Airway hyper-responsiveness to neurokinin A and bradykinin following *Mycoplasma pneumoniae* infection associated with reduced epithelial neutral endopeptidase

Jun Tamaoki, Atsushi Chiyotani, Etsuko Tagaya, Minako Araake and Atsushi Nagai

To determine whether mycoplasma infection produces airway hyper-responsiveness to tachykinins and bradykinin and, if so, to elucidate the role of neutral endopeptidase (NEP), isolated hamster tracheal segments were studied under isometric conditions in vitro. Nasal inoculation with *Mycoplasma pneumoniae* potentiated contractile responses to neurokinin A and bradykinin, causing a leftward shift of the dose-response curves to a lower concentration by 1 log unit for each agonist, whereas there was no response with acetylcholine. Pretreatment of tissues with the NEP inhibitor phosphoramidon augmented neurokinin A- and bradykinin-induced contractions in saline-treated control tissues, but did not further potentiate the responsiveness in *M. pneumoniae*-infected tissues. NEP activity in the tracheal epithelium, but not in epithelium-denuded tissues, was decreased in infected animals. These results suggest that *M. pneumoniae* infection causes airway bronchoconstrictor hyper-responsiveness to neurokinin A and bradykinin and that this effect may be associated with an inhibition of epithelial NEP activity.

**Keywords**: mycoplasma infection, neuropeptide, bronchoconstriction, neutral endopeptidase, airway epithelium

**INTRODUCTION**

*Mycoplasma pneumoniae* is one of the most common causes of community-acquired respiratory tract infections. It has been shown that *M. pneumoniae* infection frequently produces prolonged coughing and exacerbates symptoms of asthma (Seggev et al., 1986; Yano et al., 1994) and airway hyper-responsiveness (Boldy et al., 1990), but the mechanism for this remains unknown.

Tachykinins, including substance P and neurokinin A, are released from capsaicin-sensitive airway sensory nerves (Barnes, 1987) and bradykinin is produced from kininogen in the plasma and released into the airway lumen (Christiansen et al., 1983) during many inflammatory processes. These peptides produce bronchoconstriction (Lundberg et al., 1983; Fuller et al., 1987), airway microvascular leakage (Barnes, 1987), mucus and water secretion (Leikauf et al., 1985; Borson et al., 1987; Tamaoki et al., 1988) and coughing (Kohrogi et al., 1989), suggesting that they are important mediators of asthma and airway inflammation. There is increasing evidence that neutral endopeptidase (NEP; EC 3.4.24.11), a membrane-bound peptidase present in the airways of a variety of species, including humans (Johnson et al., 1985), cleaves tachykinins and bradykinin into inactive fragments (Gafford et al., 1983; Matsas et al., 1984), thereby limiting the biological actions of these peptides. Therefore, to determine whether *M. pneumoniae* infection modifies the contractile responses to tachykinins and bradykinin and, if so, whether this effect is associated with alterations in endogenous NEP activity, we studied hamster tracheal segments under isometric conditions in vitro. Of the tachykinins, we used neurokinin A in the experiments because hamster tracheal smooth muscle contracts after stimulation of NK2 receptors (Aharony et al., 1995).

**METHODS**

*M. pneumoniae* infection. Research protocols were approved by the Animal Care and Use Committee of the Tokyo Women's Medical College. All chemicals used were obtained...

**Abbreviation**: NEP, neutral endopeptidase.
from Sigma or Research Products. Specific-pathogen-free male golden Syrian hamsters, weighing 60–80 g, were anaesthetized with a mixture of nitrous oxide, halothane and oxygen and given an intranasal inoculation of *M. pneumoniae* strain FH (500 μl at 1·1 × 10⁸ c.f.u. ml⁻¹) or an equivalent volume of sterile saline (Cimolai et al., 1992). Each group was separated and housed for 10 d. To confirm that mycoplasma infection was established, antibody titres in serum samples were measured by a gelatin particle agglutination test using Serodia Myco II (Fujirebio) as described by Arai et al. (1993). A lyophilized preparation of gelatin particles sensitized with *M. pneumoniae* Mac antigen was added to a twofold dilution series of hamster sera in microplates. The plates were incubated at 25 °C for 3 h and the end point taken as the last dilution causing agglutination.

**Contractile responses.** The trachea was removed under general anaesthesia (35 mg sodium pentobarbital kg⁻¹ given intravenously) and immersed in oxygenated Krebs–Henseleit solution (118 mM NaCl, 5·9 mM KCl, 2·5 mM CaCl₂, 1·2 mM MgSO₄, 1·2 mM NaH₂PO₄, 25·5 mM NaHCO₃, 5·6 mM D-glucose). Transverse ring segments, each 3–4 mm in length, were dissected free of underlying loose connective tissue and mounted in organ chambers filled with 5 ml Krebs–Henseleit solution maintained at 37 °C and continuously aerated with a gas mixture of 95% O₂/5% CO₂ to obtain a pH of 7·4, a pCO₂ of 5·1 kPa and a pO₂ of >67 kPa. The lower end of the tracheal ring was attached to the base of the organ chamber by a loop of silk thread and the upper end was attached in the same manner to a force-displacement transducer (Nihon Kohden) for continuous recording of isometric tension by a pen recorder (Nihon Kohden). The tissues were allowed to equilibrate for 60 min and were washed with Krebs–Henseleit solution every 15 min. The resting tension was adjusted to 1 g. The contractile response was determined as the difference between peak tension developed and resting tension. To avoid possible contributions by prostaglandin E₂, which decreases bronchoconstrictor responses, indomethacin (3 × 10⁻⁶ M) was included in all solutions.

After the equilibration period, neurokinin A (10⁻¹¹–10⁻⁷ M), bradykinin (10⁻¹¹–10⁻⁵ M) or acetylcholine (10⁻⁸–10⁻⁵ M) was added to the chamber in a cumulative manner at 5 min intervals or 2 min after the stable plateau was achieved, whichever was the longer period. To study the role of NEP, the tissues were incubated for 20 min with phosphoramidon (10⁻⁶ M), a specific inhibitor of NEP (Hudgin et al., 1981) and the contractile responses to neurokinin A and bradykinin at concentrations required to produce a 50% of maximum response in control tissues (2 × 10⁻⁹ M neurokinin A; 10⁻⁷ M bradykinin) were determined.

At the end of these experiments, each tracheal segment was blotted on a gauze pad and weighed. Active tensions were normalized for tissue weight and expressed as g tension (g tissue)⁻¹. To characterize the dose-response curves, we determined the negative logarithm of molar concentration of agonist required to produce a 50% of maximum contraction (pD₂) by linear regression analysis.

**Histology and NEP activity.** The excised trachea was divided into two segments. The upper portions were assessed for histology and the lower portions for NEP activity. For histological studies, the tissues were fixed in 10% phosphate-buffered formalin and 5 μm sections were stained with Alcian blue and periodic acid–Schiff. The number of neutrophils and lymphocytes infiltrating the tracheal epithelial and sub-

![Fig. 1. Contractile responses of tracheal segments to neurokinin A, bradykinin and acetylcholine in *M. pneumoniae*-infected hamsters (○) and saline-treated control hamsters (●). Each agonist was added to the chamber in a cumulative manner and the maximal contraction in response to each concentration was determined. Values are means ± SE (n = 11 for neurokinin A; n = 10 for bradykinin and acetylcholine).](image)
cells were obtained by scraping the epithelium with a
coverglass and the remaining epithelium-denuded segments
were minced and homogenized in a polytron homogenizer for
10 s. The samples of epithelium and the tissue homogenate
were separately incubated in 125 mM NaCl containing 50 mM
HEPES buffer (pH 7.4) at 37 °C for 40 min with a radiolabelled
enkephalin analogue ([3H]Tyr-d-Ala2-Leu enkephalin,
20 nM). Degraded [3H]Tyr-d-Ala2-Leu enkephalin was chro-
matographically separated from uncleaved [3H]Tyr-d-Ala2-
Leu enkephalin and the radioactivity in each fraction was
determined in a scintillation counter. NEP activity was then
determined by calculating the ratio of cleaved enkephalin to
total [3H]Tyr-d-Ala2-Leu enkephalin, adjusted to the total
protein content of epithelial cells or tissue homogenates using
the method of Bradford (1976) and expressed as fmol
enkephalin degraded min⁻¹ (mg protein)⁻¹.

Statistical analysis. All values were expressed as mean ± SE.
Comparative statistical analysis was performed using ANOVA
followed by either Turkey's test for multiple comparisons or
Student's t test. n refers to the number of hamsters from which
tissue was taken and P < 0.05 was considered statistically
significant.

RESULTS
Antibody titres and histology

We first examined the rise of M. pneumoniae antibodies
and the development of the airway lesions in hamsters.
The indirect haemagglutination antibody titres in serum
samples 10 d after infection were 38.0 ± 3.7 in M.
pneumoniae-infected hamsters (n = 11) and 2.5 ± 0.8 (n = 11) in saline-treated control hamsters. M. pneumoniae
infection caused a marked invasion of inflammatory
cells, including neutrophils and lymphocytes,
into the tracheal mucosa, but infiltration of eosinophils
or desquamation of epithelial cells were not observed.

Contractile responses

To determine whether mycoplasma infection induces
airway hyper-responsiveness, we studied the contractile
responses of isolated tracheal segments to various
spasmogenic agonists. As demonstrated in Fig. 1, the
contractile responses to neurokinin A and bradykinin
were greater in the hamsters infected with M. pneumoniae
than in those that received saline alone. M. pneumoniae
infection caused a leftward displacement of dose-response curves for neurokinin A and bradykinin,
so that the pD2 values increased from 7.7 ± 0.2 to
8.6 ± 0.3 (P = 0.009, n = 11) and 7.0 ± 0.3 to 8.0 ± 0.4 (P
= 0.017, n = 10), respectively. However, the contractile
responses to acetylcholine were similar in tissues from
M. pneumoniae-infected and uninfected hamsters.

Because NEP plays a role in the regulation of airway responses to tachykinins and bradykinin by degrading
the peptides, we studied the involvement of this enzyme.
Incubation of control tissues with the NEP inhibitor
phosphoramidon potentiated the contractile responses
to neurokinin A (2 × 10⁻⁸ M) and bradykinin (10⁻⁷ M)
from 17.4 ± 2.7 to 32.0 ± 6.1 g (g tissue)⁻¹ (P = 0.038, n = 8) and 35.2 ± 4.5 to 60.6 ± 6.4 g (g tissue)⁻¹ (P = 0.025,
n = 9), respectively (Table 1). M. pneumoniae-infected
tissues showed greater responses to neurokinin A and
bradykinin than control tissues (P = 0.034 and P = 0.031, respectively). Neurokinin A- and bradykinin-
induced contraction in infected tissues was not signifi-
cantly different from that in the phosphoramidon-
treated control tissues and was not further potentiated
by phosphoramidon.

Tissue NEP activity

To further elucidate the role of endogenous NEP, we
measured enzyme activity by the enkephalin degradation
method. The activity of NEP in tracheal epithelial cells
was 965 ± 102 fmol min⁻¹ (mg protein)⁻¹ (n = 10) in the
saline-treated control hamsters and 367 ± 74 fmol min⁻¹
(mg protein)⁻¹ (n = 10) in the M. pneumoniae-infected
animals. There was a significant difference between
these values (P = 0.006). In contrast, NEP activity in the
epithelium-denuded tissues of M. pneumoniae-infected
animals was not significantly different from NEP activity
in epithelium-denuded tissues from control animals.

We then looked for a possible correlation between

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<tr>
<td>Neurokinin A</td>
<td>17.4 ± 2.7</td>
<td>32.0 ± 6.1*</td>
<td>36.2 ± 6.1*</td>
<td>37.1 ± 5.8*</td>
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<td>Bradykinin</td>
<td>35.2 ± 4.5</td>
<td>60.6 ± 6.4*</td>
<td>57.5 ± 6.9*</td>
<td>60.8 ± 6.0*</td>
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*P < 0.05, significantly different from control values in the absence of phosphoramidon.
airway inflammation and epithelial NEP activity. As shown in Fig. 2, there was no correlation between the NEP activity and the accumulation of neutrophils or lymphocytes in the airway mucosa.

DISCUSSION

This study demonstrates that the alteration of endogenous NEP activity may be important in airway hyper-responsiveness to tachykinins and bradykinin caused by *M. pneumoniae* infection. This conclusion is derived from the following findings. First, intranasal inoculation with *M. pneumoniae* potentiated the contractile responses of hamster tracheal segments to neurokinin A and bradykinin but not those to acetylcholine, indicating that the observed airway hyper-responsiveness is not related to a non-specific mechanism, such as the increased permeability of agonists from the airway mucosa toward the smooth muscle cells, or the loss of endogenous bronchodilating substances, including prostaglandin E₂, nitric oxide and epithelium-derived relaxing factor (Flavahan et al., 1985). Second, pharmacological blockade of NEP activity with the specific inhibitor phosphoramidon (Hudgin et al., 1981) increased the responses to neurokinin A and bradykinin in the control tissues and *M. pneumoniae*-induced hyper-responsiveness was not potentiated further by phosphoramidon. Third, we confirmed that the NEP activity of hamster trachea, as determined by the enkephalin degradation method (Llorens et al., 1982), was specifically decreased in epithelial cells by *M. pneumoniae* infection. Therefore, the potentiation of neurokinin A- and bradykinin-induced airway contraction may be attributable to the decrease in epithelial NEP activity and the consequent reduction in degradation and thus increased availability of these peptides at the airway smooth muscle receptors.

Membrane-bound NEP is present in the airway epithelium, submucosa and airway smooth muscle (Sekizawa et al., 1987a, b) and hydrolyses a variety of small peptides, including tachykinins, bradykinin, neurotensin and enkephalin (Gafford et al., 1983; Johnson et al., 1985). Because neurokinin A and bradykinin are not the only substrates for NEP, we cannot rule out the possibility that inhibition of NEP by *M. pneumoniae* infection altered responsiveness to these peptides indirectly by decreasing the breakdown of some other substrates in the vicinity of airway smooth muscle. However, it is unlikely that such an effect was due to the decreased breakdown of the enkephalins themselves as these would be expected to diminish rather than potentiate bronchoconstriction (Russel & Simons, 1985).

It has been reported that phosphoramidon and other NEP inhibitors cause a marked increase in tachykinin- and bradykinin-induced airway smooth muscle contraction (Sekizawa et al., 1987a; Dusser et al., 1988), as well as cholinergic neurotransmission (Sekizawa et al., 1987b), macromolecule and chloride secretion (Borson et al., 1987; Tamaoki et al., 1991) and microvascular leakage (Nadel, 1990). Furthermore, we and others have previously shown that influenza virus infection augments the bronchoconstrictor responses of the rat airway to tachykinins *in vitro* (Jacoby et al., 1988) and *in vivo* (Dusser et al., 1989) through desquamation of epithelial cells and the associated decrease in NEP activity. However, in contrast to these findings, apparent shedding of airway epithelium was not observed in our *M. pneumoniae*-infected hamsters and the mechanism by which *M. pneumoniae* infection inhibits NEP activity is thus uncertain. Because this enzyme is localized at the surface of epithelial cells, it may be especially sensitive to the effects of reactive molecules within the airway lumen. Therefore, in spite of the morphologically normal appearance of the airway epithelium, *M. pneumoniae* could have directly inactivated NEP. Another possibility would be that *M. pneumoniae* inactivated NEP indirectly as a result of the consequences of airway inflammation (e.g. by leading to the release of other proteolytic enzymes or toxic oxygen metabolites into
the extracellular milieu), but there was no correlation between airway epithelial NEP activity and accumulation of inflammatory cells in our experiments.

Previous studies have shown that there is a close relationship between respiratory tract infection with *M. pneumoniae* and exacerbation of asthma (Seggev et al., 1986; Yano et al., 1994) and airway hyper-responsiveness to histamines (Boldy et al., 1990). The potential ability of mycoplasma cell components to alter normal pulmonary physiology is exemplified by the cilostatic, haemagglutinating and proteolytic activities of cell extracts (Chandler & Barile, 1980). Moreover, Seggev et al. (1996) have recently shown that *M. pneumoniae*-specific IgE plays a role in the exacerbation of asthma. The results of the present study suggest that inhibition of airway epithelial NEP activity is one of the mechanisms by which *M. pneumoniae* infection causes bronchoconstrictor hyper-responsiveness to tachykinins and bradykinin, but further studies may be required to determine which toxic products released from the replicating organisms or inflammatory cells are responsible for inactivation of NEP.

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