Sensory nerve activation in airway microvascular permeability in guinea-pig late allergic response

Y. Mashito, M. Ichinose, H. Sugiura, M. Miura, N. Endoh, K. Shirato

ABSTRACT: Because both bradykinin and tachykinins have a potent inflammatory action, these molecules may be involved in the late allergic response. The role of these molecules in airway microvascular permeability during the late allergic response in sensitized guinea-pigs was investigated.

Three weeks after ovalbumin sensitization, the animals were pretreated with bradykinin B2 receptor antagonist HOE 140, neurokinin 1 receptor antagonist CP 96,345 or vehicle, 30 min before the ovalbumin inhalation challenge. The occurrence of the late allergic response was determined by a two-fold increase in the transpulmonary pressure from the baseline values. The microvascular permeability in the trachea was assessed by an index defined as the ratio of the area of vasculature labelled by Monastral blue dye (area density %).

Significant microvascular permeability and eosinophil accumulation were observed during the late allergic response. Both the bradykinin and substance P concentrations in the bronchoalveolar lavage fluid were increased during the late allergic response. Pretreatment with HOE 140 suppressed the substance P elevation. Both HOE 140 and CP 96,345 also inhibited the airway microvascular permeability during the late allergic response without affecting the eosinophil accumulation in the airways.

These findings suggest that bradykinin-mediated sensory nerve activation may play a role in microvascular permeability during the late allergic response in guinea-pigs.


Bradykinin, a 9-amino acid peptide, is formed from plasma precursors as part of the inflammatory response [1, 2]. Because bradykinin causes vasodilatation, airway microvascular leakage [3], mucus secretion, smooth muscle contraction [4] and dyspnoea [5], all of which are the cardinal features of asthma, this molecule may have an important role in the pathogenesis of asthma. There is evidence that the concentration of bradykinin is elevated in the bronchoalveolar lavage fluid (BALF) of asthmatic subjects [6] and increase after allergen challenge [7].

Among the endogenous molecules, bradykinin is the most potent stimulant for sensory C-fibres. Airway C-fibre stimulation may cause the release of tachykinins, such as substance P (SP), into the airways, resulting in neurogenic inflammation [8, 9]. In fact, in asthmatic airways, bradykinin inhalation induced bronchoconstriction and cough are largely inhibited by a tachykinin receptor antagonist [10], suggesting that the bradykinin-tachykinins pathway may play a key role in the asthmatic airways.

In allergic animal models, the role of the bradykinin-tachykinins pathway has been examined. BERTRAND et al. [11] have reported that airway microvascular permeability immediately after allergen challenge is significantly inhibited by both bradykinin B2 and neurokinin 1 (NK1) receptor antagonists, indicating the contribution of the bradykinin-tachykinins pathway during that period. In animal models, the late allergic response (LAR) is an important phenomenon because, during this period, the airways show inflammatory features that are very similar to those of asthmatic airway inflammation [12, 13]. It has been reported that bradykinin in lavage fluid increases during the LAR [14], and that a bradykinin B2-receptor antagonist inhibits the bronchoconstriction during that period [15], suggesting the involvement of bradykinin in airway inflammation during the LAR. However, the role of the bradykinin-tachykinins pathways in the LAR has not yet been fully examined.

The aim of this study was to elucidate the role of the bradykinin-tachykinins pathway in the airway inflammatory responses during the LAR. Using sensitized guinea-pigs, the concentrations of bradykinin and SP in the BALF during the LAR were measured. The occurrence of LAR was assessed while monitoring the transpulmonary pressure (Ptp) changes. The functional role of bradykinin and tachykinins in the LAR were measured using selective bradykinin B2- and NK1-receptor antagonists, respectively. As an inflammatory index, the microvascular permeability was measured by means of Monastral blue dye trapping between the postcapillary venule endothelia, as this site is important for macromolecular leakage. The accumulation of eosinophils into the airways was examined, as this also plays an important role in the inflammation during the LAR.
Effectiveness of bradykinin B2 and neurokinin 1 antagonists

The effectiveness of the bradykinin B2-receptor antagonist HOE 140 and NK1-receptor antagonist CP 96,345 in inhibiting the bradykinin- and SP-induced airway microvascular permeability, respectively, was tested in six groups (table 1). The antagonists or vehicle were administered 6 h before bradykinin or SP injection, and 1 min following the injection the vascular permeability was assessed, as described below.

Effect of bradykinin B2 and neurokinin 1 antagonists on the late allergic response

On day 21, the animals were pretreated with pyrilamine (10 mg kg⁻¹, i.p.) to prevent the animals from dying during the early phase airway response [17]. HOE 140 (s.c.), CP 96,345 (i.p.) or the vehicle thereof were also injected (table 2). Thirty minutes after the pretreatment, the animals were exposed to saline aerosol (control group) or 1% OVA aerosol (challenged group) for 1 min using the nebulizer system described above. Two hours after the inhalation, all animals were anaesthetized with urethane (2 g kg⁻¹, i.p.). A water filled oesophageal catheter (OD 1.52 mm; Natsume Seisahusho, Tokyo, Japan) was inserted to monitor the Ptp. When the Ptp reached twice that of the baseline value in the challenged group (i.e. when the LAR occurred), at ~4.5–6 h after the inhalation, the animals were examined as described below. In the control group; each experiment was performed at almost the same time point during the late phase as in the challenged animals. The dose of pyrilamine was chosen according to a previous study [17].

**Methods**

**Animal sensitization**

Male Dunkin-Hartley guinea-pigs (Funabashi Farm, Sendai, Japan) weighing 350–500 g were used. On day 0 and 1, all animals were sensitized with a subcutaneous injection of 10 mg ovalbumin (OVA) and 100 mg aluminum hydroxide [16]. On day 6, all animals were exposed to 2% OVA in saline using an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan; output 0.8 mL min⁻¹) for 3 min in a plexiglas exposure chamber (24.5 × 40.5 × 15.0 cm) under spontaneous breathing. All the experiments performed in this study were conducted with the consent of the Ethics Committee for Use of Experimental Animals of the Tohoku, University school of medicine.

Quantification of airway microvascular permeability

Monastral blue dye (particle size 20–300 nm) was sonicated for 5 min and filtrated using a 5 μm Millipore filter (Millipore, Bedford, MA, USA) just before use. One minute after the administration of Monastral blue dye (30 mg kg⁻¹, i.v.), the thorax was opened and the systemic and pulmonary circulation perfused with 1% paraformaldehyde (PFA) in 50 mM phosphate buffered saline (PBS), as previously reported [18]. The lungs were then lavaged twice from the cannulated trachea with 5 mL saline to measure the bradykinin and SP concentrations as described below. The trachea was removed. The upper part of the trachea was used for eosinophil cell counts and the lower part was immersed in 1% PFA for 2 h, washed in distilled water for 2 h and soaked in glycerol for 20 h at room temperature, followed by dehydration in 100% ethanol, followed by dehydration in 100% ethanol and then in 100% ethanol for 1 h at room temperature. The tissue was immersed in toluene for 1.5 h, then in 100% ethanol for 1 h, and hydrated in distilled water for 2 h at room temperature. The hydrated trachea was opened longitudinally.

**Table 1. Administration of bradykinin B2 (HOE 140) and neurokinin 1 (CP 96,345) receptor antagonists to guinea-pigs to test inhibition of bradykinin- and substance P-induced airway microvascular permeability**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Substance</th>
<th>Amount</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>s.c.</td>
<td>Saline</td>
<td>1 mL kg⁻¹</td>
<td>DMSO</td>
</tr>
<tr>
<td>s.c.</td>
<td>BOE 140</td>
<td>10 mg kg⁻¹</td>
<td>DMSO</td>
</tr>
<tr>
<td>s.c.</td>
<td>CP 96,345</td>
<td>1 mL kg⁻¹</td>
<td>50 mg kg⁻¹</td>
</tr>
</tbody>
</table>

DMSO: dimethy sulphoxide.

**Table 2. Administration of bradykin B2 (HOE 140) and neurokinin 1 (CP 96,345) receptor antagonists to sensitized guinea-pigs to test effect on the late allergic response**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Substance</th>
<th>Amount</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>s.c.</td>
<td>Saline</td>
<td>1 mL kg⁻¹</td>
<td>OVA</td>
</tr>
<tr>
<td>s.c.</td>
<td>BOE 140</td>
<td>0.1 mg kg⁻¹</td>
<td>OVA</td>
</tr>
<tr>
<td>s.c.</td>
<td>CP 96,345</td>
<td>1 mg kg⁻¹</td>
<td>OVA</td>
</tr>
<tr>
<td>s.c.</td>
<td>50 mg kg⁻¹</td>
<td>OVA</td>
<td></td>
</tr>
</tbody>
</table>

DMSO: dimethy sulphoxide.
along the ventral midline and flattened between two glass slides held together by clips for 24 h in 100% ethanol at room temperature, cleared in toluene for 15 min, and mounted on a glass slide. Five images of the intercartilaginous portion from each tracheal preparation were viewed with image analysing software (MacScope; Mitani Co., Fukui, Japan) using an Apple Macintosh computer (Apple Computer Inc., Cupertino, CA, USA) connected to the microscope. The Monastral blue dye trapped within the endothelia was quantified as the area density [19], that is, the percentage area of mucosa occupied by vessels stained with Monastral blue in a site just proximal to the carina.

Measurement of bradykinin and substance P concentrations in bronchoalveolar lavage fluid

Because bradykinin and SP are decomposed by angiotensin converting enzyme (ACE) or neutral endopeptidase (NEP) in only a few minutes, the BALF was boiled immediately in order to inactivate these enzymes. The BALF was then stored at -80°C until assay. The bradykinin and SP concentrations were quantified by radioimmunoassay (RIA). The BALF samples (1 mL) for bradykinin were mixed with isopropylalcohol (2.5 mL) and centrifuged at 1200 g, 4°C for 10 min. The supernatant was mixed with petroleum ether (2 mL) and the upper layer was aspirated, and this step being performed twice. The lower layer was evaporated by nitrogen gas at 55°C, mixed with tris-hydroxyxymethy-amino methane (Tris)-HCl buffer (500 mL, pH 7.0) and centrifuged at 1200 x g, 4°C for 5 min. The sample (400 μL) or bradykinin standard (Peptide Institute, Inc., Osaka, Japan) was mixed with anti-bradykinin (100 μL; SRL Inc., Tokyo, Japan) and stored overnight at 4°C. They were incubated with 100 μL [125I-l tyry]-bradykinin (Du Pont NEN Research Products, Boston, MA, USA) at 4°C for 4 h. The mixtures were diluted with 4% acetic acid (1261 Multi Gamma; Wallac Oy, Turku, Finland).

The BALF samples for SP were diluted with 4% acetic acid pH 4.0 to a final volume of 50 mL, homogenized using Polytron PT-10 (Kinematica AG, Littau, Switzerland), and centrifuged at 40,000 x g for 30 min. The supernatant was loaded onto reversed-phase C18 cartridges (Sep-Pak C18; Millipore Co., Milford, MA, USA). After washing with 20 mL of 4% acetic acid pH 4.0 and 20 mL distilled water, SP was eluted with 2 mL of 80% acetonitrile in 0.1% trifluoroacetic acid. Eluates were concentrated by spin-vacuum evaporation, lyophilized, and the eluates dissolved using 0.15 mL of assay buffer (50 mm phosphate buffer, pH 7.2, containing 3.7 mg·mL⁻¹ ethylene diamine tetra-acetic acid (EDTA) and 0.5% bovine serum albumin (BSA)) before being subjected to an RIA for SP. RIA for SP was performed using [125I-SP (Amersham International plc., Amersham, UK) and anti-SP rabbit serum (Amersham International plc.). A 0.1 mL sample was mixed with 0.5 mL assay buffer, 0.5 mL antiserum and 0.1 mL [125I-SP, and stored at 4°C for 24 h. A total of 0.2 mL dextran/charcoal suspension (0.2% dextran and 2% activated charcoal in assay buffer) was added to the reaction mixture and centrifuged at 2,000 x g, 4°C, for 10 min. The radioactivity of the supernatant was measured by a gamma counter (Model 5420A; Packard Instrument Co., Meriden, CT, USA). In this system, the sensitivity of detection of SP in saline was 1–60 fmol·mL⁻¹.

Quantification of eosinophil accumulation into the airways

The upper part of the trachea was immersed in 10% formalin for 3 days, before being embedded in paraffin and sectioned at a thickness of 4 μm. The sections were immersed in Hansel’s stain solution for 30–45 s, followed by the addition of a drop of distilled water for a further 30 s. The slides were then flooded with distilled water followed by 95% methyl alcohol. The slides were then drained [20]. Eosinophils were counted in both the epithelial layer and submucosal area using a microscope.

Drugs

HOE 140 and CP 96,345 were kindly donated by Hoechst AG (Frankfurt, Germany) and Yamanouchi Pharmaceutical Co. Ltd. (Tokuba, Japan), respectively. Bradykinin, Monastral blue dye, OVA, pyrilamine and urethane were from Sigma Chemical Co. (St. Louis, MO, USA). SP was from Funakoshi Co. (Tokyo, Japan). Aluminum hydroxide, dimethyl sulphoxide (DMSO) and paraformaldehyde were from Wako Pure Chemical Industries (Osaka, Japan). Hansel’s stain solution was from Torii Chemical Co. (Tokyo, Japan). Saline was from Ohtsuka Chemical Co. (Tokyo, Japan).

Statistical analysis

Data are expressed as mean±SEM. Multiple comparisons of mean data of dye extravasation and quantification of eosinophils among the groups were performed by one way analysis of variance (ANOVA), followed by Scheffe’s test. Comparisons of mean data of bradykinin and SP concentration in BALF among the groups were performed by the Mann-Whitney U-test. A p-value <0.05 was considered significant.

Results

Bradykinin B₂- and neurokinin 1-receptor antagonists effect on bradykinin- and SP-induced airway microvascular permeability

Bradykinin administration caused significant airway microvascular permeability. This response was completely inhibited by HOE 140 pretreatment (fig. 1a). SP also showed significant airway microvascular permeability, and this response was almost completely abolished by CP 96,345 (fig. 1b).
Bradykinin concentration in bronchoalveolar lavage fluid during the late allergic reaction

All animals showed a rapid breathing pattern and cyanosis during and immediately after OVA inhalation. About 45 min later, the animals’ breathing pattern returned to the level of the pre-inhalation challenge. At 4.5±6 h after the inhalation, the $P_{tr}$ values rose to twice that of the baseline values in OVA challenged but not in saline exposed animals.

OVA challenge caused a significant increase in the bradykinin concentration in the BALF (105±83 pg·mL$^{-1}$, $p<0.05$) compared with those of the saline exposed animals (1.8±1.1 pg·mL$^{-1}$) during the LAR (fig. 2).

Effect of bradykinin B$_2$-receptor antagonist

OVA challenge caused remarkable Monastral blue dye extravasation in the saline treated animals compared with those of the saline treated-saline exposed group during the LAR (fig. 3). Monastral blue dye trapping was observed at the vessels of 10–20 μm in width, which is compatible with the size of postcapillary venules [18].

Figure 1 shows a representative view of the eosinophil accumulation in the saline exposed and OVA challenged groups during the LAR. Figures 5 and 6 shows a representative view of the eosinophil accumulation in the saline exposed and OVA challenged groups during the LAR. OVA inhalation caused a significant eosinophil accumulation both in the epithelium (1.5±0.9–12.9±2.0 cells·100 mm epithelium$^{-2}$, $p<0.01$) and submucosa (2.2±1.2–23.6±2.9 cells·10,000 μm$^2$ submucosa$^{-1}$, $p<0.01$) during the LAR compared with the saline exposed group. HOE 140 pretreatment did not affect the eosinophil accumulation (fig. 6).

The relationship between bradykinin and SP during the LAR was then investigated. In the saline treated animals, OVA challenge caused a significant increase in the SP concentration in the BALF compared to the saline treated, saline exposed animals during the LAR (fig. 7). HOE 140 pretreatment reduced the SP concentration in the BALF in a dose-dependent manner.

Fig. 1. – a) Effect of the bradykinin B$_2$-receptor antagonist (HOE 140; 10 mg·kg$^{-1}$, i.p.) on bradykinin-induced airway microvascular permeability. S/S: saline treated, saline injected group; S/B: saline treated, bradykinin injected group; HOE/B: HOE 140 treated, bradykinin injected group. *: $p<0.01$, compared with the S/S group; #: $p<0.01$, compared with the S/B group. b) Effect of neurokinin 1 receptor antagonist (CP 96,345; 50 mg·kg$^{-1}$, i.p.) on substance P (SP)-induced airway microvascular permeability. DMSO/S: dimethyl sulphoxide (DMSO) treated, saline injected group; DMSO/SP: DMSO treated, SP injected group; CP/SP: CP 96,345 treated, SP injected group. **: $p<0.01$, compared with the DMSO/S group; ***: $p<0.01$, compared with the DMSO/SP group. All values are mean±SEM.

Fig. 2. – Bradykinin concentrations in the bronchoalveolar lavage fluid. During the late allergic responses of the animals (LAR) after ovalbumin (OVA) inhalation or saline inhalation at a time matched to the LAR. All values are mean±SEM. *: $p<0.05$, compared with the saline exposed group.

Fig. 3. – Representative view of Monastral blue labelled airway microvessels during the late allergic response in a) saline treated, saline exposed and b) saline treated, ovalbumin challenged animals. (Internal scale bar=300 μm.)
Effect of neurokinin 1-receptor antagonist

OVA challenge caused significant Monastral blue dye extravasation in the DMSO-treated animals compared with the DMSO-treated, saline exposed group during the LAR (fig. 8). CP 96,345 pretreatment almost completely inhibited the dye extravasation. Figure 9 shows the effects of CP 96,345 on eosinophil accumulation during the LAR. CP 96,345 pretreatment did not show an inhibitory effect on eosinophil accumulation.

Discussion

These data show that allergen inhalation causes significant airway microvascular permeability and eosinophil accumulation into the airway during the LAR. During this period, both the bradykinin and SP concentrations in the BALF were increased. Furthermore, each bradykinin B₂ and NK₁ receptor antagonist significantly inhibited the airway microvascular permeability during the LAR. These results suggest that the bradykinin-tachykinins pathway, that is sensory nerve activation, plays an important role in microvascular permeability during the LAR in sensitized guinea-pigs.

Bradykinin is formed from kininogens by the action of plasma and tissue kallikrein [2, 21]. In allergen inhalation challenge, it has been reported that both plasma and tissue kallikrein release and/or activation occurs [7]. ERJEFALT et al. [14] have also reported that the bradykinin concentration increases during the LAR, which is in agreement with the present results. Bradykinin has a variety of airway actions including airway smooth muscle contraction, hypersecretion and microvascular permeability via bradykinin B₂ receptors [3, 21], and is thought to be an important mediator in asthmatic airway inflammation [21]. Bradykinin-induced airway microvascular permeability has been reported to occur via two pathways. One pathway is its direct action in the endothelium and the other is an indirect mechanism that involves tachykinin release [22, 23]. In the present study, the bradykinin B₂-receptor antagonist-induced reduction in the SP concentration in the BALF was in parallel with the inhibition of microvascular permeability. Therefore, during the LAR, bradykinin seems to cause airway microvascular permeability via tachykinin release.

In the airways, tachykinins are thought to be released from sensory C-fibres via axon reflex mechanisms [9, 10]. Among the endogenous molecules, bradykinin is one of the most potent stimulators of C-fibres [3, 21]. Tachykinins cause airway microvascular leakage via activation of NK₁ receptors in the endothelial cells of post capillary venules [18, 24]. In the present study, the NK₁ receptor antagonist CP 96,345 completely inhibited the airway microvascular permeability during the LAR, strongly suggesting that NK₁ receptor activation in the endothelial cells and the resulting contraction of the cells are the final steps that lead to the airway microvascular permeability during the LAR in guinea-pigs.

In asthmatic animal models, allergen challenge causes immediate and late airway responses [12, 13]. The immediate phase response is mainly via airway smooth muscle contraction mediated by mast cell-derived mediators including histamine and leukotrienes. On the other hand, the late phase response is due to airway inflammation.
such as airway wall oedema and mucus secretion [12, 13], which is very similar to asthmatic chronic airway inflammation. To elucidate the inflammatory mechanisms, animal models including guinea-pigs [14], rats [25, 26] and dogs [27] have been developed using vascular permeability as the marker of inflammation. The present study is the first report showing that the bradykinin-tachykinins pathway, that is sensory nerve activation, is a major contributor to airway microvascular permeability, which has a key role in airway wall oedema during the LAR.

In the present study, both bradykinin B₂- and NK₁-receptor antagonists almost completely inhibited the airway microvascular permeability during the LAR. In contrast, eosinophil accumulation into the airway during this period was not affected by the antagonists. Microvascular permeability has been reported to be mainly due to endothelial contraction in the smallest postcapillary venules [18, 28]. Both bradykinin and SP are potent inducers of permeability. Previously, it has been reported that bradykinin-induced tracheal microvascular permeability is largely reduced by NK₁-receptor antagonists [22], which is compatible with the present results. On the other hand, eosinophil recruitment into the airways occurs in the largest postcapillary venules [18, 29] and is mainly regulated by chemical factors including cytokines, adhesion molecules and chemokines. Thus, the site and mechanisms of airway microvascular permeability and eosinophil accumulation seem to be different. The bradykinin-tachykinins pathway may not work in the largest postcapillary venules nor be involved in the cytokine, adhesion molecule and chemokine systems.

Monastral blue dye was used to assess the airway microvascular permeability. The particles of Monastral blue are too large to cross the endothelium of tracheal blood vessels with a normal permeability. However, when the blood vessels become more permeable, possibly due to the endothelial contraction, the dye passes through gaps in the endothelium and is trapped by the basal lamina where it remains and thus labels the site of extravasation [18, 24, 28].

In conclusion, these results have demonstrated that both bradykinin B₂- and NK₁-receptor antagonists almost completely inhibited the airway microvascular permeability but not the eosinophil accumulation during the late allergic reaction. Airway microvascular permeability is an
important factor of airway inflammation in asthma [30–33]. Further, neutral endopeptidase, which is responsible for the degradation of both bradykinin and tachykinins, is mainly localized in airway epithelial cells [34]. Because airway epithelial shedding is frequently observed in asthmatic airways, exaggerated sensory nervous pathway via inactivation of the peptide may have a key role [9]. Therefore, inhibition of the bradykinin-tachykinins pathway may become useful for asthma treatment in the future.

**Acknowledgement.** The authors thank B. Bell for reading the manuscript.

**References**


