Effect of an inhaled neutral endopeptidase inhibitor, phosphoramidon, on baseline airway calibre and bronchial responsiveness to bradykinin in asthma

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Abstract
Background – Bradykinin is a potent vasoactive peptide which has been proposed as an important inflammatory mediator in asthma since it provokes potent bronchoconstriction in asthmatic subjects. Little is known at present about the potential role of lung peptidases in modulating bradykinin-induced airway dysfunction in vivo in man. The change in bronchial reactivity to bradykinin was therefore investigated after treatment with inhaled phosphoramidon, a potent neutral endopeptidase (NEP) inhibitor, in a double blind, placebo controlled, randomised study of 10 asthmatic subjects.

Methods – Subjects attended on six separate occasions at the same time of day during which concentration–response studies with inhaled bradykinin and histamine were carried out, without treatment and after each test drug. Subjects received nebulised phosphoramidon sodium salt (10^{-3} M, 3 ml) or matched placebo for 5–7 minutes using an Inspiron Mini-neb nebuliser 5 minutes before the bronchoprovocation test with bradykinin or histamine. Agonists were administered in increasing concentrations as an aerosol generated from a starting volume of 3 ml in a nebuliser driven by compressed air at 8 l/min. Changes in airway calibre were measured as forced expired volume in one second (FEV₁) and responsiveness as the provocative concentration causing a 20% fall in FEV₁ (PC_{20}).

Results – Phosphoramidon administration caused a transient fall in FEV₁ from baseline, FEV₁ values decreasing 6-3% and 5-3% on the bradykinin and histamine study days, respectively. When compared with placebo, phosphoramidon elicited a small enhancement of the airways response to bradykinin, the geometric mean PC_{20} value (range) decreasing from 0-281 (0-015–5-575) to 0-136 (0-006–2-061) mg/ml. In contrast, NEP blockade failed to alter the airways response to a subsequent inhalation with histamine, the geometric mean (range) PC_{20} histamine value of 1-65 (0-17–10-52) mg/ml after placebo being no different from that of 1-58 (0-09–15-21) mg/ml obtained after phosphoramidon.

Conclusions – The small increase in bronchial reactivity to bradykinin after phosphoramidon exposure suggests that endogenous airway NEP may play a modulatory role in the airways response to inflammatory peptides in human asthma.

Keywords: asthma, bronchospasm, bradykinin, histamine, phosphoramidon.

Neutral endopeptidase (NEP, also known as neutral metalloendopeptidase, enkephalinase, CALLA, or E.C. 3.4.24.11) is a membrane-bound enzyme that cleaves a large variety of peptidic hormones including enkephalins, tachykinins (such as neurokinin A and substance P), and kinins (such as bradykinin and kallidin). Although NEP is widely distributed in the body, the highest activity is normally found in the lung, particularly at the level of airway epithelium and smooth muscle. A major potential physiological role for NEP in human airways has recently been proposed. Its enzymatic activity may exert a "braking effect" on airway smooth muscle contraction by inactivating peptidic hormones released under inflammatory conditions including asthma.

Kinins in general, and bradykinin in particular, are naturally occurring vasoactive peptides formed de novo in body fluids and tissues during inflammatory processes which have also been implicated in the pathophysiology of bronchial asthma and related allergic disorders. Bradykinin is generated from circulating kininogens through proteolytic cleavage by a variety of enzymes, the most important of which are tissue and plasma kallikreins. Evidence that kinin generation may be increased under conditions which prevail in areas of allergic inflammation in the airways has recently been obtained from studies in asthmatic and rhinitic subjects. In addition, bradykinin is reported to possess many pharmacological properties pertinent to the pathogenesis of asthma, including vasodilatation and increased microvascular leakage, bronchoconstriction, activation of C fibre nociceptive sensory nerve endings, and induction of non-specific bronchial hyperresponsiveness in animals.

The effector functions of bradykinin on the airways appear to be modulated by a variety of factors, including bradykinin hydrolysis by several peptidases. Little is known at present
about the potential role of lung peptidases in modulating bradykinin-induced responses in vivo. There is increasing evidence that NEP may be the most important enzyme system involved in the degradation of bradykinin in the airways.\(^1\)\(^8\)\(^9\) Although a number of animal studies have shown that the NEP inhibitor phosphoramidon\(^2\) enhances the bronchoconstrictor effect of bradykinin both in vitro\(^2\) and in vivo,\(^1\)\(^2\)\(^3\)\(^5\) the role of NEP in modulating airways responsiveness to bradykinin in man is not known.

The aim of the present study was therefore to investigate the effect of inhibiting endogenous NEP activity by inhaled phosphoramidon on bradykinin-induced bronchoconstriction and baseline airway calibre in asthmatic subjects. The strategy was based on the assumption that, if bradykinin is inactivated by NEP in the airways, then the selective enzyme inhibitor phosphoramidon should potentiate the airways responsiveness to bradykinin. The effect of phosphoramidon on histamine-induced reactivity was also assessed to rule out possible non-specific effects of this agent on airways responsiveness.

**Methods**

**SUBJECTS**

Ten asthmatic subjects (eight women) with a mean (SE) age of 29-5 (3-3) years referred to our hospital chest clinic with stable asthma as defined by the American Thoracic Society\(^2\) participated in the study (table 1). All subjects had a history of dyspnoea with wheezing or chest tightness upon exposure to airborne allergens and were non-smokers with positive skin prick tests (>2 mm weal response) to one or more of six common aeroallergens (\textit{Derma-}

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Baseline FEV(_1) (% predicted)</th>
<th>Atopy</th>
<th>PC(_{20}) histamine (mg/ml)</th>
<th>Regular medications</th>
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<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>18</td>
<td>103</td>
<td>D</td>
<td>0-33</td>
<td>S, BDP (1500 mg)</td>
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<tr>
<td>2</td>
<td>F</td>
<td>29</td>
<td>107</td>
<td>D, W</td>
<td>0-70</td>
<td>S</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>40</td>
<td>97</td>
<td>W</td>
<td>5-07</td>
<td>S</td>
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<tr>
<td>4</td>
<td>F</td>
<td>41</td>
<td>89</td>
<td>W, G</td>
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<td>S</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>32</td>
<td>98</td>
<td>W</td>
<td>6-11</td>
<td>S</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>28</td>
<td>95</td>
<td>D, W</td>
<td>0-11</td>
<td>S, BDP (2000 mg)</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>21</td>
<td>89</td>
<td>W, G</td>
<td>1-69</td>
<td>S, BDP (1000 mg)</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>23</td>
<td>101</td>
<td>D</td>
<td>0-98</td>
<td>S</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>47</td>
<td>97</td>
<td>W, G</td>
<td>8-48</td>
<td>S, BDP (1500 mg)</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>16</td>
<td>123</td>
<td>W</td>
<td>11-90</td>
<td>S, SCG</td>
</tr>
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FEV\(_1\), forced expiratory volume in one second; PC\(_{20}\) = provocation concentration producing a 20% fall in FEV\(_1\); D = \textit{Dermatophagoidees pteronyssinus}, W = wall pellitory grass; G = grass; S = salbutamol as required; BDP = beclomethasone dipropionate; SCG = sodium cro- moglicate.

* Geometric mean (range).

† Atopic, positive immediate skin test to one or more allergens.

<table>
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<th>STUDY DESIGN</th>
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<td>The study consisted of three separate phases.</td>
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**Phase 1**

In the first phase subjects (nos 1–10) attended the laboratory on two separate occasions at least 48 hours apart to undertake concentration-
response studies with inhaled histamine and bradykinin in the absence of any drug treatment. On the first occasion, after a 15 minute rest, three baseline measurements of FEV₁ were made at intervals of three minutes followed by inhalation of the corresponding diluent solution and further FEV₁ measurements repeated at one and three minutes. Provided FEV₁ had not fallen by >10% of the baseline value, a histamine concentration-response study was carried out. After administration of each histamine concentration FEV₁ was measured at one and three minutes. Increasing doubling concentrations of histamine were inhaled at five minute intervals until FEV₁ had fallen by >20% of the post-saline baseline value and the corresponding PC₂₀FEV₁ values derived. On the second occasion a bronchial provocation test with inhaled bradykinin was undertaken according to a previously described protocol. In brief, increasing concentrations of bradykinin were inhaled at approximately five minute intervals until FEV₁ had fallen by >20% of the post-diluent value and the corresponding PC₂₀FEV₁ values derived.

Phase 2
In the second phase subjects (nos 1–10) attended the laboratory on two separate visits, at least five days apart, to undertake concentration-response studies with inhaled bradykinin after receiving nebulised phosphoramidon (Sigma Chemical Co, St Louis, USA) or matched nebulised vehicle placebo administered double blind and in random order five minutes prior to challenge. Both the active and placebo solutions were freshly prepared by an independent investigator on the basis of a randomised code and then returned to the conducting physician to administer to the attending subject. On each occasion, after a 15 minute rest, three baseline measurements of FEV₁ were made at intervals of three minutes followed by inhalation of nebulised phosphoramidon (10⁻³ M, 3 ml) or nebulised vehicle placebo consisting of 0.9% sodium chloride adjusted to the same pH and tonicity as the phosphoramidon. The aerosol solutions were generated from a starting volume of 3-0 ml in an Inspiron mini-nebuliser driven by compressed air at 8 l/min and inhaled to dryness by deep tidal breathing over a 5–7 minute time period. The same nebuliser was used for all studies on all subjects. Further FEV₁ measurements were repeated two and five minutes after drug/placebo inhalation and bradykinin challenge was carried out in a similar manner to that described in phase 1.

Phase 3
The final phase of the study was carried out to determine the specificity of the effect of the drug tested on subsequent non-specific contractile stimuli. Eight subjects (nos 1, 2, 3, 6, 7, 8, 9, and 10) attended the laboratory on two further visits, at least five days apart, to undertake histamine-response studies with inhaled histamine after receiving nebulised phosphoramidon (10⁻³ M, 3 ml) or matched nebulised vehicle placebo administered in an identical fashion to that described in phase 2.

DATA ANALYSIS
Figures are presented as mean (SE) unless otherwise stated, with a level of significance of p<0.05. Pre-treatment and post-treatment baseline values of FEV₁ prior to bronchial challenges were compared between and within study days by two-factor analysis of variance (ANOVA) followed by Neuman-Keuls test, where appropriate.

Concentration-response curves were constructed by plotting the percentage change in FEV₁ from the post-diluent baseline value against the cumulative concentration of the agonist administered on a logarithmic scale and the concentration of agonist required to produce a 20% fall in FEV₁ from the post-diluent baseline value (PC₂₀FEV₁) determined by linear interpolation.

The repeatability of the bradykinin challenge procedure was determined according to the method described by Altman and Bland. Values of PC₂₀ bradykinin and histamine following treatment with phosphoramidon and placebo were logarithmically transformed to normalise their distribution and compared by the Student's t test for paired data. Concentration ratios for the effect of phosphoramidon against bronchoprovocation with each agonist were calculated by dividing the PC₂₀ value obtained after administration of active drug by that obtained after placebo and compared using the Wilcoxon signed rank test.

Any relationship was examined by least squares linear regression analysis.

**Results**

**EFF ECT OF INHALED PHOSPHORAMIDON ON AIRWAY CALIBRE**

There was no significant difference in baseline values of FEV₁ between any of the study days (table 2). However, two minutes after administration of nebulised phosphoramidon FEV₁ values were significantly lower than their predrug values, decreasing 6·3% and 5·3% from 3·12 (0·23) to 2·93 (0·22) l (p<0·01;
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Patient 6
Patient 7
Patient 8
Patient 9
Patient 10

Cumulative concentration of bradykinin (mg/ml)

Figure 1  Effect of placebo (□) and phosphoramidon (■) on the concentration-related falls in FEV₁ produced by inhaled bradykinin in 10 subjects with asthma.

n = 10) and from 3·28 (0·30) to 3·11 (0·30) l (p<0·05; n = 8) on the bradykinin and histamine study days, respectively (table 2). FEV₁ values measured five minutes after nebulised phosphoramidon was given were significantly higher than those measured at two minutes (table 2). In addition, the mean baseline values of FEV₁ following administration of phosphoramidon were significantly (p<0·05) lower than after placebo when compared at two minutes but not at five minutes (table 2). Phosphoramidon elicited a fall in FEV₁ of >5% from baseline in seven of the 10 subjects studied. No significant correlations could be established between the degree of fall in FEV₁ induced by phosphoramidon and the concentration ratio, after the active drug or baseline FEV₁ values or baseline airways responsiveness to bradykinin or histamine.

EFFECT OF INHALED PHOSPHORAMIDON ON CONCENTRATION-RESPONSE CURVE TO BRADYKININ

The challenge procedure with bradykinin in this group of patients was found to be repeatable, with a coefficient which was within 1·1 doubling dilutions (for nine of the 10 sub-
NEP blockade and bradykinin-induced bronchoconstriction

The effects of NEP blockade on bradykinin-induced bronchoconstriction were studied in asthmatic subjects. A single dose of inhaled phosphoramidon failed to alter the airways response to subcutaneous histamine, but a dose of nine subjects, which was significant, enhanced the response to histamine. These findings support the view that a functional NEP system is present in the main bronchi and mediastinum in vivo. In addition, it was interesting to note that exposure to phosphoramidon elicited a small but significant time-dependent fall in FEV₁ in the subjects studied.

In contrast to previously published work with inhaled NEP inhibitors, the present study shows changes in airway calibre following NEP blockade in man. The reasons for this discrepancy are not clear. Higher doses of this drug could have been followed by a more significant change in baseline airway calibre. Indeed, compared with previous work, the total dose of phosphoramidon used in the present investigation was considerably higher, thus explaining the reported 6% change from baseline FEV₁. Timing of FEV₁ recording after drug exposure also might have been crucial as the fall in baseline FEV₁ was significant only after minutes, thus justifying the lack of change from baseline FEV₁ at 6 minutes after NEP inhibition described in two recent investigations. The immediate bronchoconstriction seen after phosphoramidon may be the result of an irritant effect of the drug, but we cannot exclude other possibilities. Comparison between the subjects studied and those from previous studies showed no difference in their characteristics except for the use of inhaled corticosteroids. Phosphoramidon elicited a significant fall in FEV₁ only in the subjects taking inhaled steroids. The reason for this is not known, but we are currently exploring this finding in a series of controlled studies. As it has been shown that increased NEP activity is present in glucocorticoid-treated human epithelial cells, it is possible that inhaled steroids could increase NEP activity within the airways to such a level that the addition of a specific NEP inhibitor (phosphoramidon) might reveal a basal peptidergic tone. We have also shown the functional importance of endogenous NEP in modulating reactivity of the airways to exogenously administered bradykinin. That NEP inhibition has a specific effect on airways responsiveness to bradykinin is demonstrated by the lack of change in histamine reactivity after phosphoramidon. The present data are in line with the evidence provided by several authors on the potentiation of bradykinin-induced airways responses following NEP blockade in animal studies.

Although the bradykinin challenge was repeatable to within one doubling dilution of agonist in eight out of nine subjects, it must be pointed out that the 2:1-fold potentiation of bradykinin responsiveness after phosphoramidon exposure should be interpreted with caution. However, the degree of potentiation shown in the present study with NEP inhibition is similar to that reported by us and others against the airway effect of neurokinin A in asthma. Thus, despite the fact that phosphoramidon is a potent inhibitor of NEP, its effect on bradykinin-induced airway changes may be negligible because of the hydrolysis of bradykinin by other lung peptidases including carboxypeptidase N and M. This emphasises the importance of alternative enzymatic pathways in the modulation of kinin responses in man. NEP degrades a variety of peptides including bradykinin by cleaving the Pro⁷-Phe⁸ bond, thereby releasing inactive peptidic fragments. Because of its localisation on the plasma membrane, NEP may be involved in the local control of peptide hormone activity by inactivating or altering the receptor specificity of peptides at their site of action.

This view has been substantiated by a series of clinical investigations in normals and asthmatic subjects in which potentiation of the bronchoconstriction induced by the neuropeptide neurokinin A was demonstrated following NEP blockade. The mechanism of bradykinin-induced bronchoconstriction in asthma is unknown but release of mast cell mediators and potentiation of tachykinins is a possibility. The enhancing effect of phosphoramidon on bradykinin-induced bronchoconstriction might therefore have been the result of NEP directly contributing to brady-
kinin metabolism or by metabolising endo-
genous tachykinins released by bradykinin. Indeed, we and others have shown that NEP inhibition significantly enhances the airways response to inhaled neurokinin A in healthy and asthmatic subjects.28-30

In health, kinins usually exist in the cir-
culating blood at very low concentrations as a result of the balance between their production and inactivation by plasma and tissue enzymes. In various respiratory diseases kinin levels may be raised and may dramatically impair airway function. Whilst inhalation of bradykinin aero-
sols was shown to have no detectable effect on airway calibre in normal subjects, when administered to patients with asthma it resulted in a concentration-related bronchocon-
striction.14 Part of this discrepancy may be related to the loss of activity of the endogenous peptide degrading enzyme, NEP, which seems to occur in those conditions characterised by shedding of the airway epithelium—for example, acute viral infections, exposure to in-
halation irritants, or bronchial asthma.3 Three recent studies have shown that mechanical re-
moval of the respiratory epithelium increases the reactivity of the underlying smooth muscle of intact guinea pig trachea to the constrictor effects of several peptidic hormones including tachykinins and bradykinin.21,22 As the enzyme NEP 24-11 is strongly expressed on airway epithelial cells2 and is very important in the degradation of tachykinins and kinins,3 removal of the epithelial epithelium may explain the enhanced constrictor effect of bradykinin. Shed-
ing of the epithelium is commonly observed in the airways of asthmatic subjects33 and this could contribute to the increased airways response to inhaled kinins in asthma.

In conclusion, this is the first demonstration of the potential physiological importance of NEP activity in regulating airways re-
sponsiveness to bradykinin in asthma in vivo. The study shows that exogenously ad-
ministered bradykinin may be a suitable sub-
strate for NEP in asthmatic airways, and that phosphorylation given by inhalation is effective, not only in enhancing the broncho-
constrictor response of exogenously ad-
ministered bradykinin, but also in reducing the airway calibre in most of our subjects, thus suggesting that a basal endogenous peptidic tone is functionally active in asthmatic airways.

We speculate that diseases or therapies that decrease endogenous pulmonary neutral endo-
peptidase may underline the importance of such enzymatic systems in the control of kinin-
induced changes in airway function.

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