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Effects of wortmannin on airways inflammation induced by allergen in actively sensitised Brown Norway rats

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Abstract

We have investigated the effect of wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase, on the immediate-type allergic response and the late phase pulmonary inflammation induced by allergen challenge in the ovalbumin-sensitised Brown Norway rat. Intratracheal (i.t.) instillation of ovalbumin induced dose-related bronchoconstrictor responses. Administration of wortmannin (1, 10 or 100 μg kg^{-1} i.t., 1 h prior to challenge) induced a marked and dose-dependent inhibition of ovalbumin-induced bronchospasm (ED_{50} ca. 5 μg kg^{-1} i.t.). At similar doses, wortmannin also suppressed the bronchoconstrictor responses to 5-hydroxytryptamine and methacholine but the degree of blockade of these spasmogens (1.4–1.9-fold) was less than that of ovalbumin (>20-fold). Wortmannin, given intratracheally 1 h prior to allergen challenge, also suppressed the increases in bronchoalveolar lavage fluid leukocyte numbers and eosinophil peroxidase activity measured 24 h post challenge. However, relatively high doses were necessary (ED_{50} ca. 100 μg kg^{-1} i.t.). The potency of wortmannin was increased when dosed 1 h prior to and 24 h after allergen challenge and the readout was 48 h after challenge (ED_{50} 3–5 μg kg^{-1} i.t.). Thus, wortmannin is a potent inhibitor of the bronchoconstrictor response induced by allergen in the airways of actively sensitised Brown Norway rats. Inhibition of phosphatidylinositol-3-kinase, an obligatory step in mast cell activation in response to allergen, is the presumed mechanism of action. The fact that similar doses of wortmannin do not suppress the late response to allergen suggests a minimal role for the mast cell in generating the late response to allergen in this model. The striking increase in potency to inhibit the late response when dosed 1 h prior to and 24 h after allergen challenge with the readout taken at 48 h may represent an effect of wortmannin to suppress the migration of leukocytes.

Keywords: Allergic pulmonary inflammation; Brown Norway (rats); Ovalbumin; Phosphatidylinositol-3-kinase; Wortmannin

1. Introduction

Mast cells play a major role in allergy and inflammation (Wasserman, 1983; Galli, 1997; Metcalfe et al., 1997; Bakharevski and Ryan, 1999; Holt et al., 1999). Pivotal to the effector function of mast cells is their activation through the interaction of a multivalent antigen (allergen) with its specific immunoglobulin E (IgE) antibody bound to its high affinity receptor, FcεRI. Signalling through FcεRI is initiated by cross-linking of allergen bound to IgE which leads to mast cell degranulation and the release of mediators such as histamine, 5-hydroxytryptamine and proteases, the production of leukotrienes and prostaglandins and the release and synthesis of chemokines and cytokines (Church et al., 1997; Metcalfe et al., 1997). The evidence is compelling that mast cells coordinate and facilitate the development of allergic inflammation (Wasserman, 1983; Galli, 1997; Metcalfe et al., 1997).

Phosphatidylinositol-3-kinase is associated with, and activated by, a number of protein tyrosine kinases and plays an important role in signalling pathways initiated by several growth factor receptors (Vlahos, 1995; Shepherd et al., 1996; Cardenas et al., 1998). Phosphatidylinositol-3-kinase is also activated in mast cells following cross-linking of multivalent allergen bound to IgE and is involved in the signal transduction pathway responsible for degranulation and the release of bioactive mediators (Yano et al., 1993; Marquardt et al., 1996; Pendl et al., 1997; Bhattacharyya et al., 1998). Phosphatidylinositol-3-kinase is also involved in chemotaxis and the oxidative burst in leukocytes in response to a variety of stimuli (Arcaro and Wymann, 1993: Niggli and Keller, 1997; Lennartz, 1999: Rameh and Cantley, 1999; Miike et al., 2000).

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An essential factor implicating phosphatidylinositol-3-kinase in an obligatory role in inflammatory cell function is that the responses can be abolished by low concentrations of wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase (Ui et al., 1995; Cardenas et al., 1998). We report here the effect of wortmannin on the immediate-type allergic response and the late phase pulmonary inflammation induced by allergen challenge in an animal model of allergic asthma, the ovalbumin-sensitised Brown Norway rat.

A part of the results was presented at the meeting of the British Pharmacological Society held in July 1999 (Tigani et al., 1999).

2. Methods

2.1. Animals

Male Brown Norway rats weighing 200–300 g were supplied by Biological Research Laboratories (Füllinsdorf, Switzerland). They were kept at an ambient temperature of 22±2 °C under a 12-h normal phase light–dark cycle and fed on NAFAG pellets supplied by Nahr und Futtermittel, Gossau, Switzerland. Drinking water was freely available. All experiments were carried out with the approval of the Veterinary Authority of the City of Basel (Kantonales Veterinaeramt, Basel-Stadt).

2.2. Sensitisation procedure

Ovalbumin (20 μg ml^{-1}) was mixed (30 min on ice) in a blender (Polytron, Kinematica) with aluminium hydroxide (20 mg ml^{-1}) and injected (0.5 ml per animal s.c.) coincidentally with *Acullulare pertussis* adsorbat vaccine (0.2 ml per animal i.p.; diluted 1:4 with saline 0.9 % w v^{-1}). Injection of ovalbumin, together with adjuvant, was repeated 14 and 21 days later. Sensitised animals were used in experiments between days 28 and 35.

2.3. Measurement of lung function

Animals were anaesthetised with sodium pentothal (70 mg kg^{-1} i.p.) and a tracheotomy performed. Heparinised polyethylene catheters were inserted into the left carotid artery for recording mean arterial blood pressure and into the left jugular vein for drug administration. To suppress spontaneous respiration animals were given an intramuscular injection of vecuronium bromide (12 mg kg^{-1}). No experiment lasted longer than 90 min, during which time surgical anaesthesia was maintained without the need for supplementary anaesthesia. Body temperature was maintained at 37 °C with a heated pad controlled by a rectal thermistor.

Animals were ventilated (7 ml kg^{-1}, 1 Hz) via the tracheal cannula with a mixture of air and oxygen (50:50, v v^{-1}). Ventilation was monitored at the trachea by a pneumotachograph (Fleisch 0000, Zabona, Switzerland) in line with the respiratory pump and connected to a differential pressure transducer (MP 4514871, Validyne, USA). Coincident pressure changes within the thorax were measured via an intrathoracic cannula, using a differential pressure transducer (MP 4524, Validyne). From measurements of airflow and transpulmonary pressure, airway resistance (R_L, cm H_2O l^{-1} s^{-1}) was calculated after each respiratory cycle by use of a digital electronic pulmonary monitoring system (PMS, Mumed, London, UK). Mean arterial blood pressure (and heart rate by derivation) was recorded from the carotid artery by means of a pressure transducer (P23Dd, Gould, USA).

2.4. Bronchoalveolar lavage (BAL) fluid collection and analysis

Animals were killed with sodium pentobarbital (250 mg kg^{-1} i.p.). The lungs were lavaged using three aliquots (4 ml) of Hanks' balanced salt solution [(HBSS, ×10) 100 ml; ethylenediaminetetraacetic acid (EDTA), 100 mM, 100 ml; 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) 1 M, 10 ml; ddH_2O 790 ml]. The recovered solution was pooled (mean recovery 11.3±0.1 ml, $n=55$) and the total volume of recovered fluid adjusted to 12 ml by addition of HBSS.

The methods for the determination of total leukocyte numbers and differential cell counts, eosinophil peroxidase activity and protein concentration in the bronchoalveolar lavage fluid have been described in detail recently (Beckmann et al., 2001). In brief, leukocyte numbers and differential cell counts were obtained using an automatic cell analysing system (Cobas Helios 5Diff, Hoffmann-La Roche, Axon Lab, Switzerland). Eosinophil peroxidase activity was measured in a photometric assay based on the oxidation of *O*-phenylenediamine by eosinophil peroxidase in the presence of hydrogen peroxide. Protein concentrations were measured in a photometric assay based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent.

2.5. Experimental protocols

2.5.1. Studies on the acute response to allergen

Ovalbumin was administered intratracheally at doses of 3–60 mg kg^{-1}. Only one dose was given per animal. Wortmannin or vehicle was administered intratracheally 1 h prior to ovalbumin (60 mg kg^{-1}). To define the selectivity of action of wortmannin, the compound was given 1 h prior to the start of a sequence of intravenous (i.v.) injections of 5-hydroxytryptamine (3, 10 and 30 μg kg^{-1}, at 2-min intervals) followed 15 min later by methacholine (3, 10 and 30 μg kg^{-1}, at 2-min intervals).

2.5.2. Studies on the late response to allergen

The late response to allergen was quantified as the change in leukocyte numbers, protein concentration and eosinophil peroxidase activity in the bronchoalveolar lavage fluid. Animals were exposed using a nose only exposure system to an aerosol

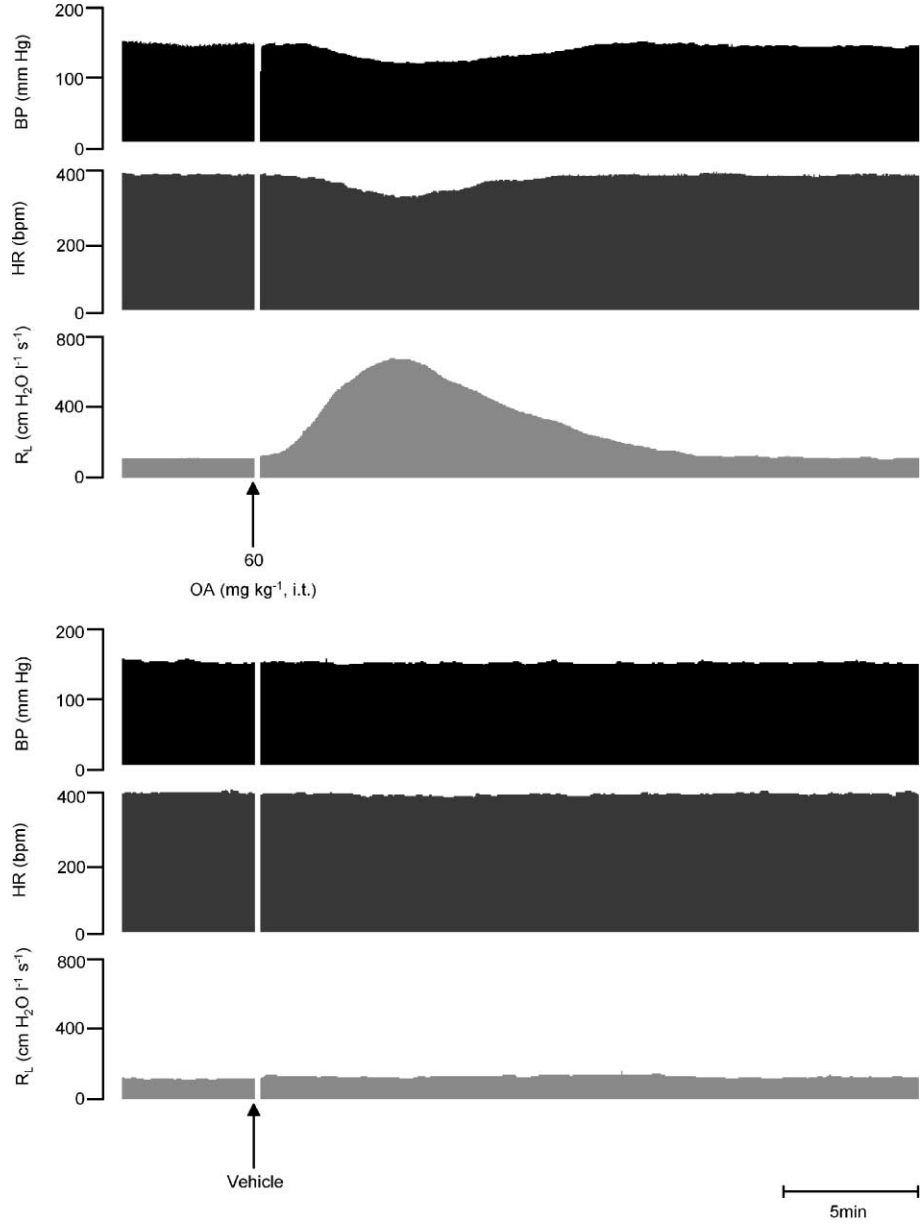

Fig. 1. Bronchoconstrictor and cardiovascular responses to intratracheal administration of ovalbumin (OA) in actively sensitised Brown Norway rats. Representative experimental tracings demonstrating the effects of intratracheal instillation of OA (60 mg kg^{-1}; upper panels) or vehicle (lower panels) on mean arterial blood pressure (BP), heart rate (HR) and airway resistance (R_L) in actively sensitised BN rats.

of ovalbumin (5 mg ml^{-1} for 60 min) generated by a Cirrhus nebuliser (DHD Medical Products, Canastota, USA). The dose administered was 0.4 mg kg^{-1} calculated using the formula:

$$\frac{f \times \text{minute volume [l]} \times \text{treatment time [min]} \times \text{concentration of compound in aerosol [mg l}^{-1}\text{]}}{\text{body weight [kg]}}.$$

The factor f refers to the assumed percentage retention of inhaled material in the lung, which was taken as 0.3.

Two dosing schedules with wortmannin were used. In the first, wortmannin was given intratracheally at doses of 1, 10 or 100 μg kg^{-1}, 1 h prior to ovalbumin challenge, and the animals were killed for bronchoalveolar lavage fluid analysis 24 h later. In the second, animals received a second dose of wortmannin 24 h after the ovalbumin challenge with the bronchoalveolar lavage fluid being collected 48 h later.

2.6. Materials

Aluminium hydroxide and EDTA were from Merck, Germany. HBSS and HEPES were obtained from Gibco BRL, UK. *A. pertussis* adsorbat vaccine was from the Vaccinal and Serotherapic Institute of Bern, Switzerland. Pentothal (thiopentalum natricum) was obtained from Abbott, Switzerland. Norcuron (vecuronium bromide) was from Organon Teknika, Holland. Ovalbumin was obtained from Fluka, Switzerland. Wortmannin ([1*S*-(1α,6*b*α,9*a*β,11α,11*b*β)]-11(acetyloxy)-1,6*b*,7,8,9*a*,10,11,11*b*-octahydro-1-(methoxymethyl)-9*a*,11*b*-dimethyl-3*H*-furo[4,3,2-de]indeno[4,5-*h*]-2-benzopyran-3,6,9-trione, 5-hydroxytryptamine creatinine sulphate and methacholine chloride were obtained from Sigma, CH. Wortmannin was dissolved at 10 mg ml^{-1} in DMSO and diluted with 0.9 % w v^{-1} NaCl to the solutions used for intratracheal administration (1, 10 or 100 μg ml^{-1}). The vehicle was 0.9 % w v^{-1} NaCl containing 1% DMSO. The volume of solution administered was 0.2 ml. All other compounds were prepared in 0.9 % w v^{-1} NaCl (pyrogen-free).

2.7. Data analysis

All data are presented as means ± S.E.M. Statistical analysis was performed on raw data by means of Student's *t* test for paired data or analysis of variance with post hoc pairwise multiple comparison procedures, using SigmaStat for Windows, version 2.03. A P value <0.05 was considered significant.

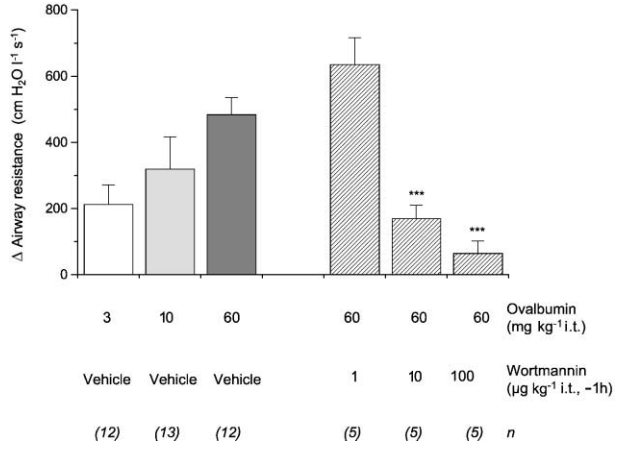

Fig. 2. Bronchoconstrictor responses to intratracheal administration of ovalbumin in actively sensitised Brown Norway rats. Effect of pretreatment with wortmannin. Columns represent mean incremental increase in airway resistance values (±S.E.M.) of the number of animals (*n*) shown in parentheses. *** $P<0.001$ indicates a significant difference between vehicle- and wortmannin-treated animals that received 60 mg kg^{-1} ovalbumin. There were no significant differences in mean baseline airway resistance values between groups or vehicle- and wortmannin-treated animals.

3. Results

3.1. Acute bronchoconstrictor responses to intratracheal administration of ovalbumin in actively sensitised Brown Norway rats: effect of pretreatment with wortmannin

Intratracheal (i.t.) instillation of ovalbumin (3–60 mg kg^{-1}) induced dose-related bronchoconstrictor responses, which peaked at 5 min and resolved within 15 min (Fig. 1). Pretreatment with wortmannin (1, 10 or 100 μg kg^{-1} i.t., 1 h prior to challenge) induced a dose-dependent inhibition of the bronchospasm induced by ovalbumin (60 mg kg^{-1}). The ED_{50} was approximately 5 μg kg^{-1} (Fig. 2). By interpolation from the ovalbumin dose–response relationship, the degree of blockade by wortmannin (10 μg kg^{-1}) was >20-fold.

To define the selectivity of action of wortmannin, its effects on the bronchoconstrictor responses to intravenously administered 5-hydroxytryptamine and methacholine were measured. Wortmannin (10 and 100 μg kg^{-1} given intratracheally, 1 h prior to starting the bronchospasm sequence) reduced dose-dependently the responses to 5-hydroxytryptamine and methacholine. Following the 10-μg kg^{-1} dose of wortmannin, the degree of blockade of 5-hydroxytryptamine and methacholine, interpolated from the control (i.e.

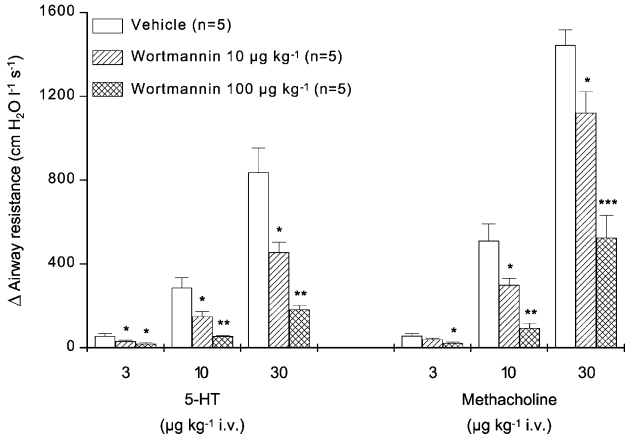

Fig. 3. Bronchoconstrictor responses to intravenous administration of 5-hydroxytryptamine (5-HT) and methacholine in actively sensitised Brown Norway rats. Effect of pretreatment with wortmannin. Columns represent mean incremental increase in airway resistance values (±S.E.M.) of the number of animals (n) shown in parentheses. Wortmannin was given intratracheally at the doses indicated 1 h prior to the start of the bronchospasmogen injection sequence. $P<0.05$, ** $P<0.01$, *** $P<0.001$ that the value is significantly different from the equivalent value in the vehicle-treated group.

vehicle-treated) dose–response curve, were 1.9- and 1.4-fold, respectively (Fig. 3).

3.2. Allergen-induced inflammatory cell infiltration and activation in the lungs of actively sensitised Brown Norway rats: effect of pretreatment with wortmannin

Challenge with ovalbumin (5 mg ml^{-1} aerosol for 60 min; calculated dose 0.4 mg kg^{-1}) led to an inflammatory response in the airways of sensitised Brown Norway rats when assessed by changes in the bronchoalveolar lavage fluid leukocyte numbers, eosinophil peroxidase activity and protein concentration measured 24 h (Fig. 4) or 48 h (Fig. 5) after challenge. Wortmannin at doses of 1 and 10 μg kg^{-1}, given intratracheally 1 h prior to allergen exposure, had no significant effects on any of the parameters of inflammation. However, following the highest dose of wortmannin (100 μg kg^{-1}), inflammatory cell numbers (eosinophils, macrophages, lymphocytes and neutrophils) and eosinophil peroxidase activity recovered from the bronchoalveolar lavage fluid of challenged animals were significantly reduced (Fig. 4). The ED_{50} for suppression of the aforementioned parameters was approximately 100 μg kg^{-1}. If the same doses of wortmannin were given twice, 1 h prior to and 24 h after allergen exposure, and the bronchoalveolar lavage fluid analysed 48 h later, inhibition of the parameters of inflammation was qualitatively similar, but occurred at substantially lower doses. The ED_{50} for suppression of all parameters was between 3 and 5 μg kg^{-1} given 1 h prior to and 24 h after allergen challenge (Fig. 5).

4. Discussion

Our results demonstrate potent inhibition by wortmannin of the acute early bronchoconstrictor response to ovalbumin in actively sensitised Brown Norway rats. At similar doses, wortmannin also inhibited the bronchoconstrictor responses to 5-hydroxytryptamine and methacholine. However, the degree of inhibition of these spasmogens (1.4–1.9-fold following the 10 μg kg^{-1} dose of wortmannin) was mark-

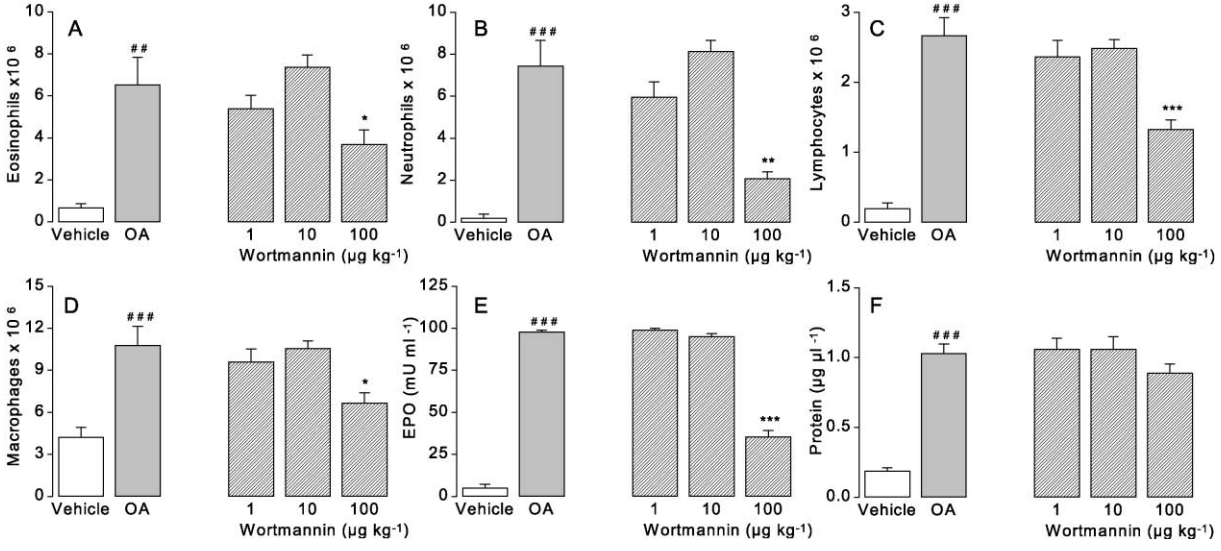

Fig. 4. Inflammatory cell infiltration and activation in the lungs of Brown Norway rats induced by ovalbumin (OA) challenge. Effect of pretreatment with wortmannin. The data (means±S.E.M.; $n=6–12$) show the effects of wortmannin on the changes in the numbers of eosinophils (A), neutrophils (B), lymphocytes (C), and macrophages (D) and eosinophil peroxidase (EPO) activity (E) and protein concentration (F) measured in bronchoalveolar lavage fluid of sensitised Brown Norway rats 24 h following ovalbumin challenge. Wortmannin was given at the doses indicated 1 h before challenge with aerosolised ovalbumin (5 mg ml^{-1} for 60 min; calculated dose 0.4 mg kg^{-1}). $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ indicates a significant difference from the animals challenged with vehicle. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ indicates significant difference from animals challenged with ovalbumin without wortmannin treatment.

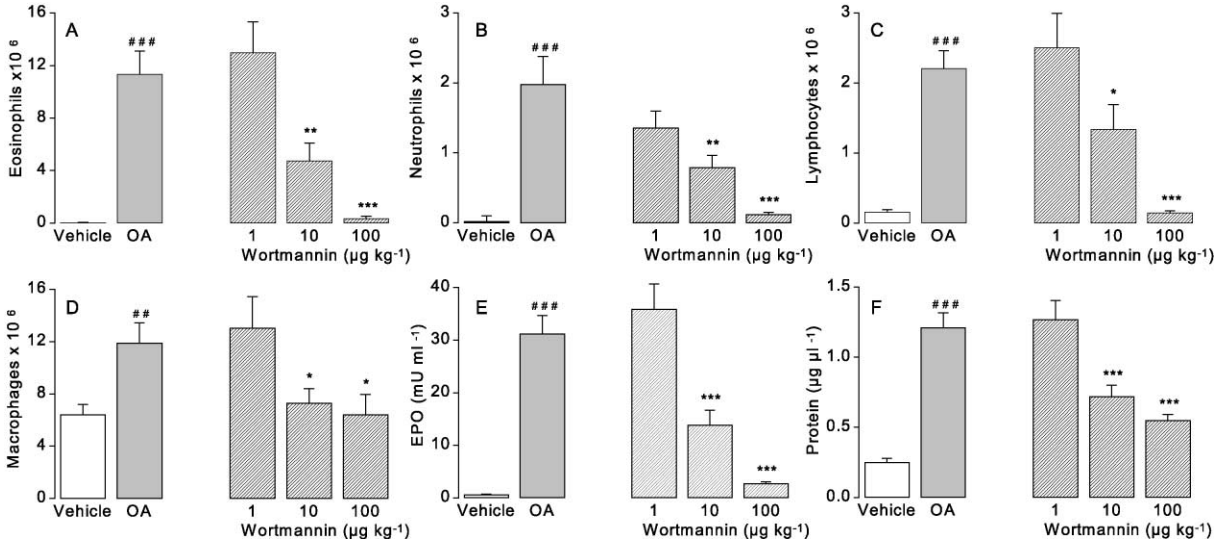

Fig. 5. Inflammatory cell infiltration and activation in the lungs of Brown Norway rats induced by ovalbumin (OA) challenge. Effect of pretreatment with wortmannin. The data (means ± S.E.M.; $n = 6–12$) show the effects of wortmannin on the changes in the numbers of eosinophils (A), neutrophils (B), lymphocytes (C), and macrophages (D) and eosinophil peroxidase (EPO) activity (E) and protein concentration (F) measured in bronchoalveolar lavage fluid of sensitised Brown Norway rats 48 h following ovalbumin-challenge. Wortmannin was given at the doses indicated 1 h before and 24 h after challenge with aerosolised ovalbumin (5 mg ml^{-1} for 60 min; calculated dose 0.4 mg kg^{-1}). $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ indicates a significant difference from the animals challenged with vehicle. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ indicates significant difference from animals challenged with ovalbumin without wortmannin treatment.

edly less than that of ovalbumin (>20-fold). Thus, non-selective suppression of bronchial contractility plays only a minor role in the inhibition of the acute response to allergen.

The acute bronchoconstrictor response to allergen in sensitised rat airways is a consequence of mast cell degranulation and the release of bronchoconstrictor mediators, in particular 5-hydroxytryptamine and leukotrienes (Elwood et al., 1994; Nagase et al., 1995, 1996; Du et al., 1996). It is, perhaps, therefore, not too surprising that the response could be blocked by low doses of wortmannin. Thus, phosphatidylinositol-3-kinase is activated following FCεRI stimulation of mast cells (Yano et al., 1993; Pendl et al., 1997) and in vitro studies have shown blockade of phosphatidylinositol-3-kinase with low concentrations of wortmannin to inhibit the immunologically triggered degranulation reaction and the release of bioactive mediators from mast cells (Yano et al., 1993; Marquardt et al., 1996; Pendl et al., 1997; Bhattacharyya et al., 1998). Our data demonstrate for the first time that the same phenomenon may occur in vivo.

When administered 1 h prior to allergen challenge, wortmannin also inhibited the late pulmonary inflammatory response to allergen, manifested as suppression of leukocyte influx into bronchoalveolar lavage fluid and a decrease in eosinophil peroxidase activity and protein concentration. However, these effects were seen only at doses much higher than those suppressing the acute bronchoconstrictor response to allergen. It cannot be ruled out that this reflects the different doses and means of administration used to elicit the acute response (i.t.) and the late response (nebulisation). However, the calculated dose delivered to the airways by nebulisation was 0.4 mg kg^{-1} and, consistent with this, the degree of inflammation in the present study was similar to that seen following intratracheal challenge with a similar dose of ovalbumin (0.32 mg kg^{-1}; Beckmann et al., 2001). This is much less than the dose used to induce the acute response (60 mg kg^{-1} i.t.). Thus, the greater potency of wortmannin against the acute response to allergen cannot be ascribed to suppression of a less powerful stimulus.

The obvious conclusion from our data is that manifestation of the late response may not be a consequence of the initial mast cell activation. However, mast cells store and produce a variety of proinflammatory chemokines and cytokines which could play a role in the development of the late inflammatory response to allergen (Church et al., 1997; Metcalfe et al., 1997). Moreover, the fact that nedocromil, a mast cell stabilising agent (Eady and Norris, 1997), blocks both the early and the late response to allergen challenge in sensitised Brown Norway rats (Du et al., 1996) would support this explanation. On the other hand, studies in mast cell-deficient mice suggest mast cell-independent mechanisms contribute to the development of eosinophilic inflammation in response to allergen (Hom and Estridge, 1994; Nagai et al., 1996; Mehlhop et al., 1997; Takeda et al., 1997). Thus, a site of action for wortmannin other than the mast cell should be considered.

At low concentrations, wortmannin can suppress leukocyte activation and the chemotactic response to various stimuli (Arcaro and Wymann, 1993: Niggli and Keller, 1997; Lennartz, 1999: Rameh and Cantley, 1999; Jones, 2000; Miike et al., 2000). Clearly, if manifested in vivo, such effects could contribute both to the suppression of leukocyte numbers and their secretion products in the bronchoalveolar lavage fluid. The data obtained with wort-

mannin administered 1 h before and 24 h after allergen challenge would support this reasoning. Thus, the influx of leukocytes and evidence of their activation and the increase in protein concentration in the bronchoalveolar fluid seen 48 h after ovalbumin challenge were inhibited by wortmannin given at doses close to those which suppress the early response to allergen.

Based on the potency and selectivity of wortmannin, it seems likely that inhibition of phosphatidylinositol-3-kinase is the basis of the potent effects on allergen-induced airway responses seen in these studies. Certainly the dose of wortmannin used to inhibit the acute and delayed responses to allergen is low (ED_{50} ca. 5 $\mu g\ kg^{-1}$ i.t.). This, plus the fact that wortmannin shows >50-fold selectivity for phosphatidylinositol-3-kinase over other mammalian kinases such as myosin-light chain kinase (Cardenas et al., 1998), strongly supports the above interpretation.

In conclusion, wortmannin is a potent inhibitor of both the immediate-type allergic response and the late-phase pulmonary inflammation induced by allergen challenge in ovalbumin-sensitised Brown Norway rats. The mast cell is the likely cellular target for inhibition of the acute response to allergen and mast cell-independent suppression of leukocyte migration may be the major factor contributing to suppression of the late response. Inhibition of phosphatidylinositol-3-kinase is the presumed mechanistic basis for the observed effects.

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