Assessment of Microvascular Leakage via Sputum Induction
The Role of Substance P and Neurokinin A in Patients with Asthma

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Microvascular leakage is an important feature of inflammation. However, the assessment of vascular leakage has seldom been used to monitor airway inflammation in asthma. The aim of this study was to determine the effect of inhaled substance P, a potent neurokinin 1 (NK1) agonist and mediator of plasma extravasation, on markers of microvascular leakage in induced sputum from patients with asthma. In a crossover study, sputum was induced before and 30 minutes after inhalation of substance P or neurokinin A (as control) by 12 subjects with atopic and mild, steroid-naive asthma. The levels of α2-macroglobulin, ceruloplasmin, albumin, and fibrinogen were determined in induced sputum as markers of leakage. Substance P induced a significant increase in the levels of α2-macroglobulin, ceruloplasmin, and albumin in induced sputum (median fold change, 3.1, 2.2, and 2.9, respectively) (p < 0.013), whereas inhaled neurokinin A was not able to induce significant changes (p > 0.31). The increase in sputum leakage markers was not associated with the cumulative dose of substance P (p > 0.12). These results indicate that NK1 receptor stimulation causes a rapid increase in microvascular leakage as shown in induced sputum in patients with asthma. This investigational model of “dual induction” (first leakage, then sputum) may therefore be useful to test the antiexudative effect of newly develop drugs, such as NK1 antagonists.

Keywords: asthma; α2-macroglobulin; microvascular leakage; sputum; substance P

Asthma is a chronic disease of the airways, characterized by variable airway obstruction and airway hyperresponsiveness to various stimuli (1). Mucosal inflammation of the airways can be considered as one of the major components of asthma (2). The role of cell activation and the subsequent release of mediators in this inflammatory process has been extensively studied (2). On the other hand, microvascular leakage and edema are also prominent features of airway inflammation in asthma (3). Remarkably, leakage has not often been measured to monitor inflammation in asthma, which is probably due to the difficulties of the measurement in bronchial tissue specimens and lumenal fluids. Thus far, there are only a few studies demonstrating the effect of treatment intervention on leakage parameters in asthma (4).

In animal models, several inducers of airway microvascular leakage have been identified (5). Among these, the tachykinin substance P (SP) appears to be one of the most potent mediators causing leakage in guinea pig airways (6). It is likely that tachykinins play a role in the pathogenesis of asthma. Tachykinins have been detected in airway sensory nerves and in secretions recovered from human airways (7). More importantly, elevated levels of SP have been demonstrated in bronchoalveolar lavage fluid and sputum, and a further increase occurs after allergen challenge in patients with asthma (8, 9). In human tissue, the distinct neurokinin (NK) 1 and NK2 tachykinin receptors have recently been identified using immunohistochemistry (10). The NK2 receptor mediates smooth muscle contraction in human airways (11), whereas the NK1 receptor primarily induces proinflammatory effects: plasma extravasation, mucus secretion, inflammatory cell chemotaxis, and activation (7). Interestingly, NK1 and NK2 receptor mRNA expression appears to be increased in subjects with asthma as compared with normal subjects (12, 13).

It is still unknown whether NK1 stimulation leads to microvascular leakage in the airways of subjects with asthma in vivo. So far, SP-induced effects on microvascular leakage have been demonstrated only in the airways of guinea pigs (14). In humans in vivo, both neurokinin A (NKA) and SP cause airway narrowing, particularly in patients with asthma (15). This may be due predominantly to NK2-mediated smooth muscle contraction (11). In addition, inhaled SP enhances airway hyperresponsiveness, as demonstrated by an increase in the maximal response to inhaled methacholine in patients with asthma in vivo (16). We postulate that this is associated with an NK1-mediated increase in microvascular permeability in the airway wall.

Therefore, in the current study, we investigated whether inhaled SP induces microvascular leakage in patients with asthma in vivo. To that end, we determined the levels of albumin, fibrinogen, ceruloplasmin, and α2-macroglobulin as markers of leakage in induced sputum before and after SP challenge in patients with asthma. Because challenges with methacholine or histamine are known to induce leakage (17), inhaled NKA was used as a control challenge, to compare the leakage markers in sputum between a NK1 and a NK2 agonist at a given degree of bronchoconstriction.

METHODS
Subjects
Twelve nonsmoking volunteers with atopic asthma (eight females and four males, 18–26 years of age) participated in the study (Table 1). All subjects had a history of episodic chest tightness and wheezing and none were using medication, except for short-acting β2-agonists as needed. Atopy was defined as one or more positive responses to a standardized skin prick test with 10 common allergens (Vivodiagnost; ALK Benelux, Houten, The Netherlands). The baseline forced expiratory volume (FEV1) was greater than 80% predicted (18), and all subjects were hyperresponsive to inhaled histamine (provocative concentration causing a 20% fall in FEV1 [PC20] < 4 mg/ml) (19). All patients were clinically stable and had no history of recent allergen exposure or respiratory chest infection. The study was approved by the medical ethics committee of the Leiden University Medical Center (Leiden, The Netherlands) and all volunteers gave written informed consent.

Design
The study had a single blind randomized, crossover design. At least 1 week before entering the study, inclusion and exclusion criteria were examined and PC20 histamine was determined.
Inhaled SP or NKA challenge was performed on two randomized study days with an interval of at least 1 week. Sputum was induced 2 days before and 30 minutes after the SP or NKA challenge. In addition, a venous blood sample was obtained before each sputum induction.

Inhalation Challenges
Histamine challenge was performed according to a standardized procedure (19) using the tidal breathing method. SP and NKA challenges were performed according to previously validated protocols (16, 20, 21). SP (Sigma, St. Louis, MO) was inhaled in serial doubling concentrations (0.25–8 mg/ml) and NKA (Bachem, Budendorf, Switzerland) was inhaled in concentrations between 8 and 1,000 μg/ml. SP and NKA were aerosolized by a jet nebulizer into a collapsible bag (Mallinkrodt, Petten, The Netherlands) (22). The aerosols were subsequently inhaled by tidal breathing for 3 to 4 minutes at 7-minute intervals.

The airflow responses to SP and NKA were measured on the basis of FEV₁ (18), determined 90 and 180 seconds after each dose (16). In addition, systolic and diastolic blood pressure were monitored after each challenge (23, 24). An equal volume of 0.1% (w/v) dithiothreitol was added to each sample. Supernatant was aspirated after centrifugation and the whole sample. The markers of microvascular leakage were expressed as medians (range) (24). The Wilcoxon signed rank test was applied to test for differences before and after, and between, challenges. Correlations were analyzed with the Spearman rank test. A p value less than 0.05 was accepted as statistically significant and all analyses were performed using SPSS (Chicago, IL) version 10.0.

RESULTS

Subjects 7 and 12 did not produce sputum after the NKA challenge, and subject 8 was not able to produce sputum after SP administration. These time points were handled as missing data. Neither SP nor NKA had a significant effect on systolic or diastolic blood pressure (data not shown), although most subjects experienced transient flushing and warmth after receiving 4 and 8 mg/ml SP. The geometric mean value (SD in doubling doses) for PC20 SP was 1.61 (1.62) mg/ml, whereas for PC20 NKA it was 130.83 (1.55) μg/ml. As a consequence, the molar concentration to reach PC20 was 7.5-fold higher for SP than for NKA. There was a moderate correlation between the PC20 values for NKA and histamine (Rs = 0.31, p = 0.04), whereas neither of these correlated with PC20 SP (Rs < 0.19, p > 0.56). The maximal drop in FEV₁ after SP and NKA challenge was similar (median [range], 25.4 [20.0–30.6] and 24.0 [20.0–28.4] % fall from baseline, respectively) (p = 0.31).

There were no significant changes in the differential cell counts 30 minutes after the SP or NKA challenge (p > 0.09). Furthermore, total cell counts per gram sputum were not altered by the challenges (p > 0.11) (Table 2).

Markers of Microvascular Leakage

There were no significant differences in the baseline levels of all sputum markers between the SP and NKA challenge days (Table 3) (p > 0.44). The concentrations of α2-macroglobulin, ceruloplasmin, and albumin in sputum increased markedly 30 minutes after SP as compared with before the challenge (median fold changes of 3.1, 2.2, and 2.9, respectively) (p < 0.013) (Figure 1 and Table 3). In contrast, there were no significant changes in these markers after NKA challenge (median fold changes of 1.0, 1.0, and 1.1) (p > 0.31) (Figure 1). Neither SP nor NKA had a significant effect on the levels of fibrinogen in sputum (median fold changes of 1.4 and 1.5, respectively) (p > 0.29) (Figure 1D). At 30 minutes after challenge, the levels of α2-macroglobulin were significantly higher after SP as compared with NKA (p = 0.028) (Figure 1A) (Table 3).

The sputum-to-serum ratios for α2-macroglobulin, ceruloplasmin, and albumin increased significantly after the SP challenge (median pre- to postchallenge values, 1.22 to 2.94, 3.83 to 7.26, and 2.94 to 10.72, respectively) (p < 0.021), but not after the NKA challenge (1.14 to 1.56, 2.77 to 5.21, and 2.94 to 5.34, respectively) (p > 0.33). The α2-macroglobulin sputum-to-serum ratio after SP challenge was higher than after the NKA challenge (p = 0.05).

TABLE 1. CHARACTERISTICS OF SUBJECTS

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Sex (F/M)</th>
<th>Age (years)</th>
<th>FEV₁ (% pred)</th>
<th>PC20his (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>23</td>
<td>86.0</td>
<td>0.20</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>23</td>
<td>87.7</td>
<td>0.65</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>22</td>
<td>96.0</td>
<td>0.74</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>23</td>
<td>100.8</td>
<td>0.98</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>22</td>
<td>100.0</td>
<td>1.37</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>21</td>
<td>91.4</td>
<td>1.76</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>24</td>
<td>102.5</td>
<td>1.82</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>18</td>
<td>102.6</td>
<td>1.89</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>20</td>
<td>80.6</td>
<td>2.03</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>26</td>
<td>82.8</td>
<td>2.19</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>24</td>
<td>82.1</td>
<td>2.73</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>24</td>
<td>105.9</td>
<td>3.90</td>
</tr>
</tbody>
</table>

|                     | 22.5 (2.11)† | 93.2 (9.13)* | 1.35 (1.15)* |

Definition of abbreviations: FEV₁ = forced expiratory volume in 1 second; PC20his = provocative concentration of histamine causing a 20% fall in FEV₁;
* Mean (SD);
† Geometric mean (SD in doubling doses).

TABLE 2. DIFFERENTIAL CELL COUNTS IN INDUCED SPUTUM

<table>
<thead>
<tr>
<th></th>
<th>Prechallenge</th>
<th>Postchallenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurokinin A</td>
<td>48.6 (9–82)</td>
<td>45.2 (24–77)</td>
</tr>
<tr>
<td>Substance P</td>
<td>52.2 (34–77)</td>
<td>35.0 (14–82)</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>36.1 (6–72)</td>
<td>38.9 (16–59)</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>27.8 (15–59)</td>
<td>41 (15–78)</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>1.3 (0–18)</td>
<td>2.8 (0–30)</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>1.6 (0–6)</td>
<td>2.0 (0–8)</td>
</tr>
<tr>
<td>Epithelial cells, %</td>
<td>6.8 (1–45)</td>
<td>5.3 (1–18)</td>
</tr>
</tbody>
</table>

Differential cell counts are expressed as percentage of nonsquamous cells. Values are presented as medians (range).
The median cumulative dose of inhaled SP was 1.88 mg (range, 0.13–6.38 mg) and for NKA it was 121.10 μg (range, 27.35–996.10 μg). These cumulative doses were not correlated with the percentage increase in sputum leakage markers (p > 0.12) or sputum-to-serum ratios (p > 0.08). Furthermore, there was no relationship between the maximal percent fall in FEV₁ after SP or NKA and the rise in leakage markers (p > 0.10) or sputum-to-serum ratios (p > 0.17).

**DISCUSSION**

This study has shown that inhaled substance P induces a rapid increase in the levels of α₂-macroglobulin, albumin, and ceruloplasmin in induced sputum in patients with asthma. In contrast, inhaled neurokinin A was not able to induce such changes. These results indicate that NK1 receptor stimulation enhances microvascular leakage in the airways of patients with asthma in vivo, whereas NK2 stimulation does not. Furthermore, it becomes apparent that induced sputum can be a suitable method of monitoring microvascular leakage as a component of airway inflammation in asthma.

![Figure 1](marker.png)
enge. This allowed comparison of the leakage markers between distinct stimuli at a given degree of bronchoconstriction.

We have chosen NKA instead of histamine or methacholine challenge, because these mediators have previously been shown to induce an increase in α2-macroglobulin (17). Moreover, NKA is a tachykinin and therefore a member of the same family of neuropeptides as SP. For each of these agonists the receptors have been demonstrated within human airways (10), and by using these two tachykinins we aimed to distinguish preferential NK1 and NK2 stimulation within the airways (7, 29). Only by using selective receptor antagonists would it be possible to prove the unique involvement of the NK1 receptor in our model (30). With selective antagonists, it has indeed been demonstrated that stimulation of the NK1 receptor induces microvascular leakage, whereas the NK2 receptor exerts its effects on bronchoconstriction in humans and animals (31, 32). We cannot exclude that the differences between SP and NKA in inducing microvascular leakage are related to the 7.5-fold difference in molar concentrations between these compounds required to reach the PC20. Therefore, we may have missed a potential positive effect of NKA because of the lower concentration nebulized. However, the absence of relationships between the doses of SP or NKA and the increase in leakage markers in sputum does not favor such an explanation. In addition, similar differences between SP and NKA in potency have been found in animal models (33).

We had chosen to start sputum induction 30 minutes after the challenges, on the basis of the study by Halldorsdottir and colleagues (17), who demonstrated an increase in α2-macroglobulin in sputum 45 minutes after a histamine challenge. It can be argued that the extravasation of plasma occurs earlier, based on the rapid effect of mediators on microvascular permeability (5). This would imply that the maximal increase in these markers is even more pronounced than we observed. The time course of leakage may also explain why we did not observe a significant SP-induced increase in the levels of sputum fibrinogen. It cannot be excluded that the leakage of fibrinogen occurred at an earlier time point and had dissipated by the time of sputum induction. Furthermore, after escaping the vasculature, fibrinogen could polymerize into fibrin and thereby cannot gain access to the airway. A recent study by Peebles and coworkers also failed to show plasma extravasation, as demonstrated by fibrinogen in bronchoalveolar lavage fluid, 24 hours after an allergen challenge in patients with asthma (34).

The SP-induced increase in permeability of the airways appears to be associated with the formation of small interendothelial pores. Subsequently, the extravasating bulk plasma moves through these pores into the airway lumen, a process that is likely to be driven by an increase in hydrostatic pressure (35). Hirata and coworkers have demonstrated that injection of SP causes the formation of intercellular gaps between endothelial cells in postcapillary and collecting venules of rat trachea (36). Microvascular leakage in vitro can be reduced with cAMP-elevating drugs, for example, phosphodiesterase inhibitors and formoterol, by inhibiting endothelial gap formation (37, 38). In healthy subjects, such antiinflammatory effects of formoterol could indeed be confirmed in sputum, indicating that this mechanism plays a role in humans in vivo (4).

What are the clinical implications of our findings? The SP-induced increase in microvascular leakage in our study suggests that the phenomenon of neurogenic inflammation can be relevant in patients with asthma in vivo. Indeed, elevated levels of SP have been found in bronchoalveolar lavage fluid and sputum of patients with asthma (8, 9). In particular, during unstable episodes of asthma, NK1 activity seems to have functional significance, for instance, during cold air, virus (39), or allergen (40)-induced plasma extravasation. Taken together, this implies that NK1 receptor antagonists may reduce microvascular leakage in asthma and thereby could have beneficial effects in the treatment of asthma. Using the current investigational model of “dual induction,” that is, induced exudation and induced sputum, it will be possible to demonstrate microvascular leakage in humans in vivo. Therefore, this method can be applied when testing the antiinflammatory effect of newly developed drugs, such as NK1 antagonists.

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References
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