Study objectives: Hypertonic saline solution inhalation is suspected to produce airway inflammation.

Design: The aim of this study was to verify this hypothesis by measuring inflammatory markers in exhaled breath condensate (EBC) collected before and after sputum induction with hypertonic and isotonic saline solution.

Patients and methods: We enrolled 10 patients with asthma, 10 patients with COPD, and 7 healthy subjects with no history of lung disease. Levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α were measured in EBC by a specific enzyme immunoassay kit. Exhaled pH was measured after deaeration/decarbonation by bubbling with argon (350 mL/min) for 10 min by means of a pH meter.

Measurements and results: Exhaled IL-6 and TNF-α concentrations were greater and pH was decreased compared to baseline after hypertonic saline solution inhalation in each group of subjects studied. No changes were observed following isotonic saline solution inhalation.

Conclusions: These findings suggest that hypertonic saline solution inhalation could cause a low-grade inflammation in airways, and levels of inflammatory markers such as IL-6, TNF-α, and pH in EBC may be a useful noninvasive way to assess and monitor airway inflammation.

Key words: airway inflammation; asthma; COPD; interleukin-6; pH; tumor necrosis factor-

Abbreviations: EBC = exhaled breath condensate; IL = interleukin; TNF = tumor necrosis factor

Hypertonic saline solution is normally used for sputum induction.1 On account of its noninvasiveness, simplicity, relative harmlessness, cost-effectiveness, and reproducibility, induced sputum is a preferred technique in the investigation of bronchial inflammation.2,3 Cell counts as well as cytokine profile are usually measured in induced sputum to assess the inflammatory state of the lower respiratory tract.2,3

The possibility that hypertonic saline solution inhalation may produce airway inflammation has been suspected.4 Induced bronchoconstriction, increased bronchial responsiveness to methacholine, as well as increased production of inflammatory cells and cytokines were found after sputum induction with hypertonic saline solution.4

Breath condensate is a completely noninvasive method for obtaining samples that reflect airway lining fluid composition.5,6 In comparison with other methods, collection and analysis of breath condensate does not influence the composition of the sample.6 Several markers have been measured in breath condensate to assess airway inflammation, such as interleukin (IL)-4,7 interferon-α,7 tumor necrosis factor (TNF)-α,8 IL-6,5 leukotriene B4,6 and pH.7

The purpose of this study was to verify the inflammatory effect of hypertonicity. With this aim in mind, we measured two soluble cytokines that were seen to have a central role in airway inflammation, IL-6 and TNF-α, in addition to the most promising inflammatory marker, pH, in the breath condensate
of subjects before and after sputum induction with hypertonic saline solution and isotonic saline solution.

Furthermore, we compared the cellular composition of induced sputum in a small group of subjects after inhalation of either hypertonic or isotonic saline solution. To evaluate the potential stimulatory role of hypertonic saline inhalation in airway inflammation, we enrolled patients belonging to three groups characterized by different degrees of inflammation: patients with asthma, patients with COPD, and healthy subjects.

**MATERIALS AND METHODS**

**Study Population**

The study population consisted of 10 patients with asthma, 10 patients with COPD, and 7 healthy subjects with no history of lung disease (Table 1). Both patient groups and control subjects were white subjects recruited from the Respiratory Disease Division of University of Foggia. Written informed consent was obtained from all subjects. The study was approved by the Institutional Ethics Committee. The diagnosis of asthma and the assessment of its severity were done at enrollment according to Global Initiative for Asthma.9 The diagnosis of COPD was based on Global Initiative for Chronic Obstructive Lung Disease guidelines.10

During the study, only inhaled short-acting β2-agonists were allowed for symptom relief. Inhaled and oral steroids were withheld for at least 2 weeks before the study. No subjects reported a history of an upper respiratory infection in the preceding 4 weeks.

At the first visit, all subjects underwent a clinical examination, lung function testing, assessment of atopic status, and methacholine bronchial challenge. Two days later, they underwent hypertonic saline solution inhalation (NaCl 4.5%) [following the procedure of sputum induction] preceded and followed by exhaled breath condensate (EBC) collection (within 15 min from saline solution inhalation). After 1 week, at the same time of day (± 1 h), a small group of these subjects (five patients with asthma, five patients with COPD, and five healthy subjects) also underwent isotonic saline solution inhalation (NaCl 0.9%). The induced sputum of these subjects was collected after either hypertonic or isotonic saline solution inhalation.

**Study Design**

This study was designed to investigate the effects of hypertonic saline solution inhalation on airway inflammation through analysis of EBC.

**Table 1—Subject Characteristics**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>COPD</th>
<th>Asthma</th>
<th>Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects, No.</td>
<td>10</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Age, yr</td>
<td>62 ± 6</td>
<td>47 ± 10</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>Male/female gender, No.</td>
<td>6/4</td>
<td>4/6</td>
<td>5/2</td>
</tr>
<tr>
<td>FEV1, % predicted</td>
<td>75.0 ± 6.9</td>
<td>65.9 ± 9.8</td>
<td>112 ± 18</td>
</tr>
<tr>
<td>FVC, % predicted</td>
<td>85.5 ± 6.9</td>
<td>72.7 ± 6.5</td>
<td>119 ± 9</td>
</tr>
<tr>
<td>PD20, mg</td>
<td>&gt; 1,600</td>
<td>553 ± 362</td>
<td>&gt; 16.00</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD. PD20 = provocative dose of methacholine causing a 20% fall in FEV1.

**Hypertonic and Isotonic Saline Solution Inhalation**

FEV1 and FVC were measured before and 10 min after salbutamol inhalation (two puffs; 200 µg), and subjects then inhaled hypertonic (4.5%) or isotonic (0.9%) saline solution nebulized for increasing time periods, 1 min, 2 min, 4 min, 8 min, and 16 min according to Spanevello et al.11 FEV1 was repeated 1 min after each inhalation period.11 Saline solutions were nebulized by an ultrasonic nebulizer (DeVilbiss 65; DeVilbiss Corporation; Somerset, PA).

**Induced Sputum**

Sputum was collected in the small group of patients (five asthma patients, five COPD patients, and five healthy subjects) who underwent hypertonic saline solution and later isotonic saline solution inhalation. The sputum was processed according to Spanevello et al.11 All sputum counts and measurements were performed blind to the clinical details. Definition of an adequate selected sputum was one in which there were < 20% squamous cells and viability > 50%.

**Assessment of the Atopic Status**

Skin-prick tests for aeroallergens were performed and interpreted as previously described.12

**Methacholine Bronchial Challenge**

A methacholine bronchial provocation test was performed in all subjects enrolled according to Sterk et al.11 2 days before saline solution inhalation. Bronchial challenges were performed in all subjects at the same hour of the day under the same environmental conditions.

**Lung Function**

Pulmonary function tests were performed during the first visit and before saline solution inhalation using a spirometer (SensorMedics; Yorba Linda, CA).

**EBC**

EBC was collected using a condenser (EcoScreen; Jaeger; Wurzburg, Germany). The subjects breathed through a mouthpiece and a two-way nonbreathing valve that also served as a saliva trap. They were asked to breathe at a normal frequency and tidal volume, wearing a nose clip, for a period of 10 min. If the subjects felt saliva in their mouth, they were instructed to swallow it. Condensate, at least 1 mL, was collected as ice at ~20°C, transferred to Eppendorf tubes, and immediately stored at −70°C. Samples were analyzed within 3 months of collection. To exclude saliva contamination, the amylase activity was analyzed in EBC.

**Measurement of TNF-α and IL-6**

A specific enzyme immunoassay (Cayman Chemical; Ann Arbor, MI) was used to measure TNF-α and IL-6 concentrations in EBC. The intra-assay and interassay variability was ≤ 10%. The detection limit of the assays was 1.5 pg/mL and 1.5 pg/mL, respectively. The reproducibility of the repeated measurements of exhaled TNF-α and IL-6 was confirmed by the Bland and Altman test and by the coefficient of variation.14
A stable pH was achieved in all cases after deaeration/decarbonation of EBC specimens by bubbling with argon (350 mL/min) for 10 min, as previously reported. The pH was then measured within 5 min of condensate collection by means of a pH meter (Corning 240; Corning, Science Products Division; Acton, MA) with a 0.00 to 14.00 pH range and a resolution/accuracy to the order of 0.01 ± 0.02 pH.

Statistical Analysis

Data were expressed as mean ± SEM. Mann-Whitney tests were used to compare groups and correlations between variables were performed using the Spearman rank correlation test. Significance was defined as p < 0.05.

RESULTS

Hypertonic and isotonic saline solution inhalation was performed without observing any significant adverse effect or a decrease of FEV₁ > 20%. Repeated FEV₁ did not show significant reduction after each inhalation period. All asthmatic subjects were identified as atopic (positive to perennial allergens) and positive to methacholine bronchial provocation test (provocative dose [± SD] of methacholine causing a 20% fall in FEV₁ of 553 ± 362 μg).

Total and Differential Sputum Cell Counts

There was no significant difference between isotonic or hypertonic saline solution inhalation in the quality of samples as judged by the viability and the extent of squamous cell contamination. Likewise, there were no statistically significant differences regarding the total and differential cell counts between sputum induced by hypertonic and isotonic saline solutions (Table 2).

IL-6

IL-6 was measurable in the EBC of all subjects. Baseline concentrations of exhaled IL-6 were significantly greater in asthmatic and in COPD patients than in healthy subjects (p < 0.001). We observed higher concentrations of this cytokine after hypertonic saline inhalation than baseline both in asthmatic subjects (8.1 ± 1.9 pg/mL vs 7.3 ± 1.5 pg/mL, p < 0.01) and in COPD subjects (7.8 ± 1.2 pg/mL vs 7.0 ± 1.2 pg/mL, p < 0.05), and in healthy control subjects (3.9 ± 0.9 pg/mL vs 3.1 ± 0.7 pg/mL, p < 0.01) [Fig 1]. However, exhaled IL-6 concentrations did not show any increment after isotonic saline inhalation (6.8 ± 1.7 pg/mL vs 6.9 ± 1.3 pg/mL).

Exhaled IL-6 correlated positively with TNF-α (r = 0.7, p < 0.001) and negatively with pH (r = −0.5, p < 0.01). IL-6 changes from baseline between asthmatic patients (0.9 ± 0.3), COPD patients (1.6 ± 0.6), and control subjects (1.0 ± 0.2) were not significant, with confidence intervals of 95%.

Reproducibility of exhaled IL-6 measurements was assessed in 10 nonsmoking normal subjects (6 men; mean age, 35 ± 7 years). In the majority of measurements, the differences between the two IL-6 values remained within ± 2 SD (mean difference, −0.03 ± 0.24 pg/mL). The coefficient of variation for IL-6 measured was 5.9%.

TNF-α

TNF-α was measurable in the EBC of all subjects. Baseline concentrations were significantly greater in asthmatic and in COPD patients with respect to healthy subjects (p < 0.001). We observed higher concentrations of TNF-α after hypertonic saline solution inhalation than baseline both in asthmatic subjects (9.4 ± 2.0 pg/mL vs 7.8 ± 0.7 pg/mL, p < 0.05) and in COPD subjects (9.1 ± 1.6 pg/mL vs 8.2 ± 1.3 pg/mL, p = 0.1), and in healthy control subjects (4.9 ± 0.5 pg/mL vs 4.2 ± 0.6 pg/mL, p < 0.005) [Fig 2]. However, exhaled TNF-α concentrations did not show any increment after isotonic saline solution inhalation (7.3 ± 1.6 pg/mL vs 7.4 ± 1.8 pg/mL). Exhaled TNF-α correlated negatively with pH (r = −0.6, p < 0.005).

TNF-α changes from baseline between asthmatics

<table>
<thead>
<tr>
<th>Variables</th>
<th>Asthmatic Subjects (NaCl 0.9%)</th>
<th>Asthmatic Subjects (NaCl 4.5%)</th>
<th>COPD Subjects (NaCl 0.9%)</th>
<th>COPD Subjects (NaCl 4.5%)</th>
<th>Healthy Subjects (NaCl 0.9%)</th>
<th>Healthy Subjects (NaCl 4.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count, × 10⁶/mL</td>
<td>2.7 ± 0.9</td>
<td>2.9 ± 1.8</td>
<td>3.2 ± 2.8</td>
<td>3.3 ± 1.2</td>
<td>2.7 ± 1.5</td>
<td>2.6 ± 2.4</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>62.3 ± 5.1</td>
<td>64.2 ± 6.9</td>
<td>45.3 ± 17.2</td>
<td>42.3 ± 9.9</td>
<td>76.1 ± 16</td>
<td>73.7 ± 12</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>21.1 ± 6.6</td>
<td>20.6 ± 4.6</td>
<td>39.0 ± 5.4</td>
<td>40.4 ± 8.3</td>
<td>16.1 ± 6.8</td>
<td>19.3 ± 8.3</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>6.9 ± 3.4</td>
<td>7.3 ± 2.9</td>
<td>1.4 ± 0.4</td>
<td>1.8 ± 0.2</td>
<td>0.7 ± 0.3</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>0.11 ± 0.28</td>
<td>0.9 ± 3.4</td>
<td>2.4 ± 14</td>
<td>2.1 ± 3.7</td>
<td>1.1 ± 2.1</td>
<td>1.7 ± 3.7</td>
</tr>
<tr>
<td>Epithelial cells, %</td>
<td>9.9 ± 2.6</td>
<td>8.1 ± 2.9</td>
<td>12.1 ± 2.7</td>
<td>13.0 ± 3.3</td>
<td>12.7 ± 3.7</td>
<td>11.1 ± 3.7</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD.
(1.8 ± 0.6 pg/mL), COPD (1.5 ± 0.3 pg/mL), and control subjects (0.7 ± 0.1 pg/mL) were not significant, with 95% confidence intervals. Reproducibility of exhaled TNF-α measurements was assessed in 10 nonsmoking normal subjects (6 men; mean age, 35 ± 7 years). In the majority of measurements, the differences between the two TNF-α values remained within ± 2 SD (mean difference, 0.07 ± 0.19 pg/mL). The coefficient of variation for TNF-α measured was 3.3%.

**Exhaled pH**

pH was measurable in the EBC of all subjects. Baseline concentrations were significantly lower in asthmatic and COPD patients than in healthy subjects (p < 0.001) [Fig 1].

We observed a lower value of pH after hypertonic saline solution inhalation than at baseline in asthmatic subjects (7.5 ± 0.2 vs. 7.6 ± 0.1, p < 0.05), in COPD subjects (7.5 ± 0.1 vs. 7.6 ± 0.2, p < 0.05), and in healthy subjects (7.8 ± 0.1 vs. 7.9 ± 0.1, p < 0.01) [Fig 3]. However, exhaled pH concentrations did not show any increment after isotonic saline solution inhalation (7.6 ± 0.1 vs. 7.6 ± 0.2). pH changes from baseline among asthma patients (0.2 ± 0.0), COPD patients (0.1 ± 0.0), and control subjects (0.2 ± 0.0) were not significant, with a confidence interval of 95%.

The reproducibility of exhaled pH measurements was assessed in 10 healthy subjects (6 men; mean age, 35 ± 7 years) [Fig 4]. In most measurements, the mean difference between the two measurements was −0.01 ± 0.4. The coefficient of variation for exhaled breath condensate pH was 0.4%.
This study suggests that hypertonic saline solution inhalation itself causes a low-grade inflammation in airways. Measured exhaled inflammatory cytokines were in fact greater after hypertonic saline solution inhalation than at baseline in each group of subjects studied. In addition, pH became more acid. However, no changes were observed after isotonic saline solution inhalation.

Concentrations of IL-6, TNF-α, and pH in the EBC correlated. By contrast, no differences were observed in total and differential cell counts between inductions with hypertonic or isotonic saline solution.
This is the first work using EBC to study the effect of hypertonicity on airway inflammation. Compared to BAL, the biological sample is not diluted. The procedure does not affect airway function or cause inflammation.\textsuperscript{16,17} We chose EBC analysis to evaluate the inflammatory effects of hypertonic saline inhalation because the noninvasive technique of EBC collection allows for repeated sampling without concern of inducing inflammation. Since there were insufficient numbers of cells in these biological samples, we chose to study airway inflammation by measuring soluble mediators such as cytokines.

We chose to measure two soluble mediators, IL-6 and TNF-\(\alpha\). IL-6 is an inflammatory marker with known properties of neutrophil recruitment and activation and ability to direct T-helper type 2 immune response.\textsuperscript{18,19} TNF-\(\alpha\) is a cytokine known to mediate and augment the inflammatory reaction.\textsuperscript{20} These cytokines have been previously measured and shown to be reproducible by Purokivi et al.\textsuperscript{21} who used IL-6 and TNF-\(\alpha\) to study the inflammatory effect of saline solution inhalation in induced sputum repeated after 48 h.

Our results are in agreement with previous reports\textsuperscript{22,23} on the effects of the hypertonic saline solution on increased production of inflammatory cytokines in vitro and in vivo. Our findings confirm the involvement of neutrophils in saline solution-induced inflammation, as described by Nightingale et al.\textsuperscript{24} In this study, we observed an increase of inflammatory cytokines levels of the same degree after hypertonic inhalation in each group of subjects enrolled. This finding is very important because it excludes other inflammatory stimuli as causes of airway inflammation.

A similar response for exhaled pH confirms the cytokine data on hypertonic-induced inflammation. Acidification of EBC was in fact found after sputum induction with hypertonic saline solution but not with isotonic saline solution in all groups of subjects. Decreasing exhaled pH as a result of airway inflammation was previously proposed by Hunt et al\textsuperscript{25} and verified by normalization of EBC pH values after antiinflammatory treatment. The inflammatory cells mainly involved in the pH acidification appear to be neutrophils.\textsuperscript{26} The granules of these cells contain myeloperoxidase that are released into the airways and catalyze a reaction between hydrogen peroxide and chloride ions to form hypochlorous acid (HOCl).\textsuperscript{27} This highly volatile acid seems to play a key role in the acidification of EBC.\textsuperscript{26}

In accordance with previous findings of our group, there was a negative correlation between exhaled pH and other neutrophilic inflammatory markers (IL-6 and TNF-\(\alpha\)), confirming our previous hypothesis that exhaled pH is a marker of neutrophilic inflammation.\textsuperscript{7} We believe that the correlation observed between exhaled IL-6, TNF-\(\alpha\), and pH may suggest that each of these markers are related to the intensity of ongoing inflammation.

However, contrasting data are reported in literature on the effect of hypertonic saline solution inhalation on airway inflammation.\textsuperscript{17,28,29} The discrepancy seen between different studies most probably reflects methodologic differences and different study populations. Subjects who have preexistent airway inflammation may hide the inflammatory effect of hypertonic saline solution. To obviate this problem, we chose to study three groups of subjects with different inflammatory status of airways: asthmatics, patients with COPD, and healthy subjects. Regarding this last group, available data are very limited even though healthy subjects are a particularly useful target of study, not having preexisting airway inflammation.\textsuperscript{20} The increases in exhaled IL-6 and TNF-\(\alpha\) compared to baseline that we observed in healthy subjects therefore represents important evidence that hypertonicity generates low-grade airway inflammation. In this study, an increase in preexistent airway inflammation was also found in asthmatic and COPD subjects after inhalation of hypertonic saline solution. These findings suggest that hypertonicity may also act to further increase the baseline airway inflammation in subjects affected by respiratory disorders.

In this study, we confirmed that hypertonicity itself is responsible for airway inflammation since exhaled cytokines did not increase after sputum induction with isotonic saline solution. This finding suggests that airway inflammation observed after hypertonic inhalation is not secondary to mechanical stimulation of sputum induction but to the hypertonicity. Our supposition is confirmed by Beier et al,\textsuperscript{31} who showed a decrease of exhaled nitric oxide after hypertonic but not isotonic saline solution.

Several proposals have been suggested to explain the mechanism of airways inflammation stimulated by hypertonic saline solution. The most important theory describes an increase in vascular permeability through the release of neuropeptides from sensory nerves. This vascular permeability has been demonstrated, to date, only in rats.\textsuperscript{4} Knowledge of this matter is, however, contrasting, as well as in short supply.

We analyzed the effects of hypertonicity after 15 min of saline solution inhalation. The increase in exhaled inflammatory markers at this time point could be transient and may not have clinical significance. The finding therefore needs to be confirmed after a longer period.

In this study, we also investigated the effect of hypertonicity on differential cell counts in the in-
duced sputum of a small group of subjects enrolled after hypertonic saline solution inhalation and 7 days later after isotonic saline solution inhalation. We performed the two inductions at a 7-day interval in order to avoid having any influence of the first induction on the cellular composition of the second sputum sample, as previously suggested. In accordance with previous reports, we did not find any significant difference with respect to total and differential cell counts between the two methods of sputum induction. This finding indicates that inhalation of hypertonic saline solution per se might stimulate inflammatory mediator release in the airways but not induce changes in inflammatory cells.

In conclusion, our results provide evidence that hypertonic saline inhalation causes a low-grade inflammation in airways that may be demonstrated through measurement of levels of inflammatory markers, such as IL-6, TNF-α, and pH, in EBC. This study further confirms the usefulness of EBC analysis as a noninvasive and repeatable way of monitoring airway inflammation.

**References**

