Endobronchial adenosine monophosphate challenge causes tachykinin release in the human airway

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Background: Adenosine 5 monophosphate (AMP) has been shown to cause bronchoconstriction and a sensation of chest tightness when inhaled by asthmatic subjects. This response is attenuated after repeated inhalation of bradykinin, suggesting that AMP may act in part by the release of neuropeptides.
Objective: This study examined neuropeptide release in the human airway after endobronchial AMP challenge.
Methods: Endobronchial AMP challenge was performed in 20 subjects and tachykinin levels were measured after endobronchial AMP challenge and after placebo endobronchial challenge with saline.
Results: All subjects coughed immediately after adenosine challenge. There was a significant increase in neurokinin A and substance P levels ($P < .01$, $P < .01$ respectively) when post-saline and post-AMP levels were compared. There was, however, no significant change in calcitonin gene related peptide levels ($P = .37$).
Conclusion: This study demonstrates that endobronchial AMP challenge causes tachykinin release in the human airway *in vivo*. (J Allergy Clin Immunol 2005;116:312-7.)

Key words: *Tachykinin, neuropeptide, adenosine, endobronchial challenge*

Abbreviations used
AMP: Adenosine 5′ monophosphate
CGRP: Calcitonin gene related peptide
NEP: Neutral endopeptidase
NKA: Neurokinin A
NKB: Neurokinin B
NPK: Neuropeptide K
PC_{20} AMP: Provocative concentration of AMP causing a 20% fall in FEV_1
SP: Substance P

Adenosine is a naturally occurring purine nucleoside that functions as a constituent of nucleic acid, as an intracellular and autocoid mediator.[1] Elevated levels have been found in bronchoalveolar lavage fluid of asthmatic subjects as compared to normal subjects, suggesting that adenosine may be a mediator in asthma.[2] Inhalation of adenosine monophosphate (AMP), which is rapidly dephosphorylated to adenosine *in vivo*,[3] causes bronchoconstriction in atopic asthmatic and non-asthmatic subjects but not in non-atopic, non-asthmatic subjects.[4] The related nucleoside guanosine has no effect, suggesting that this is a specific receptor mediated effect.[5]

Atopic asthmatic and non-asthmatic subjects cough and bronchoconstrict in response to stimuli such as inhaled AMP and sulphur dioxide; however, asthmatics tend to respond to lower concentrations.[6] Cough and chest tightness are both common symptoms in asthma and are related to stimulation of sensory nerves.[6]

Evidence exists that while AMP acts mainly via primed mast cells, the agent also stimulates vagal nerves. Pretreatment with ipratropium (an anti-cholinergic agent) has a bronchoprotective effect on the response to AMP, suggesting activation of cholinergic nerves in the response to AMP.[7,8] Inhalation of AMP and bradykinin cause a greater sensation of chest tightness than does inhalation of methacholine, for the same degree of bronchoconstriction, suggesting that the former acts on sensory pathways.[9] Repeated inhalation of bradykinin attenuates the response to inhaled AMP suggesting that both of these agents act in part via liberation of neuropeptides from sensory nerves.[10]

Hong et al[11] have shown that pulmonary C fibers (the nerve fibers containing neuropeptides) in the rat are activated after right atrial injection of adenosine, implicating these nerves in the response to AMP in this model. The purpose of this study was to examine neuropeptide release *in vivo* in the human airway after endobronchial AMP challenge.

METHODS

Subjects

Ethical approval was granted by the Research Ethics Committee of the Queen's University of Belfast. All subjects gave written informed consent. All subjects were non-smokers and had not received any anti-histamines or inhaled or oral steroids in the preceding six months. Asthmatics were recruited if they (1) had a prior clinical

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diagnosis of asthma and a history of intermittent shortness of breath or wheeze, (2) were atopic (reacted to at least one allergen on skin prick testing), and (3) had FEV_1 greater than 60% predicted. All other subjects had no symptoms suggestive of asthma; atopic non-asthmatic subjects had at least one positive skin prick test as aforementioned.

All subjects attended on two occasions. At the screening visit, informed consent was obtained and clinical assessment, skin prick testing, and AMP inhalation challenge were performed. At the subsequent visit (at least 72 hours after screening visit), bronchoscopy and endobronchial AMP challenge were performed.

Skin prick testing

Skin prick testing was performed using a standardized puncture technique,[12] using allergen preparations of house dust mite, cat, and dog protein and grass pollen (Dome-Hollister-Stier, Epernon Cedex, France). A positive reaction was taken as a wheal size of 3 mm or more.

Inhalational challenging

Spirometry was performed according to American Thoracic Society Guidelines[13] using a Vitalograph spirometer (Buckingham, UK). AMP (Sigma-Aldrich Ltd., Poole, UK) was freshly prepared in 0.9% saline in doubling concentrations ranging from 0.391 mg/mL to 400 mg/mL. The AMP provocation test was performed using the two-minute tidal breathing method of Cockcroft et al[14] using a Medix Turbonebuliser (Leicestershire, UK) with an output of 0.13 mL/min. PC_{20} AMP was calculated by linear interpolation.

Bronchoscopy

AMP was freshly made up on the morning of the bronchoscopy in 0.9% saline from a stock solution of 400 mg/mL. At bronchoscopy, subjects were given intravenous Midazolam (up to 14 mg) to achieve mild sedation and the hypopharynx was anaesthetised using 4% lignocaine spray. Vocal cord and tracheal anaesthesia was achieved using 4 mL of 4% lignocaine introduced trans-cricoidally. Oxygen was routinely applied at 2 L/min via nasal cannulae. Heart rate, ECG, and oxygen saturations were monitored throughout the procedure. The bronchoscope (240 IT Olympus Optical Co. Ltd. Tokyo, Japan) was introduced orally and 2-mL aliquots of 2% lignocaine were used as necessary to anesthetize the airways below the carina to suppress coughing.

The site of the subsequent endobronchial challenge was randomized prior to bronchoscopy. Subjects were randomly assigned a number, which determined the site of the active challenge to either the right middle or upper lobes, and randomization was constrained to achieve balance. The placebo challenge was automatically assigned to the opposite site from the active challenge.

The bronchoscope was initially wedged in a segmental orifice of the site randomized for the placebo challenge and a baseline bronchial wash with 20 mL of saline was performed and aspirated back after minimum dwell time. A placebo challenge of 5 mL of saline was administered to the same segment and the segment closely observed for any visible reaction. After 3 minutes, a second bronchial wash using 20 mL of saline was performed and aspirated back after minimal dwell time.

The active (AMP) challenge was then performed in the other site. Again a baseline bronchial wash was performed using 20 mL saline and immediately aspirated back under gentle suction. Then the active challenge with 5 mL AMP was performed. The initial AMP concentration administered was one tenth that which caused a 20% fall in FEV_1 on the prior inhalational challenge or if the subject had been unresponsive to adenosine one tenth of the maximum concentration during the inhalation challenge (400 mg/mL). Up to two subsequent AMP doses were given at quadrupling concentrations, the maximum administered endobronchial dose being 400 mg/mL. There was a time lapse of 3 minutes after each concentration given to observe for any visual reaction using the analogue outlined in Table I. The endobronchial challenge was terminated either when there was a visible reaction to AMP, when the maximum concentration of AMP had been administered or when it was necessary to terminate the challenge for reasons of patient comfort. Three minutes after the final concentration of adenosine had been administered, a further bronchial wash of 20 mL of saline was performed and aspirated after a minimum dwell time. Subjects remained under observation for a period of at least two hours after the procedure.

TABLE I. Visual analogue reaction for grading of response after endobronchial challenge

Analogue score	Reaction observed
0	No reaction
1	Subject coughs after instillation of AMP (no coughing after saline challenge)
2	Subject coughs/immediate bronchial pallor then hyperemia/increased mucus secretion after instillation of AMP
3	Bronchoconstriction observed after instillation of AMP

Adapted from Polosa et al.[1]

Processing of samples

A total cell count was measured using a modified Neubauer hemocytometer and was expressed as the number of cells $\times 10^5$/mL of BAL. Cell viability was assessed by Trypan blue exclusion staining. Viable cells are expressed as a percentage of total cell numbers. Samples were centrifuged at 200 $\times$ g for 10 minutes at 4°C to separate any debris and added to a protease inhibitor cocktail (see Appendix) and stored at −70°C for subsequent analysis.

Neuropeptide measurement

NKA was measured using radioimmunoassay, utilizing a N-terminal specific anti-serum that was raised in guinea pigs to synthetic human NKA (Amersham Bioscience UK Ltd product number IM168, Buckinghamshire, UK). It cross-reacts fully with NKB and NPK but less than 0.1% with SP. The detection limit for the assay is 2 ng/L.

CGRP immunoreactivity was measured using a commercial CGRP human radioimmunoassay (RIA) kit (catalogue number RIK009, Peninsula Laboratories, San Carlos, Calif). This antibody is a rabbit anti-human CGRP peptide (II) antibody. The label was ^{125}I-Tyr^0-CGRP (catalog number Y6011). The limit of detection for this assay is 2 ng/L and the antibody cross-reacts 100% with human CGRP (II), human CGRP, and rat CGRP. It cross-reacts <0.001% with rat calcitonin C-terminal adjacent peptide and less than 0.02% with insulin, glucagon, somatostatin, SP, vasoactive intestinal peptide, and gastrin releasing peptide.

Substance P (SP) was measured using a commercially available ELISA (catolog number DE1400, R&D Systems, Minneapolis, Minn). It shows no significant cross-reactivity with NKA, neurokinin B (NKB), and neuropeptide K (NPK). The limit of detection of this assay is 8 pg/mL.

For radioimmunoassays, lavage fluid was extracted using a previously validated technique.[16] In brief, cleared plasma and traysolol were added to equal volume of lavage fluid followed by precipitation of large molecular weight proteins in 60% alcohol and the sample centrifuged (30 min, $\times$ 1500 *g*). Thiomersal was added to the supernatant. This was then decanted, the extract was evaporated to

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