Airway inflammation in asthma : from concept to the clinic

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Chapter
4

Assessment of microvascular leakage via sputum induction: the role of substance P and neurokinin A in patients with asthma

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Abstract

Background 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 NK1-agonist and mediator of plasma extravasation, on markers of microvascular leakage in induced sputum in patients with asthma.
Methods In a cross-over study, sputum was induced before and 30 min. after inhalation of substance P or neurokinin A (as control) in 12 atopic and mild, steroid-naive asthmatic subjects. The levels of alpha-2-macroglobulin, ceruloplasmin, albumin, and fibrinogen were determined in induced sputum as markers of leakage.
Results Substance P induced a significant increase in the levels of alpha-2-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 ($p>0.12$).
Conclusion 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 anti-exudative effect of newly develop drugs, such as NK1-antagonists.

Introduction

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 airways 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 luminal fluids. Thus far, there are only 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). Amongst 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 BAL fluid and sputum, and a further increase occurred following allergen challenge in patients with asthma (8;9). In human tissue, the distinct NK1- 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 pro-inflammatory effects: plasma extravasation, mucus secretion, inflammatory cell chemotaxis and activation (7). Interestingly, NK1- and NK2-receptor mRNA expression appears to be increased in asthmatics as compared to normals (12;13).

It is still unknown whether NK1-stimulation leads to microvascular leakage in the airways of asthmatics *in vivo*. So far, SP-induced effects on microvascular leakage have only been demonstrated 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 predominantly be due 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 asthmatic patients *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 asthmatic patients *in vivo*. To that end, we determined the levels of albumin, fibrinogen, ceruloplasmin and alpha-2-macroglobulin as markers of leakage in induced sputum before and after SP challenge in patients with asthma. Since challenges with methacholine or histamine are known to induce leakage (17), inhaled NKA was used as a control challenge, in order to compare the leakage markers in sputum between a NK1- and NK2-agonist at a given degree of bronchoconstriction.

Methods

Subjects

Twelve non-smoking atopic asthmatic volunteers (8 female, 20-26 years) participated in the study (Table 1). All subjects had a history of episodic chest tightness and wheezing and none was using medication except for short-acting ß$_2$-agonists as needed. Atopy was determined by a positive response to a standardized skin prick test with 10 common allergens (Vivodiagnost, ALK, Benelux). The baseline forced expiratory volume (FEV_1) was > 80% predicted (18) and all subjects were hyperresponsive to inhaled histamine (provocative concentration causing a 20% fall in FEV_1 (PC_{20}) <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 and all volunteers gave a written informed consent.

Design

The study had a single blind randomized, cross-over design. At least one week before entering the study, inclusion and exclusion criteria were examined and PC_{20} histamine was determined. Inhaled SP or NKA challenge was performed on two randomized study days with an interval of at least one week. Sputum was induced 2 days before and 30 minutes following 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 a previously validated protocol (16;20;21). SP (Sigma, St. Louis, USA) was inhaled in serial doubling concentrations (0.25 - 8 mg/ml) and NKA (Bachem, Budendorf, Switzerland)

Table 1. Characteristics of the subjects

Subject no	Sex (F/M)	Age (years)	FEV_1 (%pred)	PC_{20} his (mg/ml)
1	F	23	86.0	0.20
2	F	23	87.7	0.65
3	F	22	96.0	0.74
4	F	23	100.8	0.98
5	M	22	100.0	1.37
6	M	21	91.4	1.76
7	F	24	102.5	1.82
8	F	18	102.6	1.89
9	F	20	80.6	2.03
10	M	26	82.8	2.19
11	M	24	82.1	2.73
12	F	24	105.9	3.90
		22.5 (2.11)*	93.2 (9.13)*	1.35 (1.15)†

FEV_1 = forced expiratory volume in one second; PC_{20}his = provocative concentration of histamine causing a 20% fall in FEV_1; * mean (SD), † geometric mean (SD in doubling doses).

in concentrations between 8 and 1000 mg/ml. SP and NKA were aerosolized by a jet-nebulizer into a collapsible bag (Mallinckrodt, Petten, The Netherlands) (22). The aerosols were subsequently inhaled by tidal breathing for 3-4 min. at 7 min. intervals. The airway responses to SP and NKA were measured using FEV_1 (18), at 90 and 180 seconds after each dose (16). Additionally, systolic and diastolic blood pressure were monitored after each dose. Both challenges were discontinued when PC_{20} was reached (FEV_1 dropped by 20% from baseline).

Sputum induction and processing

Prior to induction, each subject inhaled 200 µg salbutamol. Sputum was induced by inhalation of NaCl 4.5% during 3x5 minutes intervals, according to a recommended protocol validated in our lab (23;24). Sputum samples were processed according to a validated method (24). An equal volume of 0.1% w/v dithiotreitol was added to the whole sample. Supernatant was aspirated following centrifugation and the total cell counts were determined. Differential cell counts were expressed as a percentage of 250 non-squamous cells (24).

Measurement of microvascular leakage

Fibrinogen was measured in coded sputum samples using a commercially available ELISA (Kordia, Leiden, The Netherlands) and albumin was determined by rate nephelometry (24). The concentrations of alpha-2-macroglobulin and ceruloplasmin in sputum and serum were measured by ELISA. Commercially available antibodies for alpha-2-macroglobulin (code A033) and ceruloplasmin (code A031) were obtained from DAKO (DAKO, Glostrup, Denmark) (25;26). To correct for protein concentrations in blood, sputum-to-serum ratios were determined as follows: sputum-protein (mg/l)/serum-protein (g/l) (26).

Analysis

The PC_{20} for histamine, SP and NKA were log transformed before statistical analysis and expressed as geometric mean (SD in doubling doses) The markers of microvascular leakage were expressed as median (range). Wilcoxon Signed Rank test was applied to test for differences before and after, and between challenges. Correlations were analysed using Spearman rank test. A p-value of < 0.05 was used as statistical significance and all analysis were performed using SPSS 10.0.

Results

Two subjects (nos. 7 and 12) did not produce sputum after the NKA challenge, whilst subject 8 was not able to produce sputum following SP. 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), though most subjects experienced transient flushing and warmth after 4 and 8 mg/ml SP. The geometric mean value (SD in doubling doses) for PC_{20} SP was 1.61 (1.62) mg/ml, whereas for PC_{20} NKA was it 130.83 (1.55) mg/ml. As a

consequence, the molar concentration to reach PC_{20} was 7.5 fold higher for SP than for NKA. There was a moderate correlation between the PC_{20} values for NKA and histamine (Rs = 0.59, p = 0.04), whereas neither of these correlated with PC_{20} SP (Rs < 0.19, p > 0.56). The maximal drop in FEV_1 following 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 the NKA challenge days (Table 3) (p > 0.44). The concentrations of alpha-2-macroglobulin, ceruloplasmin and albumin in sputum increased markedly 30 minutes following SP as compared to before the challenge (median fold change 3.1, 2.2 and 2.9, respectively) (p < 0.013) (Figure 1; Table 3). In contrast, there were no significant changes in these markers after NKA challenge (median) fold change 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 change 1.4 and 1.5, respectively) (p > 0.29) (Figure 1d). At 30 min following challenge, the levels of alpha-2-macroglobulin were significantly higher after SP as compared to NKA (p = 0.028) (Figure 1a) (Table 3). The sputum-to-serum ratios for alpha-2-macroglobulin, ceruloplasmin and albumin increased significantly after the SP challenge (median prior to post challenge 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 (1.14 to 1.56, 2.77 to 5.21 and 2.94 to 5.34) (p > 0.33). The alpha-2-macroglobulin sputum-to-serum ratio following SP challenge was higher than after the NKA challenge (p = 0.05). The cumulative dose of inhaled SP was (median (range)) 1.88 (0.13-6.38) mg and for NKA 121.10 (27.35-996.10) mg. 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 % fall in FEV_1 following SP or NKA and the rise in leakage markers (p>0.10) or sputum-to-serum ratios (p>0.17).

Table 2. Differential cell counts in induced sputum

	Neurokinin A		Substance P	
	pre	post	pre	post
macrophages (%)	48.6 (9-82)	45.2 (24-77)	52.2 (34-77)	35.0 (14-82)
neutrophils (%)	36.1 (6-72)	38.9 (16-59)	27.8 (15-59)	41 (15-78)
eosinophils (%)	1.3 (0-18)	2.8 (0-30)	2.2 (0-21)	1.8 (0-32)
lymphocytes (%)	1.6 (0-6)	2.0 (0-8)	1.6 (0-10)	2.4 (0-13)
epithelial cells (%)	6.8 (1-45)	5.3 (1-18)	5.6 (3-35)	5.2 (2-25)

Differential cell counts are expressed as percentage of non-squamous cells. Values are presented as median (range)

Table 3. Markers of microvascular leakage in induced sputum

	Neurokinin A		Substance P	
	pre	post	pre	post
α2-macroglobulin	4.8	6.2	3.2	14.9*†
(µg/ml)	(0.4-25.5)	(0.4-41.1)	(0.4-22.1)	(0.4-139.9)
Ceruloplasmin	0.56	0.65	0.62	1.70*
(µg/ml)	(0.2-3.3)	(0.2-5.2)	(0.2-3.5)	(0.2-4.9)
Albumin	132.7	238.8	141.2	471.6*
(µg/ml)	(19.6-1889.6)	(25.8-1795.6)	(21.6-629.4)	(83.4-1834.0)
Fibrinogen	13.1	12.9	12.2	16.4
(µg/ml)	(1.0-28.7)	(1.6-44.1)	(1.9-41.4)	(3.6-44.2)

* p-value < 0.013 compared with pre-challenge; † p-value = 0.028 compared with post NKA-challenge. Data are expressed in median (range)

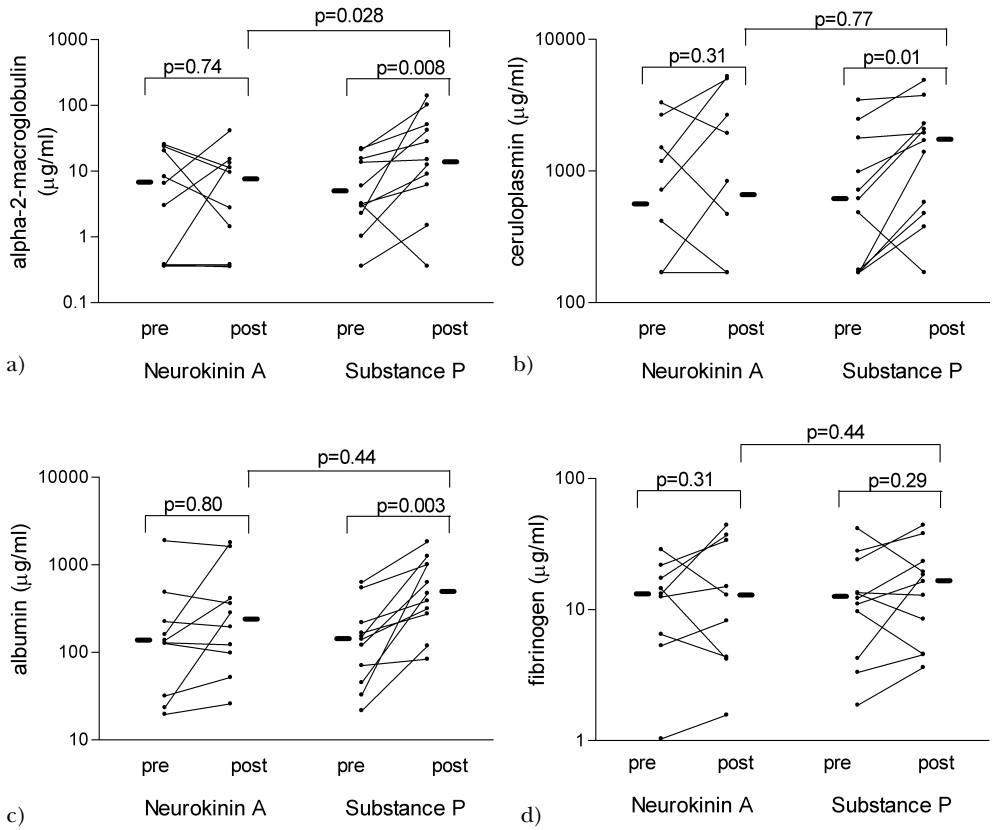

Figure 1. Markers of microvascular leakage in induced sputum pre and 30 min. post inhalation of neurokinn A or substance P.

a) alpha-2-macroglobulin in µg/ml; b) ceruloplasmin in µg/ml; c) albumin in µg/ml;
d) fibrinogen in µg/ml)

Discussion

This study has shown that inhaled substance P induces a rapid increase in the levels of alpha-2-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 airways inflammation in asthma.
This is the first study examining the effects of SP on microvascular leakage in humans *in vivo*. Similar results have been found in previous animal studies. Lötvall *et al.* have demonstrated in guinea pigs, that microvascular leakage in the airways occurred after aerosolized SP (14), which appears to be inhibited by a selective NK1 receptor antagonist (27). In addition, the presently observed absence of leakage following NKA inhalation is also in agreement with results reported in guinea pig airways (28). Interestingly, our data demonstrate that induced exudation can be measured using induced sputum. This confirms and extends the findings by Halldorsdottir *et al.*, who have shown that inhaled histamine induces a comparable increase in the levels of alpha-2-macroglobulin in induced sputum (17).

It seems unlikely that the present findings can be explained by measurement errors, since the data were obtained by using validated methods of performing SP and NKA challenge (16;21), sputum induction and processing (24) and determination of markers of microvascular leakage (25). All subjects were non-smokers, and were having clinically stable asthma without using steroids. Furthermore, we have applied a cross-over, randomized design, using NKA as a control challenge. 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 alpha-2-macroglobulin (17). Moreover, NKA is a tachykinin and therefore 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). Though, only when using selective receptor antagonists, it would be possible proof 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 man and animals (31;32). We can not 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 PC_{20}. Therefore, we may have missed a potential possitive effect of NKA due to the lower concentration nebulized. However, the absence of relationships between the doses of SP or NKA and the increase of leakage

markers in sputum does not favour such 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 at 30 minutes after the challenges, based on the study of Halldorsdottir *et al.* (17), who demonstrated an increase in alpha-2-macroglobulin in sputum 45 minutes following 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 can not be excluded that the leakage of fibrinogen occurred at an earlier time point and had dissipated by the time of the sputum induction. Furthermore, after escaping the vasculature, fibrinogen could polymerize into fibrin and thereby cannot gain access to the airway. A recent study of Peebles *et al.*, also failed to show plasma extravasation, as demonstrated by fibrinogen in BAL, 24 hours following 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 is moving through these pores into the airway lumen, which is likely to be driven by an increase in hydrostatic pressure (35). Hirata *et al.* 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 using cAMP elevating drugs e.g. PDE inhibitors and formoterol by inhibiting endothelial gap formation (37;38). In healthy subjects, such anti-exudative effect 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 asthmatics *in vivo*. Indeed, elevated levels of SP have been found in BAL 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 implicates 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 anti-exudative effect of newly developed drugs, such as NK1-antagonists.

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Chapter 4