colleagues, in which formoterol, another long-acting inhaled β₂-agonist, also inhibited the rise in serum ECP 24 h after exposure to allergen, but neither budesonide nor formoterol inhibited sputum eosinophils or CD25-positive lymphocytes. Perhaps, Calhoun demonstrated that salmeterol reduced BAL eosinophils by approximately 40% 48 h after segmental allergen challenge.

Conversely, other investigators have not shown anti-inflammatory effects by salmeterol. Soler and colleagues showed that salmeterol provided bronchoprotection to histamine, but not adenosine 5’-monophosphate (AMP), a direct mast cell stimulator. Subjects underwent bronchoprovocation testing with histamine and AMP, each 14 h after salmeterol, salbutamol, or placebo in a randomized, double-blind, crossover fashion. The PC₂₀ for histamine was significantly improved with salmeterol, but the PC₂₀ for AMP was no different among salmeterol, salbutamol, and placebo. These results suggest a lack of a cell stabilizing effect of salmeterol, favoring a functional antioxidant effect at the level of airway smooth muscle. Wempe and coworkers found no effect of bambuterol on the circadian increase in blood eosinophils and ECP levels in asthmatics with nocturnal worsening of symptoms. Gardiner and colleagues evaluated BAL fluid from subjects with asthma after 8 weeks of salmeterol. They found no change in the BAL total cell count, differential count, lymphocyte subsets, and mast cell tryptase. Their study differs from ours in that all subjects were using 400 to 1,000 μg beclomethasone dipropionate daily for at least 2
ACKNOWLEDGMENT: The authors would like to acknowledge Dr. Steven Ackerman for assistance with the eosinophil studies and John Trudeau for assistance with the macrophage studies.

REFERENCES