Elevated tachykinin levels in induced sputum from asthmatics and cough patients with acid reflux.

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**ABSTRACT**

**Introduction:** Acid reflux may aggravate airway disease including asthma and chronic cough. One postulated mechanism concerns a vagally mediated oesophageal-tracheobronchial reflex with airway sensory nerve activation and tachykinin release.

**Objective:** We tested the hypothesis that patients with airways disease and reflux have elevated airway tachykinin levels compared to those without reflux.

**Methods:** Thirty-two patients with airways disease (16 mild asthmatics and 16 non asthmatics with chronic cough) underwent 24 hour esophageal pH monitoring. Acid reflux was defined as increased total oesophageal acid exposure (% total time pH < 4 of > 4.9% at the distal probe). All subjects underwent sputum induction; differential cell counts and concentrations of substance P (SP), Neurokinin A (NKA), albumin and α2-macroglobulin were determined.

**Results:** SP and NKA levels were significantly higher in reflux patients compared to those without (SP; 1434(680) pg/ml versus 906(593) pg/ml, p = 0.026, NKA, 81(33) pg/ml versus 52(36) pg/ml, p = 0.03). Significantly increased tachykinin levels were also measured when asthmatic patients with reflux were compared to asthmatics without, (SP; 1508 (781) pg/ml versus 737(512) pg/ml, p = 0.035, NKA; median [IQR],108 [85-120] pg/ml versus 75 [2-98] pg/ml, p = 0.02). Among asthmatics there was significant positive correlation between distal oesophageal acid exposure and SP levels (r = 0.59, p=0.01) and NKA levels (r = 0.56, p=0.02). Non-significant increases in SP and NKA were measured in coughers with reflux, (SP; 1534.71 (711) pg/ml versus 1089(606) pg/ml, p = 0.20, NKA, 56 (43) pg/ml versus 49(17) pg/ml, p = 0.71). No significant difference in differential cell counts or any other biochemical parameter was noted between study groups.

**Conclusion:** This study demonstrates elevated airway tachykinin levels in asthmatics and cough patients with co-existent acid reflux. This suggests airway sensory nerve activation in this population.
INTRODUCTION

There is an increased prevalence of acid reflux among asthmatics and patients with chronic cough compared to the general population (1-3). It has been suggested that acid reflux may trigger exacerbations of asthma and chronic cough although the precise mechanism by which this may occur is unclear. Microaspiration of gastric contents (the reflux theory) is one possibility but this has not been consistently demonstrated (4). As an alternative, a vagally mediated oesophageal-tracheobronchial reflex (the reflex theory) was first postulated because oesophageal acidification was shown to increase respiratory resistance in dogs, which could be ablated with bilateral vagotomy (5). Subsequent studies in adult asthmatics have confirmed that oesophageal acidification increases respiratory resistance and bronchial hyperresponsiveness (6; 7). In subjects with chronic cough, acid perfusion into the distal oesophagus, induces coughing episodes which can be attenuated by pre-treatment with an inhaled anti-cholinergic (8).

Activation of afferent sensory nerves in the oesophagus may stimulate a subpopulation of neurons in the central nervous system (CNS) that mediate defensive reflexes including cough and bronchospasm. However, evidence from neuronal tracing studies in guinea pigs has documented distinct neural projections between the oesophagus and airway (9). Physiological studies have indicated that these are nonadrenergic noncholinergic (NANC) neurones and their activation evokes ‘axonal’ reflexes with release of neuropeptides into the airway and subsequent neurogenic inflammation (10). The tachykinins, substance P (SP) and neurokinin A (NKA) are the neuropeptides most often associated with axonal reflexes and are potent mediators of cough, bronchospasm, microvascular leakage and mucus secretion (11). In support of this hypothesis, animal studies consistently demonstrate that intra-oesophageal acid perfusion stimulates release of tachykinins into the airways (12; 13). To date, there have been no findings to suggest evidence for neurogenic inflammation in the airways of respiratory patients with reflux disease. The aim of this study was to test the hypothesis that asthmatic and chronic cough patients with acid reflux have elevated levels of tachykinins (SP and NKA) in induced sputum. In addition, we measured biomarkers of microvascular leakage ($\alpha_2$-macroglobulin and albumin) and the inflammatory cell profile in these subjects.

METHODS

Subjects

Asthmatic and cough subjects were recruited from patients attending the general respiratory clinic and specialist cough clinic respectively at the Regional Respiratory Centre, Belfast City Hospital who had expressed an interest in the study and met the inclusion criteria. All subjects were lifetime non-smokers aged between 18 and 75 years, and had no history of previous gastrointestinal surgery. Subjects with asthma had a prior history of typical symptoms together with objective evidence of variable airflow obstruction, as indicated by one or more of the following: peak expiratory flow (PEF) variability (greater than 20% maximum within-day amplitude from twice daily PEF measurements over two weeks); or more than 15% improvement in $FEV_1$ 10 minutes after administration of 200 $\mu$g of albuterol; or evidence of methacholine airway hyperresponsiveness ($PC_{20FEV_1} < 16$ mg / ml). Subjects with non-asthmatic chronic cough had a cough lasting longer than 8 weeks, normal spirometry and negative methacholine challenge test ($PC_{20FEV_1} > 16$ mg/ml).
Definition of Acid reflux
As we wished to compare subjects with and without oesophageal reflux, all subjects, had 24-h ambulatory pH monitoring performed, as previously described (14). In brief, a pre-calibrated dual probe monocrystalline antimony electrode (Synectics Medical, Enfield, UK) was placed (distal probe - 5 cm above the manometrically determined upper border of the lower oesophageal sphincter and proximal probe – 10 cm above the distal probe). Subjects documented respiratory symptoms both in a diary card and by pressing an event marker on the digitrapper. Data were analysed using the Esophogram program (Gastrosoft, USA) and based on previously defined normal values (15), subjects were classified as having significant acid reflux (% total time pH < 4 of > 4.9% at distal probe). Using this cut-off value, we recruited 32 subjects; 16 subjects with mild asthma (8 with acid reflux) and 16 with non-asthmatic chronic cough (8 with acid reflux). These subjects were asked to attend on 2 occasions. At visit 1, a physical examination and spirometry were performed. At the second visit, four weeks later, induced sputum was performed and processed as described below. At visit 1, subjects were asked to discontinue acid suppression therapy at least 1 week prior to Visit 2 and asthmatic subjects on inhaled steroids were asked to discontinue these from visit 1 i.e. 4 weeks prior to sputum induction. These subjects were only eligible for inclusion, if they remained stable during this 4-week period. In order to recruit 32 patients, approximately 50-60 patients were screened. The principal investigators (LMcG and LGH) and the research fellow (RNP) performed the subject screening.

Ethical approval for this study was obtained from the Research Ethics Committee, Queen’s University Belfast and all patients gave informed consent to participate.

Induced sputum
This was performed as previously described (16). In brief, sputum was induced using 4.5% sterile hypertonic saline using a high-output ultrasonic nebuliser (DeVilbiss ultra-neb 2000: Sunrise Medical, Wollaston, West Midlands, UK). The sputum sample was processed using established methods (17) and samples processed for differential cell count and supernatants aliquotted and frozen (-70 °C) for later analysis. Samples were stored in a protease inhibitor cocktail (18). Slides were stained with DiffQuik to obtain a differential cell count made by a blinded observer counting 500 cells.

Tachykinin measurements
SP was measured using a commercially available enzyme linked immunosorbent assay (ELISA) (R & D systems, UK). It has no significant cross reactivity with NKA, Neurokinin B, or Neuropeptide K. The limit of detection of this assay is 8 pg/ml. NKA was measured using a radioimmunoassay utilising an N-terminal specific anti-serum (SK-570), which was raised in guinea pig to synthetic human NKA. It cross-reacts fully with neurokinin B and neuropeptide K but less than 0.1% with SP. The detection limit for the assay is 5 pg/ml. The coefficient of variation for the NKA assay was inter-assay 10.8% and intra assay 6.8% over the relevant range. Samples were Sep-pakked (C18 Sep-pak, Waters,) and after elution freeze dried and reconstituted in buffer prior to assay. Using these extraction and assay methods, spiking studies demonstrated that peptide recovery was >90%.
Albumin & α2-Macroglobulin
Albumin was measured using rate nephelometry (IMMAGE microalbumin method) (Beckman Coulter Inc) (19). Using this assay method, albumin recovery was > 90%. α2-macroglobulin was measured by rate nephelometry using the Dade Behring ProSpec system (Dade Behring Marburg GmbH, Marburg, Germany), achieving a sensitivity of 0.65 mg/l (20).

Statistical analysis
All statistical analyses were performed by using SPSS version 11 for Windows (SPSS inc, Chicago, IL, USA). Normality was determined using a Kolmogorov-Smirnov test. Parametric data is quoted as mean (standard deviation) and comparison between subjects with and without GORD was made using independent t-tests. Non-parametric data are quoted as median (interquartile range) and analysed using the Mann-Whitney U test. All tests were two-tailed and p values < 0.05 were considered significant. Correlation analysis was performed using Spearmans rank test.

RESULTS
The study subjects’ characteristics are presented in table 1. Distal oesophageal pH parameters were significantly different between subjects with and without reflux (p < 0.05). There were no significant differences in any other demographic parameter displayed between asthmatics and coughers or subjects with and those without reflux. The majority of coughers (7 of 8) with acid reflux had typical reflux symptoms whereas 4 of the 8 asthmatics with acid reflux were asymptomatic.

Table 1. Demographic details of all asthma and chronic cough study patients (n=32).

<table>
<thead>
<tr>
<th></th>
<th>ASTHMA (reflux)</th>
<th>ASTHMA (non-reflux)</th>
<th>COUGH (reflux)</th>
<th>COUGH (non-reflux)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Age (years) [mean (SD)]</td>
<td>59.4 (9.18)</td>
<td>51.0 (14.09)</td>
<td>62.0 (5.66)</td>
<td>61.5 (6.46)</td>
</tr>
<tr>
<td>Gender (n=male)</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>FEV1 (litres/min) [mean (SD)]</td>
<td>2.38 (0.59)</td>
<td>2.66 (1.2)</td>
<td>2.66 (0.54)</td>
<td>2.38 (0.48)</td>
</tr>
<tr>
<td>FEV1 (% pred) [mean (SD)]</td>
<td>83.8 (11.82)</td>
<td>83.3 (14.35)</td>
<td>103.8 (11.54)</td>
<td>103.3 (15.63)</td>
</tr>
<tr>
<td>Number prescribed ICS</td>
<td>7</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Methacholine Pc20 (mg/ml) [Median (IQR)]</td>
<td>2.0 (0.2-14.6)</td>
<td>3.0 (1.98-5.23)</td>
<td>&gt; 16 mg/ml</td>
<td>&gt; 16 mg/ml</td>
</tr>
<tr>
<td>% time pH &lt; 4 at distal probe</td>
<td>13.9 (7.4-16.18)</td>
<td>2.25 (1.43-3.15)</td>
<td>7.75 (5.65-9.58)</td>
<td>2.8 (1.95-4.23)</td>
</tr>
</tbody>
</table>
Tachykinin levels

When all 32 patients were categorised according to the presence of acid reflux, there were increased SP levels in those with reflux (mean 1434 (680) pg/ml) compared to the non-refluxers (mean 906 (593) pg/ml) (p =0.026), [see figure 1]. NKA levels in those with reflux were also increased (81(33) pg/ml) compared to non-refluxers (52(36) pg/ml) (p = 0.03) [see figure 2]. Significantly increased substance P levels were also seen in asthmatic subjects with acid reflux (mean 1508 (781) pg/ml compared to those without reflux (737 (512) pg/ml [p = 0.035]). Similar elevations in NKA levels in asthmatics with acid reflux were also seen (median [IQR] 108 [85-120] pg/ml) compared to those without reflux (median [IQR] 75 [2-98] pg/ml) (p = 0.02). In the asthmatic subjects there was a significant positive correlation between the degree of distal acid reflux (% time pH < 4) and SP levels (r = 0.59, p = 0.01) (figure 3) and NKA levels (r = 0.56, p = 0.02) (figure 4). There was no significant correlation between the degree of proximal acid reflux and neuropeptide levels (SP; r = 0.19, p = 0.38, NKA; r = 0.26, p = 0.24). There was no significant correlation between SP and NKA levels (r = 0.30, p = 0.10).

In the cough group, SP levels were also increased in those with reflux compared with non-refluxers although this did not reach statistical significance (mean SP 1535 (711) pg/ml compared to 1089 (606) pg/ml (p = 0.20). There was no significant difference in NKA levels; (56 (43) pg/ml in those with reflux compared to 50 (18) pg/ml in those without [p = 0.71].

Differential cell counts

Total cell counts, cell viability and differential cell counts for all study subjects are displayed in table 2.

<table>
<thead>
<tr>
<th>Data given as median (interquartile range)</th>
<th>ASTHMA (reflux) n = 8</th>
<th>ASTHMA (non-reflux) n = 8</th>
<th>COUGH (reflux) n = 8</th>
<th>COUGH (non-reflux) n = 8</th>
</tr>
</thead>
</table>

Definition of abbreviations: FEV₁ = Forced expiratory volume in 1 second; SD = Standard deviation; IQR = Inter-quartile range; ICS = inhaled corticosteroids.
<table>
<thead>
<tr>
<th></th>
<th>1.1(0.9-2.0)</th>
<th>0.7(0.5-1.0)</th>
<th>1.6(0.9-2.7)</th>
<th>1.1(0.9-1.7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count x10⁶/ml</td>
<td>86.3(79.7-92.4)</td>
<td>80.2(75.4-89.8)</td>
<td>74.9(68.6-81.3)</td>
<td>85.8(76.1-91.8)</td>
</tr>
<tr>
<td>Cell viability (%)</td>
<td>13.4 (10.1-22.1)</td>
<td>32.9 (13.9-51.5)</td>
<td>12.9 (8.9-25.7)</td>
<td>21.5 (16.1-28.0)</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>0.0 (0.0-0.4)</td>
<td>0.4 (0.1-1.1)</td>
<td>0.0 (0.0-0.2)</td>
<td>0.0 (0.0-0.1)</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>12.9 (8.9-25.7)</td>
<td>21.5 (16.1-28.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>3.2 (0.6-5.8)</td>
<td>1.2 (0.5-3.5)</td>
<td>9.2 (0.2-38.4)</td>
<td>5.5 (1.1-9.55)</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>0.0 (0.0-0.2)</td>
<td>0.4 (0.2-0.8)</td>
<td>0.0 (0.0-0.7)</td>
<td>0.0 (0.0-1.1)</td>
</tr>
<tr>
<td>Epithelial cells (%)</td>
<td>0.7(0.5-1.0)</td>
<td>1.6(0.9-2.7)</td>
<td>74.9(68.6-81.3)</td>
<td>85.8(76.1-91.8)</td>
</tr>
</tbody>
</table>

On comparing all subjects (asthmatic and cough patients), there was no difference in neutrophil counts in those with reflux compared to those without reflux (median [IQR] 86 [65-92] % in those with reflux, compared to those without reflux (median [IQR] 74 [63-82] %, p = 0.14). In the asthma patients alone however, there was a trend towards a significant increase in % neutrophils in the asthma patients with reflux compared to those without, (median [IQR] 82 [7-87] % compared to 55 [39-79] % (p =0.07)). In the cough patients, there was no significant difference observed in any inflammatory cell type.

**Albumin and α2-macroglobulin**
There was no significant difference in sputum levels of albumin or α2-macroglobulin when refluxers were compared with non-refluxers (median [IQR] albumin 218.7 g/l (122.2-305.1) mg/l versus 177.8 (177.8-549.9) mg/l in the non-refluxers (p = 0.79), median [IQR] α2-macroglobulin 12.7 (0.31-39.8) mg/l versus 9.6 (0.31-62.6) mg/l in the non refluxers (p = 0.954).

**DISCUSSION**
The main finding of this study is that substance P and neurokinin A levels are elevated in induced sputum samples from patients with airways disease and acid reflux compared to those without. This suggests that gastro-oesophageal reflux may contribute to airway inflammatory events possibly by sensory nerve stimulation and the subsequent release of tachykinins into the airway.

A number of mechanisms whereby reflux of acid may aggravate respiratory disease have been proposed. Aspiration of refluxed gastric contents including acid may cause direct inflammatory stimulation of the airway, although this has not been consistently demonstrated in radioisotope studies of asthmatic patients with known reflux disease (4). However, the observation that co- incidental oesophageal and tracheal acidification can be accompanied by falls in peak expiratory flow measurements suggests that in some circumstances, aspiration may be a factor (21).

Alternatively, a vagally mediated esophageo-tracheobronchial reflex has been proposed, and a series of in vivo animal studies have clearly demonstrated that intra-oesophageal acid instillation causes bronchoconstriction and airway microvascular leakage due to tachykinin release into the lungs (12; 13). Most recently, these effects have been significantly reduced in rabbits by pre-treatment with nociceptin/orphanin FQ (N/OFQ), an endogenous peptide ligand for the N/OFQ peptide (NOP) receptor which is believed to have a direct inhibitory effect on tachykinin release from nerve fibres (10). The net conclusion from these animal studies is that acidification of the oesophagus activates local axonal reflexes resulting in neurogenic inflammation in the airway and such events may be pharmacologically attenuated.
In the present study, when all study subjects were considered together, SP and NKA levels were significantly elevated in those with clearly documented acid reflux compared to those without evidence of reflux disease. This finding was maintained when asthmatic subjects alone were considered and although tachykinin levels remained elevated in cough patients with acid reflux compared to those without, this was not statistically significant. One possible explanation for this latter finding is that asthmatics have a heightened response to the effects of acid reflux.

In our study, the SP concentrations measured were of a similar magnitude to those reported in the airways of respiratory patients in other studies (22). We believe the presence of elevated tachykinins in the airways of respiratory patients with acid reflux is likely to be of pathophysiological importance. In a previous study we have reported the local release of tachykinins into the airway of asthmatic subjects who developed coughing following endobronchial challenge with adenosine monophosphate (18). Tachykinins have a variety of effects within the human airway and have been implicated in inducing important airway events including smooth muscle contraction, mucus gland secretion, vascular permeability and stimulation and recruitment of inflammatory cells. Increased levels of substance P immunoreactive nerves in the airways of coughers have been reported (23). When inhaled by asthmatics, both substance P (24) and NKA (25) cause bronchoconstriction in a dose dependent manner. Inhaled SP is known to induce a rapid increase in airway microvascular leakage in asthmatics (26). Substance P is known to have a proinflammatory effect on inflammatory cells, causing mast cell degranulation (27) and increasing neutrophil adhesion to bronchial epithelial cells (28). In our current study, there was a non-significant increase in airway neutrophil numbers in asthmatic subjects with acid reflux.

In this study, there was no correlation between NKA or SP levels, which may reflect the observation that both neuropeptides may be located independently of one another (29). There is evidence of tachykinin production in bronchial epithelial cells (30) and airway inflammatory cells (31). It is not possible from our study to determine the cellular source of these elevated neuropeptides although a non-neuronal source may be more relevant to microaspiration rather than a neuronal reflex.

Although the direct effect of gut refluxate into the airway is known to induce airway symptoms (21) and trigger the release of neuropeptides (32), the subjects in this study had no evidence of significant proximal reflux. However, several lines of evidence suggest that stimulation of the distal oesophagus alone is sufficient to evoke changes in airway function. These include the oesophageal acidification experiments in guinea pigs and rabbits described above, during which great care was take to avoid pulmonary aspiration (12; 33) and in human studies where heightened bronchial hyperreactivity (7), cough reflex hyperreactivity (34) and increased cough frequency (8) were triggered with distal oesophageal stimulation alone. In our current study, we used dual probe oesophageal pH monitoring, which enabled the measurement of extent of both distal and proximal acid reflux. In asthmatic subjects we observed a significant positive correlation between the extent of distal oesophageal acid exposure and the level tachykinins in the airway although we acknowledge that this association may be due mainly to the asthmatic refluxers. No such association was noted with the extent of proximal acid exposure, which may reflect the relatively few acid reflux episodes to this level. Although this study was not designed to establish the precise mechanisms whereby acid reflux may aggravate respiratory disease, the results do support the notion that acid in the distal oesophagus may activate airway sensory nerves.

Despite the recognition that respiratory disease and acid reflux commonly co-exist and that gastro-oesophageal reflux may trigger exacerbations of asthma and cough, there is little
convincing evidence that acid suppression treatment is beneficial in controlling symptoms. Recent systematic reviews have suggested that medical treatment of gastro-oesophageal reflux in either asthmatics or patients with chronic cough does not consistently improve symptoms or lung function (35; 36). However, these metaanalyses have recognised that existing studies vary greatly in respect of sample size, study design and intensity of medical therapy. Recently, a large multi-centre placebo-controlled study of intensive acid suppression over 16 weeks in asthmatics suggested small improvements in lung function, but only in those with reflux symptoms and nocturnal respiratory symptoms (37). It appears therefore that a subgroup of patients with airway disease may benefit from acid suppressive therapy.

To date, treatment has focussed largely on acid suppression with little attention given to volume reflux and the effects of non-acidic refluxate. Weakly acidic and non-acidic reflux events are known to trigger cough events in humans (38). Furthermore, distention of the oesophagus by refluxate induces airway protective reflexes (39). Whether such responses evoke neurogenic inflammation within the airway is unknown.

In conclusion, we have demonstrated elevated tachykinin levels in induced sputum from asthmatics and cough patients with acid reflux. This was most apparent in the asthmatic refluxers. These findings may suggest sensory nerve activation in the airways of such patients and could provide alternative therapeutic options for reflux-associated respiratory disease.

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**Competing interests**
The authors declare they have no competing interests in relation to this manuscript.
Legends for figures

Figure 1. Sputum Substance P levels (pg/ml) in asthmatic and cough patients (n=32).

Figure 2. Sputum Neurokinin A levels (pg/ml) in asthmatic and cough patients (n=32).

Figure 3. Correlation of sputum substance P levels and distal oesophageal acid exposure times for asthmatics (n = 16)

Figure 4. Correlation of sputum neurokinin A levels and distal oesophageal acid exposure times for asthmatics (n = 16)
REFERENCES


Figure 1. Sputum Substance P levels (pg/ml) in asthmatic and cough patients (n=32).
Figure 2. Sputum Neurokinin A levels (pg/ml) in asthmatic and cough patients (n=32).
Figure 3. Correlation of sputum substance P levels and distal oesophageal acid exposure times for asthmatics (n = 16)

\[
\begin{align*}
\text{Distal acid exposure (\% time pH < 4)} & \\
\text{Substance P (pg/ml)} & \\
0 & 500 & 1000 & 1500 & 2000 & 2500 & 3000
\end{align*}
\]

\[r = 0.59\]
\[p = 0.01\]
Figure 4. Correlation of sputum neurokinin A levels and distal oesophageal acid exposure times for asthmatics (n = 16)

\[ r = 0.56 \]
\[ p = 0.02 \]
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