Bradykinin-induced lung inflammation and bronchoconstriction: role in PIV-3-induced inflammation and airways hyperreactivity


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Inhaled bradykinin causes bronchoconstriction in asthmatic subjects but not non-asthmatics. To date, animal studies with inhaled bradykinin have only been performed in anaesthetized guinea-pigs and rats, where it causes bronchoconstriction through sensory nerve pathways. In the present study, airways function was recorded in conscious guinea-pigs by whole body plethysmography. Inhaled bradykinin (1mM, 20 s) caused bronchoconstriction and influx of inflammatory cells to the lungs, but only when the enzymatic breakdown of bradykinin by angiotensin converting enzyme and neutral endopeptidase was inhibited by captopril (1mg/kg i.p.) and phosphoramidon (10mM, 20 min inhalation), respectively. The bronchoconstriction and cell influx were antagonised by the B2 kinin receptor antagonist, MEN16132, when given by inhalation (1 and 10μM, 20 min), and are therefore mediated via B2 kinin receptors. However, neither intraperitoneal MEN16132 nor the peptide B2 antagonist, icatibant, by inhalation, antagonized these bradykinin responses. Sensitization of guinea-pigs with ovalbumin was not sufficient to induce airways hyperreactivity (AHR) to the bronchoconstriction by inhaled bradykinin. However, ovalbumin challenge of sensitized guinea-pigs caused AHR to bradykinin as well as to histamine. Infection of guinea-pigs by nasal instillation of parainfluenza-3 virus produced AHR to inhaled histamine and lung influx of inflammatory cells. These responses were attenuated by the bradykinin B2 receptor antagonist, MEN16132, and by VA999024, an inhibitor of tissue kallikrein (KLK1), the enzyme responsible for lung synthesis of bradykinin. These results suggest that bradykinin is involved in virus-induced inflammatory cell influx and AHR.
Introduction

In healthy humans, inhalation of bradykinin has little or no effect, but in asthmatics it produces a bronchoconstriction (Polosa et al., 1993). Asthmatic subjects show a greater degree of airways hyperreactivity to bradykinin than to methacholine after allergen challenge (Berman et al., 1995). Kinins exert their pharmacological effects through two main kinin receptor subtypes, named B₁ and B₂ receptors (Leeb-Lundberg et al., 2005). B₁ receptors are characterized by binding of [des-Arg⁹]-bradykinin, although there are more selective agonists such as [lys-des-Arg⁹]-bradykinin (Leeb-Lundberg et al., 2005). B₁ receptors are absent in the lungs under normal conditions but their expression is induced by inflammation (Christiansen et al., 2002). B₂ kinin receptors show high affinity for bradykinin and are constitutively expressed in the airways (Leeb-Lundberg et al., 2005).

In guinea-pigs, inhaled bradykinin has been shown to produce bronchoconstriction which can be blocked by the bradykinin B₂ receptor antagonist HOE140, now called icatibant (Sakamoto et al., 1994; Wirth et al., 1993). To date, the effects of bradykinin on the airways have been largely examined after intravenous, inhalation or intratracheal administration to anaesthetised animals, such as guinea-pigs (Ichinose and Barnes, 1990a, b; Wirth et al., 1993; Miura et al., 1994; Sakamoto et al., 1994; Valenti et al., 2005) and rats (Ellis et al., 2004). One study showed that intravenously administered bradykinin caused bronchoconstriction in unanaesthetized guinea-pigs (Chodimella et al., 1991). In these earlier studies there are a number of discrepancies in the effects of bradykinin, for example, a lack of blockade of allergen-induced microvascular leakage and bronchoconstriction (Sakamoto et al., 1996) and differences in the sensitivity to bradykinin between bronchoconstriction and microvascular
leakage (Ricciardolo et al., 1994). A possible reason for the variable effects of bradykinin in the lung is its rapid breakdown by neutral endopeptidase (NEP) and/or angiotensin converting enzyme (ACE). There do not appear to have been any studies on the airways effects of inhaled bradykinin in conscious animals, where the effect of anaesthetic is eliminated. In this study, we therefore attempted to identify a bronchoconstriction with inhaled bradykinin and to examine the effects of phosphoramidon and captopril, inhibitors of NEP and ACE, respectively. As bradykinin is thought to act on B2 kinin receptors in the airways (Ellis and Fozard, 2002), we also compared the blockade of the bradykinin-induced bronchoconstriction with two distinct B2 receptor antagonists, icatibant and MEN16132. Icatibant is a selective peptidic antagonist of B2 receptors (Leeb-Lundberg et al., 2005), its affinity (pKᵢ) for displacement of radioligand binding to human B₁ and B₂ receptors expressed in CHO cells being <5 and 10.2, respectively (Regoli et al., 1998). Intravenous, intratracheal or aerosol administered icatibant has been shown to inhibit the bronchoconstriction to intravenously administered bradykinin in anaesthetized guinea-pigs (Tramontana et al., 2001; Valenti et al., 2005). Inhaled icatibant also inhibited the bronchoconstriction and microvascular leakage after inhaled bradykinin in anaesthetized guinea-pigs (Sakamoto et al., 1994). MEN16132 is a nonpeptide selective antagonist of B₂ kinin receptors, the affinity values (pKᵢ) of which for B₁ and B₂ receptors are <5 and 10.5, respectively (Cucchi et al., 2005). MEN16132 has been shown to block bronchoconstriction to intravenous bradykinin in anaesthetized guinea-pigs (Valenti et al., 2005).

In addition to a role for B₂ receptors in bronchoconstriction by bradykinin in asthmatics, kinin levels are increased in the bronchoalveolar lavage fluid (BALF) of asthmatic subjects after
allergen challenge (Christiansen et al., 1992; Polosa and Holgate, 1990). Furthermore, B₁ but not B₂ kinin receptor expression is up-regulated in sensitized rat lungs (Huang et al., 1999), in murine airways under interleukin-4 (IL-4) stimulation (Bryborn et al., 2004) and B₁ in asthmatic airways inflammation (Christiansen et al., 2002).

Allergen sensitization and challenge has been repeatedly shown to cause airways hyperreactivity (AHR) in guinea-pigs to a range of spasmogens, including histamine and methacholine (Toward and Broadley, 2004; Smith and Broadley, 2007). AHR to intravenous bradykinin has been demonstrated in anaesthetized rats after allergen challenge (Ellis et al., 2004), but there are few studies examining AHR to inhaled bradykinin in conscious animals. In this study, we therefore determined whether ovalbumin exposure in ovalbumin-sensitized guinea-pigs produced airways hyperreactivity to inhaled bradykinin. AHR is also a consequence of viral infection of the airways and has been shown along with an influx of inflammatory cells after inoculation of guinea-pigs with parainfluenza-3 virus (PIV-3) (Toward et al., 2005). Folkerts et al. (2000) showed that the bradykinin B₂ antagonist, icatibant, can inhibit AHR induced by PIV-3 but not the inflammatory cell influx. We therefore determined whether bradykinin was involved in the PIV-3-induced AHR and inflammatory cell influx and whether it was mediated by the B₂ kinin receptor by treating PIV-3 inoculated guinea-pigs with the B₂ kinin receptor antagonist, MEN16132. Since the primary synthesis of bradykinin in the lung is via tissue kallikrein (KLK1) (Christiansen et al., 1992), we also examined the effects of inhibiting bradykinin production on PIV-3-induced inflammation and AHR, by using a potent KLK1 inhibitor, VA999024 (also known as FE999024 and CH-2856). VA999024 has
previously been shown to be effective in reducing eosinophilia in an ovalbumin challenge model in guinea-pigs (Evans et al., 1996).

**Methods**

**Animal welfare and ethics.** Male Dunkin Hartley guinea pigs (250-350 g) were obtained from Harlan UK Ltd (Oxon UK). The animals were housed at 20\(^0\)C ± 2\(^0\)C with 12 hour alternating light/dark cycles at approximately 50% humidity. The animals were fed on commercial guinea pig diet pellets (Harlan UK Ltd., Oxon, UK) supplemented with ascorbic acid and water allowed *ad libitum*. The animals were provided with cardboard tubes and received hay every day for environmental enrichment. The animals were acclimatised for at least 1 week before the commencement of any experiments. Animal welfare and experimentation were undertaken in accordance with the Animal Scientific Procedures Act 1986 under Home Office personnel and project licences. The guinea pigs were without infections of the respiratory airways as evaluated by the health monitoring quality control report by Harlan UK Ltd.

**Growth of virus.** African green monkey kidney epithelial (VERO) cells (European Collection of Cell Cultures, ECCAC, Wiltshire, UK) were grown to confluence in a humidified incubator (Sanyo, Osaka, Japan) maintained at 95% with an atmosphere of 95% O\(_2\), 5% CO\(_2\) at 37\(^0\)C in T75 culture flasks containing 10ml of Dulbecco’s modified essential medium (DMEM), 10% heat inactivated foetal bovine serum (FBS) and 1% L-glutamate. Antibiotics were not used. The medium was removed and the cells were trypsinized and 1ml of foetal calf serum (FCS) was added to deactivate the trypsin. The cells were then pelleted by centrifugation (Jouan
CR412, Jouan Inc., Winchester, Virginia, USA) at 1200 r.p.m. for 6 min. The supernatant was removed and the cells were resuspended in 2ml of DMEM. Human PIV-3 virus (Strain:DEL/139/05) (ECCAC) was added to the cells and mixed by pipetting up and down three times. The cell/viral suspension was then placed in the incubator for 30 min, and swirled by hand every 5 mins to ensure the virus had access to every cell. 3 ml of the cell/viral mix was then added to the T75 flask and topped up with 7 ml of DMEM and the flask was replaced in the incubator.

After 4 to 5 days the cytopathic effects (CPE) of viral infection became obvious, with large syncitia (multi-nuclear cells) and dead cells floating in the medium. The virus was then harvested. The cells were frozen at –80°C for 5 min and allowed to thaw at room temperature to break open the cell membranes. This freeze-thaw cycle was repeated twice and the resulting suspension centrifuged (Jouan CR412, Jouan Inc., Winchester, Virginia, USA) at 4000 r.p.m. (504 g) for 5 min to remove cellular debris. The supernatant was then transferred into sterile 1ml vials and stored at –80°C. For control inoculation uninfected infected VERO cells were subjected to the same procedure.

**Calculation of viral titre (TCID₅₀).** The tissue culture infective dose (TCID₅₀/ml) refers to the quantity of virus producing cytopathic effects in 50% of infected wells. 450 μl of DMEM was added to 10 wells of a 24 well plate. A vial of frozen virus was thawed and 50 μl was added to the first well and mixed by pipetted up and down three times. Serial 1 in 10 dilutions were added to the remaining wells and the plate placed on ice. A T75 cell culture flask of confluent VERO cells was trypsinised and after the addition of FCS to deactivate the trypsin, the cells
were centrifuged (Jouan CR412) at 1000 r.p.m. (224 g) for 6 min, the supernatant removed and the cells resuspended in 5ml of DMEM. 50 μl of each viral dilution from the 24 well plate was added to each of the wells of the corresponding column of a 96 well plate. 50 μl of the VERO cell suspension was then added to each well and the plate placed in the 37°C incubator. The cells were then checked daily for cytopathic effects. After 8 to 10 days, when no additional wells had shown any sign of infection for two days, the medium was removed and 50 μl of crystal violet was immediately added to each well and left for 30 min. The crystal violet was then washed off with water and the plate was left to dry. Each well was then examined by manual microscopy for signs of infection. Destroyed cells showed very little staining but uninfected and control cells showed complete staining. The viral concentration was then determined as the TCID$_{50}$/ml using the Karber formula (Reed and Muench, 1938). TCID$_{50}$/ml

\[ \text{TCID}_{50} = 10^{\log \text{dilution above 50% infection} - (I \times \log H)} \]

where $I = \text{interpolated value of 50\% endpoint (percentage of wells infected above 50\%)} - 50 / \text{percentage of wells infected above 50\%} - \% \text{ of wells infected below 50\%}$ and H = dilution factor.

**Inoculation of guinea-pigs with virus.** Guinea-pigs were inoculated with PIV-3 (3.16 x $10^8$ or 6.32 x $10^6$ infectious units per ml) by intranasal instillation, performed in a Class II safety cabinet (Captair Madcap 804, Erlab, Wiltshire, UK). The guinea-pigs were held in a supine position with the head firmly supported. The virus or virus-free medium was then pipetted into the nostrils using a Gilson Pipette. Each guinea-pig was given a 250 μl inoculation; 125 μl in one nostril, which was then repeated after 15 min in the other nostril. This procedure was then repeated 24 h later. A fresh vial of virus (or virus free media) was thawed and warmed by hand.
to 37°C immediately prior to inoculation to ensure no decrease in the viral titre and to facilitate delivery.

Guinea-pigs were treated with a daily 20 min inhalation exposure of the B2 bradykinin receptor antagonist, MEN16132 (10 µM in saline) or sterile 0.9% saline (control) or with the tissue kallikrein inhibitor, VA999024 (1, 3 or 10 mg/kg in saline) or sterile 0.9% saline by bilateral subcutaneous injections every 12 h. Dosing with MEN16132 or VA999024 commenced on day 1 and ended on day 8. On days 3 and 4, the animals received the viral inoculations 3 h after drug or saline treatments. On days 2 and 8, histamine reactivity tests were performed and on day 8 the lungs were lavaged.

**Measurement of respiratory function.** Airway function was monitored in conscious spontaneously breathing animals by whole body plethysmography and recorded as specific airway conductance (sGaw). Guinea-pigs were held by a neckpiece in a restrainer. The animal’s snout was covered by a mask with a rubber diaphragm creating an airtight seal. This was attached to a pneumotachograph (Mercury FIL, Glasgow) before the animal, in its restrainer, was placed in the plethysmograph chamber, which was then sealed by a plate secured to the front of the chamber. Prior to each experiment, the guinea-pigs were handled and familiarized with the restrainer and the plethysmograph chamber to reduce stress-related factors.

Pressure transducers (Pioden Type 1, Pioden Controls Ltd. Canterbury, UK), UP1 and UP2, attached to the pneumotachograph and plethysmograph chamber, respectively, measured changes in respiratory flow and box pressure. These transducers were attached to a
computerised recording system comprising of AcqKnowledge® software with a Biopac® data acquisition system, as previously described by Smith and Broadley (2007). The resulting waveforms were analysed by comparing the gradients of the flow and box pressure at a point where flow tended towards zero; i.e. end tidal volume (at the end of expiration and beginning of inspiration). Each recording period was 5 s long, and from this, a minimum of 5 breaths were analysed. Using these values and taking into account air pressure and the weight of each guinea-pig, average sG aw was determined. Between recordings, animals were removed from the plethysmograph and returned to the holding cage.

**Airway reactivity to histamine and bradykinin.** Airway reactivity to aerosolised histamine (1 mM, 20 s) and bradykinin (0.1, 0.3 or 1 mM, 20, 40 or 60 s) delivered to the snout of the guinea-pigs was measured by whole body plethysmography. sG aw was measured before exposure to histamine or bradykinin (baseline), immediately after transferring to the plethysmograph (zero time) and at 5 and 10 min thereafter. From previous experience in these laboratories, 1 mM of histamine causes minimal bronchoconstriction having been found to be a threshold concentration in naïve guinea-pigs (Smith and Broadley, 2007). Airways reactivity to histamine or bradykinin was measured 24 h before and 24 h after allergen challenge or the day before viral inoculation and on the fourth day after the second viral inoculation.

The effects of bradykinin were examined 1 h after administration of captopril (1 mg/kg i.p.), phosphoramidon (0.1 mg/kg i.p. or 1 M by inhalation for 20 min) or combined captopril (1 mg/kg i.p.) and phosphoramidon (1 mM, 20 min exposure). The selective B2 kinin receptor antagonists, icatibant (10 µM inhalation exposure) and MEN16132 (30, 100 and 300 nM/kg,
i.p., or 1 and 10 µM by 20 min inhalation exposure), were administered 1 h before inhalation exposure to 1 mM bradykinin in guinea-pigs treated with captopril (1 mg/kg).

**Bronchoalveolar lavage.** Within 30 min of the hyperreactivity test, the guinea-pigs were sacrificed with a lethal overdose of the anaesthetic (pentobarbital sodium, Euthatal, 400mg/kg) by bilateral intraperitoneal injection. The trachea was then cannulated by insertion of a nylon intravenous cannula (Sims Portex Ltd, Kent, UK). An incision was made below the level of the diaphragm upwards to expose the ribcage. The ribcage was then removed exposing the lungs and trachea. The trachea and lungs were then removed from the thoracic cavity and the heart and any fat and connective tissue were then removed. The right bronchi was clamped shut with Spencer-Wells forceps so the lavage could be performed on the left lung only.

Saline (0.9%, 10 ml/kg) was then injected into the left lung with a syringe, via the tracheal cannula. After 3 min the fluid was withdrawn by means of the syringe, while gently massaging the lung. This procedure was repeated and the two recovered bronchoalveolar lavage fluid samples were combined and placed on ice for total and differential cell counts. Only plasticware was used for the collection process to minimise adherence of the cells to the surface of the tube.

**Total and differential cell counts.** Total cell counts (cells/ml) were determined using a Neubauer haemocytometer (Marienfield, Germany) viewed under a light microscope (X10, Nikon, Tokyo, Japan). Differential cell counts were then undertaken to determine the levels of alveolar leucocytes. A 100 µl sample of the BALF was centrifuged using a cytospin (ThermoShandon Ltd. Cheshire, UK) at 1000 r.p.m. (112 g) for 7 min, onto a glass microscope
slide and air-dried. The slides were then stained with 1.5% Leishman’s stain (Sigma-Aldrich, Dorset, UK) for 7 min. Using a light microscope (X100, Nikon, Tokyo, Japan), a minimum of 200 consecutive cells were examined, using standard morphological criteria to determine alveolar macrophages, eosinophils, lymphocytes and neutrophils. Using the corresponding total cell count, numbers of each subtype of leucocytes were determined.

**Ovalbumin sensitization and exposure.** On days 1 and 5 guinea pigs were sensitized with i.p. injection of 100 µg of ovalbumin and 100 mg of aluminium hydroxide in 1 ml. The suspension was placed on a magnetic stirrer for at least 2 h before use and to ensure adequate distribution, the dose was divided and administered bilaterally. On day 15 the animals were placed in a steel exposure chamber (40 cm diameter, 15 cm height) and given a 60 min inhalation exposure of ovalbumin (0.01%) dissolved in saline (0.9 % NaCl). Any animals that looked to be in respiratory distress were immediately removed from the exposure chamber and the exposure considered complete. Lung function measurements were taken by whole body plethysmography immediately before challenge and at 0, 15, 30, 45 and 60 min after, then hourly up to 12 h and finally at 24 h. Histamine or bradykinin reactivity was determined 24 h before and 24 h after OA challenge. Histamine reactivity was also tested in saline challenged guinea-pigs. Ovalbumin sensitized animals were all treated with dimethylsulfoxide (50% in saline i.p.) at 24 h before, 30 min before and 6 h after ovalbumin or saline challenge. This served as a control for drug intervention studies using this solvent not reported in this paper.

**Data handling and statistical analysis.** Baseline $sG_{aw}$ level was determined as the average of two $sG_{aw}$ readings taken before each challenge. Subsequent readings taken after challenge with
histamine, bradykinin or ovalbumin were expressed as a percentage of the baseline value. Statistical comparisons of histamine or bradykinin responses were made between values before and after treatments (e.g. ovalbumin challenge and/or viral inoculation, or drug treatment), using a Student’s paired $t$-test.

The ovalbumin model of allergy shows distinct early and late phases of airway reactivity, however due to natural variation each animal can display these reactions at different time points. The average maximum decrease from baseline in each animal between 0 and 6 h and between 7 and 12 h is shown in a separate graph to demonstrate the early and late phase respectively alongside the graph showing the time course.

Statistical comparisons between mean values from more than two groups were made by analysis of variance (ANOVA) followed by a Dunnett’s post hoc test. A $p$ value of <0.05 was considered significant. All values are presented as mean ± standard error of the mean (S.E.M.).

**Drugs, materials and solutions.** Aluminium hydroxide, bradykinin acetate, captopril, dimethyl sulfoxide, histamine diphosphate, icatibant, ovalbumin and phosphoramidon disodium salt were obtained from Sigma-Aldrich, Dorset, UK. Saline was purchased from Baxter Healthcare, Newbury, UK. FBS was obtained from Perbio Science UK Ltd (Chester, UK) and DMEM, L-glutamate and trypsin from Invitrogen (Paisley, UK). MEN16132 (4-((S)-amino-5-(4-[[2,4-dichloro-3-(2,4-dimethyl-8-quinolyoxy)methyl]phenylsulfonamido]-tetrahydro-2H-4-pyranylcarbonyl)piperezino)-5-oxopentyl)(trimethylammonium chloride hydrochloride) was kindly provided by Dr Christopher Fincham, Menarini Ricerche, Pomezia,
Italy. VA999024 (H-(4-chloro)DPhe-2’(1-naphthylalanine)-(3-aminopropyl)guanidine) (Evans et al., 1996) was kindly provided by Dr D. Michael Evans, Vantia Ltd, Southampton, UK. All drugs were dissolved in saline.

Results

Bradykinin exposures. After 20 second, nose only exposures to 0.01, 0.1 and 1 mM bradykinin in guinea-pigs, there was no significant decrease in sGaw from baseline (data not shown). Similarly, when guinea-pigs were pretreated with captopril (1mg/kg, i.p.), there was no significant reduction in sGaw after 0.01 and 0.1 mM bradykinin exposure. However, there was a significant decrease in sGaw after a 1 mM bradykinin exposure of captopril treated guinea-pigs (Fig. 1A). After captopril (1 mg/kg) treatment, a 0.3 mM bradykinin exposure produced modest decreases in sGaw after 20 (-8.7 ± 7.0 %) and 40 (-5.9 ± 6.3 %) second exposures and a significant decrease (p<0.05) after a 60 s (-17.9 ± 6.4 %) exposure (Fig. 1B).

After phosphoramidon (0.1 mg/kg, i.p.) there was no significant decrease (-3.8 ± 4.4%) in sGaw after a 1 mM bradykinin exposure (p>0.05), but after a 10 mM, 20 min inhalation exposure of phosphoramidon, bradykinin (1 mM) caused a significant decrease (21.7 ± 3.2%) in sGaw (p<0.05)(Fig. 1C). After combined phosphoramidon (10 mM, 20 min inhalation exposure) and captopril (1 mg/kg i.p.) there was a significant 22.3 ± 3.2% decrease (p<0.05) in sGaw after a 1 mM bradykinin exposure. However, this response was no greater than with the same doses of phosphoramidon or captopril alone (Fig. 1C).
Icatibant (10 µM) by 20 min inhalation exposure had no effect on the decrease in sG\textsubscript{aw} seen after a 1 mM bradykinin exposure in captopril (1 mg/kg, i.p.) treated guinea-pigs (Fig. 2A). MEN16132 (30, 100 and 300 nM/kg) administered by i.p. injections failed to block the decrease in sG\textsubscript{aw} seen after a 1 mM bradykinin exposure in captopril (1 mg/kg, i.p.) treated guinea-pigs (Fig. 2B). The bronchoconstrictor responses (-11.1 ± 4.3, -13.6 ± 4.3 and -11.2 ± 7.1%) were not significantly different (p>0.05) from the saline control (-17.3 ± 2.4%). After MEN16132 (1 and 10 µM) by a 20 min inhalation exposure, however, there was complete blockade of the decrease in sG\textsubscript{aw} seen after a 1 mM bradykinin exposure in captopril (1 mg/kg, i.p.) treated guinea-pigs (Fig. 2C).

**Bronchoalveolar lavage.** In animals receiving no treatment, the numbers of macrophages, eosinophils, neutrophils and lymphocytes in the BALF were as follows; total cells: 1.95 ± 0.09 (×10\textsuperscript{6}/ml), macrophages: 1.68 ± 0.09 (×10\textsuperscript{6}/ml), eosinophils: 0.20 ± 0.04 (×10\textsuperscript{6}/ml), neutrophils 0.04 ± 0.03 (×10\textsuperscript{6}/ml) and lymphocytes: 0.03 ± 0.01 (×10\textsuperscript{6}/ml) (Fig. 3). In animals treated with captopril (1 mg/kg, i.p.) which received 1 mM bradykinin prior to lavage there was no significant increase (p<0.05) to any of the cell types (Fig. 3). However, in animals treated with captopril (1 mg/kg, i.p.) and phosphoramidon (10 mM, 20 min inhalation exposure) which received a 1 mM bradykinin inhalation exposure prior to lavage there were significant (p<0.05) increases in total cells, macrophages, eosinophils and neutrophils compared to the same experiment without phosphoramidon. There was no increase in lymphocytes (Fig. 3).
In animals treated with captopril (1 mg/kg, i.p.), phosphoramidon (10 mM, 20 min inhalation exposure), the increases in cells due to 1 mM bradykinin inhalation were blocked by MEN16132 (10 µM, 20 min inhalation exposure). Total cells, eosinophils, neutrophils and lymphocytes were significantly reduced \( (p<0.05) \), with a modest decrease in macrophages, compared to bradykinin exposure without MEN16132 (Fig. 3).

**Ovalbumin sensitized animals.** In ovalbumin-sensitized guinea-pigs, ovalbumin exposure caused an immediate \( 25.5 \pm 4.5\% \) decrease in \( sG_{aw} \) from baseline. After 4 h there was a partial recovery, which was followed by a secondary \( 19.6 \pm 8.3\% \) decrease in \( sG_{aw} \) at 8 h (Fig. 4A). In ovalbumin-sensitized guinea-pigs before ovalbumin exposure, there was no significant decrease in \( sG_{aw} \) from baseline levels after a 1 mM bradykinin exposure (Fig. 4B). 24 h after ovalbumin exposure of sensitized guinea-pigs, however, there was a significant decrease in \( sG_{aw} \) after a 1 mM bradykinin exposure \( (p<0.05) \) (Fig. 4B).

In these animals, where inhaled bradykinin (1 mM, 20 s) was used to test for reactivity 24 h after ovalbumin challenge, there were significant increases \( (p<0.05) \) in total cells, macrophages, eosinophils, neutrophils and lymphocytes compared to saline challenged ovalbumin sensitized and histamine-exposed guinea-pigs (Fig. 4C). The numbers of macrophages, eosinophils, neutrophils and lymphocytes in the BALF of saline-challenged animals were as follows; total cells: \( 4.38 \pm 0.42 \times 10^{6}/\text{ml} \), macrophages: \( 2.31 \pm 0.22 \times 10^{6}/\text{ml} \), eosinophils: \( 1.95 \pm 0.34 \times 10^{6}/\text{ml} \), neutrophils \( 0.03 \pm 0.01 \times 10^{6}/\text{ml} \) and lymphocytes: \( 0.1 \pm 0.02 \times 10^{6}/\text{ml} \) (Fig. 4C). Compared to guinea-pigs in which histamine was used to test for reactivity 24h after the ovalbumin challenge, the levels of macrophages after
the bradykinin reactivity test were significantly less (29.9 ± 10.1%) with modest decreases in total cells and eosinophils and small increases in neutrophils and lymphocytes (Fig. 4C).

**Effects of PIV-3 inoculation.** Prior to inoculation with virus, there was no significant decrease in sGaw following inhalation exposure to histamine (1 mM, 20 s nose only) (Fig. 5A). After viral inoculation (3.16 x 10^8 infectious units per ml), in the saline treated guinea-pigs, there was a significant 31.5 ± 6.3% reduction (p<0.05) in sGaw value immediately after histamine exposure compared to the baseline value. sGaw recovered after 5 and 10 min (Fig. 5A). In animals receiving no virus, but the medium only, the numbers of macrophages, lymphocytes, eosinophils and neutrophils in the BALF were as follows; total cells: 1.54 ± 0.17 (×10^6/ml), macrophages: 1.37 ± 0.15 (×10^6/ml), eosinophils: 0.12 ± 0.01 (×10^6/ml), lymphocytes: 0.06 ± 0.02 (×10^6/ml) and neutrophils 0.03 ± 0.01 (×10^6/ml) (Fig. 6). In PIV-3 virus inoculated guinea-pigs treated with saline (daily for 8 days, 20 min exposures), there were significant increases (p<0.05) in total cells (55.0 ± 6.4%), macrophages (51.3 ± 6.7%), eosinophils (69.1 ± 7.3%) and neutrophils (66.3 ± 8.8%) and a modest increase in lymphocytes compared with medium controls.

**Effects of MEN16132 and VA999024 on PIV-3 responses.** Treatment of guinea-pigs with MEN16132 (10 µM daily for 8 days, 20 min inhalation exposures) abolished the bronchoconstriction to histamine after viral inoculation as there was only a -5.6 ± 4.6% reduction in sGaw after histamine exposure (Fig. 5B). After MEN16132 (10 µM daily for 8 days, 20 min inhalation exposures), there were significant decreases (p<0.05) in total cells
(29.3 ± 5.5%), macrophages (29.5 ± 7.3%), neutrophils (42.1 ± 25.2%) and lymphocytes (43.9 ± 8.4%) and a modest decrease in eosinophils (Fig. 6).

VA999024 (1, 3 and 10 mg/kg s.c.) was also examined in animals inoculated with PIV-3 (6.32 x 10^6 infectious units per ml). Prior to inoculation of guinea-pigs with PIV-3, there was no significant decrease in sG_{aw} following histamine inhalation (Fig. 7A). In PIV-3 inoculated guinea-pigs treated with saline (s.c.) for 8 days, there was a significant bronchoconstriction to histamine seen as a significant reduction in the peak sG_{aw} value at 10 min (-13.5 ± 3.5%) after histamine exposures (Fig. 7A). After treatment of PIV-3 inoculated animals with VA999024 (1 mg/kg s.c.), there was still a significant bronchoconstriction (p<0.05) to histamine (-19.5 ± 4.2% reduction in sG_{aw}). However, after treatment with higher doses of 3 and 10 mg/kg, there was no significant bronchoconstriction to histamine (Fig. 7A). The bronchoalveolar lavage of animals receiving no virus (medium controls) showed the numbers of macrophages, lymphocytes, eosinophils and neutrophils in the BALF as follows; total cells: 1.54 ± 0.17 (×10^6/ml), macrophages: 1.37 ± 0.15 (×10^6/ml), eosinophils: 0.12 ± 0.01 (×10^6/ml), lymphocytes: 0.06 ± 0.02 (×10^6/ml) and neutrophils 0.03 ± 0.01 (×10^6/ml) (Fig. 7B). In guinea-pigs inoculated with PIV-3 (6.32 x 10^6) and treated with saline for 8 days, there were significant increases (p<0.05) in total cells, macrophages, eosinophils and lymphocytes (Fig. 7B). These increases in leukocytes were inhibited in a dose-related manner by treatment with VA999024, the highest dose producing significant reductions in total cells, macrophages and lymphocytes (Fig. 7B).
Discussion

Bradykinin inhalation exposures up to 1mM produced no significant bronchoconstriction in conscious guinea-pigs. This contrasts with observations made in anaesthetized guinea-pigs where intravenous (Wirth et al., 1993; Tramontana et al., 2001; Ichinose and Barnes, 1990a), intratracheal (Ichinose and Barnes, 1990b) or inhaled (Sakamoto et al., 1994) bradykinin exerted bronchoconstriction. The lack of effect of inhaled bradykinin in conscious guinea-pigs appears to be due to the rapid breakdown of bradykinin as there was a significant bronchoconstriction after treatment with captopril, an ACE inhibitor. ACE (also known as kininase II) and NEP catalyse the breakdown of bradykinin into inactive metabolites, while kininase I produces the active metabolite [des-Arg⁹]-bradykinin (Decarie et al., 1996). [des-Arg⁹]-bradykinin has about 4-fold selectivity of binding to B₁ receptors over B₂ receptors (Leeb-Lundberg et al., 2005) but does not cause any bronchoconstriction in asthmatic or normal subjects (Polosa and Holgate, 1990), suggesting that B₁ kinin receptors are not involved in the bronchoconstriction. ACE mediates the breakdown of bradykinin in the circulation and the lung endothelial cells are a rich source of this enzyme, as are the airways (Dusser et al., 1988).

Intraperitoneal treatment with phosphoramidon had no effect but after inhalation exposure there was a bronchoconstriction to bradykinin similar to that seen after captopril treatment. NEP is expressed in the respiratory epithelium (Baraniuk et al., 1995), so inhalation exposure would enable phosphoramidon to have an instant effect, whereas after i.p. injection the drug may not be reaching the lung in a concentration sufficient to inhibit NEP. Captopril and phosphoramidon were shown to potentiate the bronchoconstriction by airways-instilled
bradykinin in anesthetized guinea-pigs (Ichinose and Barnes, 1990b) and intravenous bradykinin in conscious guinea-pigs (Chodimella et al., 1991). The kininase I inhibitor, DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid (MGTA), failed to alter the bronchoconstriction in both these studies. This suggested that both ACE and NEP degrade bradykinin but kininase I is not involved. In the present study, when capropril and phosphoramidon treatment were combined, the bronchoconstriction by bradykinin was similar to that seen with treatment with each drug individually. A similar observation was made by Ichinose et al. (1990). This could be because the maximum bronchoconstriction to bradykinin has been reached. It was interesting, however, that there was also no prolongation of the bronchoconstriction when the two inhibitors were combined.

Thus, either ACE or NEP had to be inhibited to prevent bradykinin breakdown before a bronchoconstriction to inhaled bradykinin could be observed in conscious guinea-pigs. Kininase I is probably not involved in the metabolism of inhaled bradykinin as this enzyme is localized to the blood (Proud and Kaplan, 1988). However, a second kininase I-type enzyme, carboxypeptidase M, which is also inhibited by MGTA, is highly expressed in the lungs (Nagae et al., 1993) including guinea-pig lungs (Chodimella et al., 1991). The lack of effect of MGTA alone, however, suggests that neither of these enzymes is involved in the breakdown of bradykinin. The mechanism of the bronchoconstriction by the inhaled route is probably via a neural mechanism. Atropine, to block parasympathetic muscarinic pathways and capsaicin, to deplete sensory neuron tachykinins, blocked the responses in anesthetized guinea-pigs to instilled bradykinin but not intravenous bradykinin (Ichinose et al., 1990). Thus, it is possible that the potentiation of inhaled bradykinin by ACE and NEP inhibitors was also due to
enhancement of tachykinins, since these are also susceptible to breakdown by these enzymes (Dusser et al., 1988). Intravenous bradykinin mediates bronchoconstriction mainly by release of cyclooxygenase products, since it was attenuated by indomethacin (Ichinose et al., 1990).

The B₂ kinin receptor antagonist MEN16132 (1 and 10µM inhalation exposure) blocked the bronchoconstriction to 1mM bradykinin exposure indicating the involvement of B₂ kinin receptors rather than B₁ receptors. Icatibant (10µM inhalation exposure), however, had no effect. These results are similar to those by Valenti et al. (2005), who have shown that MEN16132 is more potent and longer lasting than icatibant in inhibiting the bradykinin-induced bronchoconstriction and microvascular leakage in anaesthetized guinea-pigs. Our result differs from those of Wirth et al. (1993), however, who found that both aerosolized and intravenous icatibant inhibited the bronchoconstriction to bradykinin in anaesthetized guinea-pigs. It is difficult to compare our dose of icatibant with those used by Wirth et al. (1993) since they delivered directly into the lungs whereas our delivery was via box exposure. Thus, mode of delivery and the fact that our animals were conscious may explain the discrepancy. MEN16132 administered i.p. had no effect in our study, possibly because the drug was not absorbed by this route and did not reach the lungs in enough concentration to have an effect, although in the study of Valenti et al. (2005) it was effective by intravenous administration.

After bradykinin inhalation exposure with captopril treatment, there was no significant increase in inflammatory cells in the lung but after inhalation exposure with both captopril and phosphoramidon there were significant increases in total cells, macrophages, eosinophils and neutrophils. This suggests that the threshold for leucocyte influx was higher than for
bronchoconstriction, since both degradative enzymes were required to be blocked. After treatment with the B\textsubscript{2} receptor antagonist MEN16132 (10µM inhalation exposure), the increases in total cells, eosinophils, neutrophils and lymphocytes were significantly inhibited and there was a modest inhibition of the macrophage increase. This study shows that bradykinin can produce an influx of inflammatory cell but only after its metabolism in the lung is prevented by inhibition of both ACE and NEP. Although bradykinin is not thought to have much direct effect on activation and recruitment of inflammatory cells, it is thought to work indirectly through the release of monocyte and neutrophil chemotactic activity from the airway epithelial cells (Koyama et al., 1995), to cause release of neutrophil, monocyte and eosinophilic factors from alveolar macrophages (Sato et al., 1996) and also to stimulate eotaxin release from human lung fibroblasts (Sato et al., 2000). The inhibition of the bradykinin-induced inflammatory cell influx by MEN16132 indicates that it is mediated through the bradykinin B\textsubscript{2} receptor.

Bradykinin exposure had no effect in the absence of enzyme inhibitors in guinea-pigs that were sensitized but not challenged with ovalbumin. Thus, sensitization alone is not sufficient to induce a bronchoconstriction to bradykinin. This contrasts with another indirect bronchoconstrictor, adenosine, which shows no bronchoconstriction in normal subjects (Cushley et al., 1983) and unsensitized guinea-pigs (Smith and Broadley, 2008) but produces a bronchoconstriction in asthmatic subjects and sensitized guinea-pigs. However, in sensitized guinea-pigs that received an ovalbumin exposure at 24 h after challenge there was a significant bronchoconstrictor response to bradykinin. This was achieved without inhibition of ACE or NEP. This indicates that ovalbumin challenge induces airway hyperreactivity to bradykinin.
Since ovalbumin challenge causes airway hyperreactivity to other directly acting spasmogens, including histamine and methacholine (Toward and Broadley, 2004; Smith and Broadley, 2007), these results demonstrate that the hyperreactivity is extended to the indirect spasmogen, bradykinin. Whether this is because of a common mechanism cannot be deduced from the present study. It is likely due to epithelial damage caused by the ovalbumin challenge. This would expose the sensory nerves through which bradykinin acts after inhalation (Ichinose et al., 1990) or result in loss of the epithelium-derived NEP and ACE. The hyperreactivity could also arise from a loss of an epithelial derived relaxing factor, such as NO, which can be released by bradykinin (Ricciardolo et al., 1994). If this opposing relaxing component is lost, it would allow the bronchoconstrictor effects of bradykinin to emerge. Allergen-induced airways hyperreactivity to inhaled bradykinin does not appear to have been demonstrated previously in guinea-pigs, although it has been demonstrated in Brown Norway rats after intravenous administration (Ellis et al., 2004). These results therefore provide a basis for the appearance of bronchoconstriction in asthmatics but not in normal subjects after bradykinin inhalation (Polosa and Holgate, 1990).

Inoculation of guinea-pigs with parainfluenza-3 virus caused AHR to inhaled histamine and inflammatory cell influx into the BALF, in agreement with previous studies (Toward et al., 2005; Folkerts et al., 2000). The B2 receptor antagonist, MEN16132, and the tissue kallikrein inhibitor, VA999024 (Evans et al., 1996), abolished these effects after PIV-3 inoculation. The inhibition of the PIV-3-induced inflammatory cell influx with MEN16132 and VA999024 shows that bradykinin is involved in the virus-induced inflammation and that it is mediated through B2 kinin receptors. Folkerts et al. (2000) also showed that the B2 receptor antagonist,
Icatibant, administered subcutaneously inhibited the AHR to intravenously administered histamine in anaesthetized guinea-pigs treated with PIV-3. However, they did not show any reduction in cell influx after icatibant. This discrepancy could be due to the different route of delivery of MEN16132 in our study or the fact that this compound has greater efficacy when administered locally to the lungs. In their study, there was a tendency for an increase in bradykinin levels in BALF after PIV-3 infection, which supports the idea that bradykinin is involved in the AHR and cell influx caused by PIV-3 infection. In experiments using histamine to test for AHR, it is of interest that the histamine challenge may itself release bradykinin. Thus, inhibition of the AHR to histamine by MEN16132 could have been due to blockade of released bradykinin rather than the underlying bradykinin-mediated AHR. However, this would require that the bronchoconstrictor responses to histamine were entirely due to bradykinin release and clearly this is very unlikely as inhaled bradykinin does not cause bronchoconstriction unless its breakdown is inhibited.

In summary, this study has demonstrated a bronchoconstriction and influx of inflammatory cells in conscious guinea-pigs after inhaled bradykinin. However, these responses only occur when breakdown of bradykinin is inhibited by blockade of ACE and NEP. These responses are mediated via B₂ kinin receptors since they were antagonised by inhaled MEN16132. Neither intraperitoneal MEN16132 nor inhaled icatibant antagonized bradykinin, indicating the importance of the route of injection of these agents in bradykinin research. Sensitization of guinea-pigs is not sufficient to induce airways hyperreactivity to the bronchoconstriction by inhaled bradykinin. However, ovalbumin challenge of sensitized guinea-pigs causes AHR to bradykinin as well as to histamine and methacholine. Viral infection of guinea-pigs with PIV-3
also produces AHR to inhaled histamine and influx of inflammatory cells. These responses are attenuated by the B$_2$ receptor antagonist, MEN16132 by inhalation, and the tissue kallikrein inhibitor, VA999024, suggesting that bradykinin is involved in virus-induced inflammatory cell influx and AHR.

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**Footnotes**

We are grateful to Vantia Ltd, Southampton, UK for financial support.
Legends for Figures

**Fig. 1.** Effects of inhaled bradykinin on $sG_{aw}$ in conscious guinea-pigs after treatment with captopril or phosphoramidon. **A.** Effects of 0.1, 0.3 and 1 mM bradykinin exposures for 20 s after treatment with captopril (1 mg/kg, i.p.). **B.** Effects of 20, 40 and 60 s, 0.3 mM bradykinin exposures after captopril (1 mg/kg, i.p.) treatment. **C.** Effect of phosphoramidon (10 µM, 20 min inhalation exposure) and combining phosphoramidon with captopril (1 mg/kg, i.p. on bradykinin (1 mM, 20 s exposure). Each point represents the mean ± S.E.M. (n=6) change in $sG_{aw}$ expressed as a percentage of the baseline $sG_{aw}$ values. Negative values represent bronchoconstriction. * indicates a significant ($p<0.05$) difference from baseline $sG_{aw}$ after bradykinin exposure, and # indicates a significant ($p<0.05$) difference between the changes in $sG_{aw}$ values with and without phosphoramidon or captopril treatment as determined by ANOVA followed by a Dunnet’s post hoc test.

**Fig. 2.** Effects of bradykinin B2 antagonists icatibant and MEN6132 on bronchoconstrictor responses to bradykinin (1 mM, 20 s exposures) after treatment with captopril (1 mg/kg, i.p.). **A.** Effects of icatibant (10 µM, 20 min inhalation exposure) or saline (20 min inhalation exposure). **B.** Effects of MEN16132 (30, 100 and 300 nmol/kg, i.p.) or saline (i.p.). **C.** Effects of MEN16132 (1 and 10 µM by 20 min inhalation exposures) or saline (20 min inhalation exposure). Each point represents the mean ± S.E.M. (n=6) change in $sG_{aw}$ expressed as a percentage of the baseline $sG_{aw}$ values. Negative values represent bronchoconstriction. * denotes a significant ($p<0.05$) difference between the changes in baseline $sG_{aw}$ value with and
without MEN16132 treatment as determined by ANOVA followed by a Dunnett’s *post hoc* test.

**Fig. 3.** Total and differential cell (macrophage, eosinophil, neutrophil and lymphocyte) counts of BALF removed from guinea-pigs receiving no treatment or receiving bradykinin (1 mM, 20 s exposure) with captopril (1 mg/kg, i.p.) with or without 10 mM phosphoramidon. The final group received phosphoramidon and 10 µM MEN16132. Each bar represents the mean ± S.E.M. (n=6) of total or differential count of cells (x10⁶ per ml). * denotes a significant (*p*<0.05) difference compared to the corresponding group without phosphoramidon and + denotes a significant (*p*<0.05) difference compared to the group without MEN16132 as determined by ANOVA followed by a Dunnett’s *post hoc* test.

**Fig. 4.** Effect of bradykinin in ovalbumin-sensitized guinea-pigs. A. Guinea-pigs were exposed to ovalbumin (60 min, (0.01%) and changes in sGₐw were monitored for 12 h and at 24 h. B. Changes in sGₐw in response to bradykinin (1 mM, 20 s exposure) measured 24 h before and 24 h after the inhalation exposure to ovalbumin. * denotes a significant (*p*<0.05) difference between the changes to baseline sGₐw values before and after ovalbumin exposure, as determined by a Student’s *t*-paired test.. In A and B, each point represents the mean ± S.E.M. (n=6) change in sGₐw expressed as a percentage of the baseline sGₐw values. Negative values represent bronchoconstriction. In A, the separate graph on the right shows the mean ± S.E.M. peak falls in sGₐw occurring between 0-6, 7-12 and at 24 h. C. Total and differential cell (macrophage, eosinophil, lymphocyte and neutrophil) counts in BALF removed from ovalbumin sensitized and challenged animals. Each point represents the mean ± S.E.M. (n=6)
of total or differential count of cells (x10⁶ per ml). * denotes a significant (p<0.05) difference compared to the corresponding saline challenged and histamine-exposed group. + denotes a significant (p<0.05) difference compared to the ovalbumin challenged and histamine exposed group, as determined by ANOVA followed by a Dunnett’s post hoc test.

**Fig. 5.** Effect of histamine (1 mM 20 s nose only exposure) on airway function before and after PIV-3 virus (3.16x10⁶ infectious units per ml) instillation in A. saline (20 min inhalation exposures) treated guinea-pigs and B. MEN16132 (10 µM, 20 min inhalation exposures) treated guinea-pigs. Each point represents the mean ± S.E.M. (n=6) change in sGaw expressed as a percentage of the baseline sGaw values. Negative values represent bronchoconstriction. * denotes a significant (p<0.05) difference between the changes from baseline sGaw values before and after virus instillation, as determined by a Student’s paired t-test.

**Fig. 6.** Total and differential cell (macrophage, eosinophil, lymphocyte and neutrophil) count in BALF removed from virus free medium inoculated animals or PIV-3 inoculated animals treated with saline or MEN16132 (10 µM, 20 min exposure for 4 days). Each point represents the mean ± S.E.M. (n=6) of total or differential count of cells (x10⁶ per ml). * denotes a significant (p<0.05) difference compared to the corresponding no virus control. + denotes a significant (p<0.05) difference compared to the corresponding saline group, as determined by ANOVA followed by a Dunnett’s post hoc test.

**Fig. 7.** Effects of treatment of guinea-pigs with VA999024 (1, 3 and 10 mg/kg s.c.) or saline on A. airway responses to histamine (1 mM 20 s nose only exposure) before and after PIV-3 virus
(6.32 x 10^6 infectious units per ml) instillation, and B. total and differential cell (macrophage, eosinophil, lymphocyte and neutrophil) counts in BALF removed from virus-free medium-inoculated animals or PIV-3 inoculated animals treated with saline or VA999024. Responses to histamine are the mean ± S.E.M. (n=6) peak changes in sGaw expressed as a percentage of the baseline sGaw values. Negative values represent bronchoconstriction. * denotes a significant (p<0.05) difference between the changes in the maximum decrease in sGaw from baseline before and after viral inoculation, as determined by a Student’s paired t-test. Total or differential cell counts are the mean ± S.E.M. (n=6) cells (x10^6 per ml) in medium-treated controls and in virus-inoculated guinea-pigs treated with saline or VA999024. * denotes a significant (p<0.05) difference compared to the corresponding medium control. + denotes a significant (p<0.05) difference compared to the corresponding saline group, as determined by ANOVA followed by a Dunnett’s post hoc test.
Figure 1

A

- Captopril 1mg/kg, bradykinin 0.1mM
- Captopril 1mg/kg, bradykinin 0.3mM
- Captopril 1mg/kg, bradykinin 1mM

B

- Bradykinin 20 second exposure
- Bradykinin 40 second exposure
- Bradykinin 60 second exposure

C

- Saline
- Phosphoramidon 0.1mg/kg i.p.
- Phosphoramidon 10mM inhalation
- Phosphoramidon 10mM inhalation and Captopril 1mg/kg
**Figure 2**

**A**

- Captopril 1mg/kg, saline
- Captopril 1mg/kg, icatibant 10μM

**B**

- Captopril 1mg/kg, saline
- Captopril 1mg/kg, MEN16132 30nmol/kg
- Captopril 1mg/kg, MEN16132 100nmol/kg
- Captopril 1mg/kg, MEN16132 300nmol/kg

**C**

- Captopril 1mg/kg with saline
- Captopril 1mg/kg with MEN16132 1μM
- Captopril 1mg/kg with MEN16132 10μM
Figure 3

- No treatment
- Bradykinin 1mM, Captopril 1mg/kg
- Bradykinin 1mM, Captopril 1mg/kg, Phosphoramidon 10mM
- Bradykinin 1mM, Captopril 1mg/kg, Phosphoramidon 10mM, MEN16132 10μM

Million cells per ml

- Total cell count
- Macrophages
- Eosinophils
- Neutrophils
- Lymphocytes
Figure 4

A

Ovalbumin Exposure

Time (mins)

% sGAW

-40

-30

-20

-10

0

10

20

30

0min

15min

30min

45min

1hr

2hr

3hr

4hr

5hr

6hr

7hr

8hr

9hr

10hr

11hr

12hr

13hr

14hr

15hr

B

Pre-ovalbumin exposure

Post-ovalbumin exposure

% sGAW

baseline

0mins

5mins

10mins

Time (mins)

C

Saline challenged/histamine exposed

OA challenged/histamine exposed

OA challenged/bradykinin exposed

Million cells per ml

Total cells

Macrophages

Eosinophils

Neutrophils

Lymphocytes

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Figure 5

A

Saline Treated

- After viral inoculation
- Before viral inoculation

B

MEN16132 Treated

- After viral inoculation
- Before viral inoculation

% change in sGaw vs. time (mins)

baseline 0 5 10 15 20

% change in sGaw
Figure 6

![Graph showing cell counts](image_url)

- Media control
- Virus and saline
- Virus and MEN16132 10μM

Legend:
- Million cells per ml
- Total cells
- Macrophages
- Eosinophils
- Lymphocytes
- Neutrophils

* indicates a significant difference from the media control group.
Figure 7

A

Changes in $s_{Gaw}$ from baseline

Before viral inoculation
After viral inoculation

* * *

B

Total cell count
Macrophages
Eosinophils
Lymphocytes
Neutrophils

Media control
Virus and saline
Virus and VA999024 1mg/kg
Virus and VA999024 3mg/kg
Virus and VA999024 10mg/kg

* * *

Saline
VA999024 1mg/kg
VA999024 3mg/kg
VA999024 10mg/kg

Million cells per ml

* *

* *