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Role of endogenous adenosine in the acute and late response to allergen challenge in actively sensitized Brown Norway rats

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1 Endogenous adenosine has been suggested to amplify the response of airway mast cells to allergen *in vivo*. We have sought evidence for this by monitoring the acute and late-phase response to allergen in Brown Norway (BN) rats actively sensitised to ovalbumin (OA) and treated either with adenosine deaminase (ADA) linked covalently to polyethylene glycol (PEG-ADA; Adagen®) to decrease adenosine availability or with erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), an inhibitor of ADA, plus *S*-(4-nitrobenzyl)-6-thioinosine (NBTI), an inhibitor of facilitated adenosine transport, to increase adenosine availability.

2 The cardiovascular effects of adenosine (0.01–3 mg kg^{-1} i.v.) were significantly reduced in PEG-ADA-treated animals and augmented in EHNA/NBTI-treated animals. The difference in sensitivity to adenosine in the treated groups was 33- and 15-fold, at the level of 30% reduction in blood pressure and heart rate, respectively.

3 The acute response to allergen, given either intravenously or intratracheally, was quantified as bronchoconstriction. The late phase to allergen was measured as the influx and activation of immunoinflammatory cells into the bronchoalveolar lavage fluid 24 h after challenge.

4 Despite evidence of a substantial difference in adenosine availability following pretreatment with PEG-ADA or EHNA/NBTI, there were no differences in either the acute or late response to allergen in the actively sensitised BN rat.

5 Our data suggest no role for endogenous adenosine in determining the response to allergen under our experimental conditions.
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Keywords: Allergic asthma; Brown Norway rat; endogenous adenosine; adenosine deaminase; ovalbumin; erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA); *S*-(4-nitrobenzyl)-6-thioinosine (NBTI)

Abbreviations: ADA, adenosine deaminase; BN, Brown Norway; BP, blood pressure; Δ, change in; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; HR, heart rate; i.t., intratracheal; NBTI, *S*-(4-nitrobenzyl)-6-thioinosine; OA, ovalbumin; R_L, airway resistance

Introduction

A role for adenosine in allergic asthma has long been advocated (Phillips & Holgate, 1995; Jacobson & Bai, 1997; Marquardt, 1997; Polosa & Holgate, 1997; Feoktistov *et al*., 1998; Forsythe & Ennis, 1999; Fozard & McCarthy, 2002; Holgate, 2002; Polosa, 2002). A key premise is that adenosine plays a role in determining the response of the airways to allergen (Feoktistov *et al*., 1998; Meade *et al*., 2001; Polosa, 2002). Adenosine is elevated under inflammatory conditions and in the asthmatic lung (Driver *et al*., 1993) and is released from mast cells following allergen challenge (Marquardt *et al*., 1984; Lloyd *et al*., 1998). Moreover, the bronchoconstrictor response to adenosine is strikingly upregulated in asthmatics and the response is entirely mast cell mediated (Fozard & Hannon, 2000; Meade *et al*., 2001; Holgate, 2002). Significantly, adenosine receptor activation potentiates the release of mediators from human and rodent mast cells induced by allergen (Church *et al*., 1983; Peachell *et al*., 1991; Konnaris *et al*., 1996; Fozard & Hannon, 2000; Meade *et al*., 2001). Meade *et al*. (2001) concluded from their review of the evidence that endogenous adenosine may amplify the response of airway mast cells to allergen *in vivo*.

In fact, although there is a wealth of circumstantial evidence supporting a role for endogenous adenosine in the airway response to allergen, there is little direct evidence in its support. An exception would be the work of Nyce & Metzger (1997), who selectively depleted rabbits of their pulmonary adenosine A_1 receptors by treatment with an antisense oligodeoxynucleotide and showed an attenuated bronchoconstrictor response to acute challenge with allergen.

We were interested in providing further evidence for or against an involvement of endogenous adenosine in the response to allergen. To this end, we have monitored the acute and late response to allergen in Brown Norway (BN) rats actively sensitised to ovalbumin (OA) and treated either with adenosine deaminase (ADA) linked covalently to polyethylene glycol (PEG-ADA; Adagen®) to decrease adenosine availability or with erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), an inhibitor of ADA, plus *S*-(4-nitrobenzyl)-6-thioinosine (NBTI), an inhibitor of facilitated adenosine transport, to increase adenosine availability.

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Methods

Animals

Male BN rats weighing 250–300 g were supplied by Iffa-Credo (L'Arbresle, France). Groups of up to five animals were housed in sawdust-lined drawer cages and kept at a temperature of $22\pm2^{\circ}C$ under a 12 h normal-phase light–dark cycle. They were fed on NAFAG® pellets supplied by Nahr und Futtermittel AG, Gossau, Switzerland and drinking water was available *ad libitum*. All experiments were carried out with the approval of the Veterinary Authority of the City of Basel (Kantonales Veterinaeramt, Basel-Stadt).

Sensitisation protocol

This procedure is based on the method described by Tarayre *et al*. (1992), using low doses of allergen, in this case OA, and the adjuvant aluminium hydroxide to optimise specific IgE production. OA ($20\,g\,ml^{-1}$) was mixed (30 min on ice) in a blender (Polytron, Kinematica Ltd) with aluminium hydroxide ($20\,mg\,ml^{-1}$) and injected (0.5 ml per animal s.c.). Injection of OA with aluminium hydroxide was repeated 14 and 21 days later. Sensitised animals were used in experiments between days 28 and 35.

Measurement of lung function and cardiovascular effects in anaesthetised animals

Animals were anaesthetised with sodium pentothal ($70\,mg\,kg^{-1}$ i.p.) and a tracheotomy was performed. Heparinised polyethylene catheters were inserted into the left carotid artery for recording mean arterial blood pressure (MABP) and into the left jugular vein for drug administration. To prevent spontaneous respiration, the animals were given an intramuscular injection of vecuronium bromide ($12\,mg\,kg^{-1}$). No experiment exceeded 90 min, during which time surgical anaesthesia was maintained without the need for supplementary anaesthetic. 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, U.S.A.). Coincident pressure changes within the thorax were measured *via* an intrathoracic cannula, using a differential pressure transducer (MP 4524, Validyne, U.S.A.). From measurements of airflow and transpulmonary pressure, airway resistance was calculated (R_L, $cm\,H_2O\,l^{-1}\,s^{-1}$) after each respiratory cycle using a digital electronic pulmonary monitoring system (PMS, Mumed, London, U.K.). MABP and heart rate (HR) by derivation was recorded from the carotid artery by means of a pressure transducer (P23Dd, Gould, U.S.A.). Signals were produced as traces using Lung Function Recording Systems software, version 6.0.

Bronchoalveolar lavage fluid collection and analysis

Animals were killed with sodium pentobarbital ($250\,mg\,kg^{-1}$ i.p.). The lungs were lavaged using three 4 ml aliquots of modified Hank's balanced salt solution (HBSS). The recovered solution was pooled (representative 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 and myeloperoxidase activities and protein concentration in the bronchoalveolar lavage (BAL) 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). Myeloperoxidase activity was measured in a photometric assay based on the oxidation of *O*-dianiside dihydrochloride by myeloperoxidase in the presence of hydrogen peroxide. 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.

Experimental protocols

Cardiovascular sensitivity to adenosine Cardiovascular responses to adenosine ($0.01–3\,mg\,kg^{-1}$ intravenous (i.v.)) were established in groups of actively sensitised animals pretreated 20–24 h prior to experimental intervention with PEG-ADA ($500\,U\,kg^{-1}$ i.m.; 1 U is defined as the amount necessary to convert 1 μM of adenosine to inosine per min at 25°C) or 1 h previously with EHNA ($10\,mg\,kg^{-1}$ i.p.) plus NBTI ($1\,mg\,kg^{-1}$ i.p.) or the respective vehicles. Adenosine doses were administered at 5 min intervals.

Acute response to allergen Bronchoconstrictor responses to 5-HT ($3–30\,\mu g\,kg^{-1}$ i.v.), methacholine ($3–30\,\mu g\,kg^{-1}$ i.v.) and OA ($0.1–10\,mg\,kg^{-1}$ i.v. or $5–45\,mg\,kg^{-1}$ intratracheal (i.t.)) were established in groups of actively sensitised animals pretreated as before. 5-HT was administered with 2 min between doses, followed 10 min later by methacholine with 2 min between doses. At 10 min after the third methacholine dose, OA was administered cumulatively; that is, the dose was increased after 5 min or at the plateau of the preceding response.

Late response to allergen The pulmonary inflammatory response induced by allergen was quantified as the change in leukocyte numbers, myeloperoxidase and eosinophil peroxidase activities and protein concentration in the BAL fluid. Animals were exposed using a nose-only exposure system to an aerosol of OA ($5\,mg\,ml^{-1}$ for 60 min) generated by a Cirrhus nebuliser (DHD Medical Products, Canastota, U.S.A.). The dose administered was approximately $0.4\,mg\,kg^{-1}$, calculated using the formula: ($f\times$ minute volume (l) $\times$ treatment time (min) $\times$ concentration of compound in aerosol ($mg\,l^{-1}$)/body weight (kg)). The factor f refers to the assumed percentage retention of inhaled material in the lung which was taken as 0.3. At 24 h after OA aerosol exposure, BAL was performed. PEG-ADA ($500\,U\,kg^{-1}$) or vehicle was given intramuscularly (i.m.) 24 h prior to the OA exposure. EHNA ($10\,mg\,kg^{-1}$) and NBTI ($1\,mg\,kg^{-1}$) or vehicles were coadministered intraperitoneally (i.p.) 1 h prior to the OA exposure.

Materials

HBSS (10 ×), and 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (HEPES) were obtained from Gibco BRL, U.K. Ethylenediaminetetraacetic acid (EDTA; pH 7.4) and aluminium hydroxide were obtained from Merck, Germany. Pentothal (thiopentalum natricum) and Forene (Isofluran 100%) were obtained from Abbott, Switzerland. Norcuron (vecuronium bromide) was obtained from Organon Teknika, Holland. OA (albumin from hen egg white) was obtained from Fluka, Switzerland. Methacholine, 5-hydroxytryptamine creatinine sulphate, adenosine hemisulphate, EHNA, and NBTI were obtained from Sigma, Switzerland. Polyethylene glycol-modified ADA (PEG-ADA, Adagen®) was kindly provided by Enzon Inc. (Piscataway, NJ, U.S.A.). All compounds were made up in 0.9% w v^{-1} NaCl.

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 assuming unequal variances. Where appropriate, data were corrected for multiple comparison using the Hommel–Hochberg test. A *P*-value <0.05 was considered significant.

Results

Cardiovascular sensitivity to adenosine

The results are presented in Figures 1 and 2. Representative experimental tracings in Figure 1a clearly show the significant inhibitory effect of PEG-ADA on the MABP response to adenosine, and the potentiation following the EHNA/NBTI pretreatment. Taking both shifts into account at the 20% decrease in blood pressure (BP) level, the difference in adenosine availability is 33-fold. At the 60% decrease in BP level, the difference in adenosine availability is eight-fold. Baseline MABP values taken immediately before the administration of adenosine were significantly increased compared to vehicle in the PEG-ADA-treated animals ($P=0.03$; see legend to Figure 1).

Similar results were obtained with the effects of adenosine on HR after the three pretreatments (Figure 2). Again, significant inhibition and potentiation of the HR response to adenosine was seen after PEG-ADA and EHNA/NBTI pretreatment, respectively. Similarly, taking both shifts into account, the adenosine availability at the 20 and 60% decrease in HR levels is 32- and four-fold, respectively. Baseline HR values were not significantly altered between the three treatment groups prior to adenosine administration.

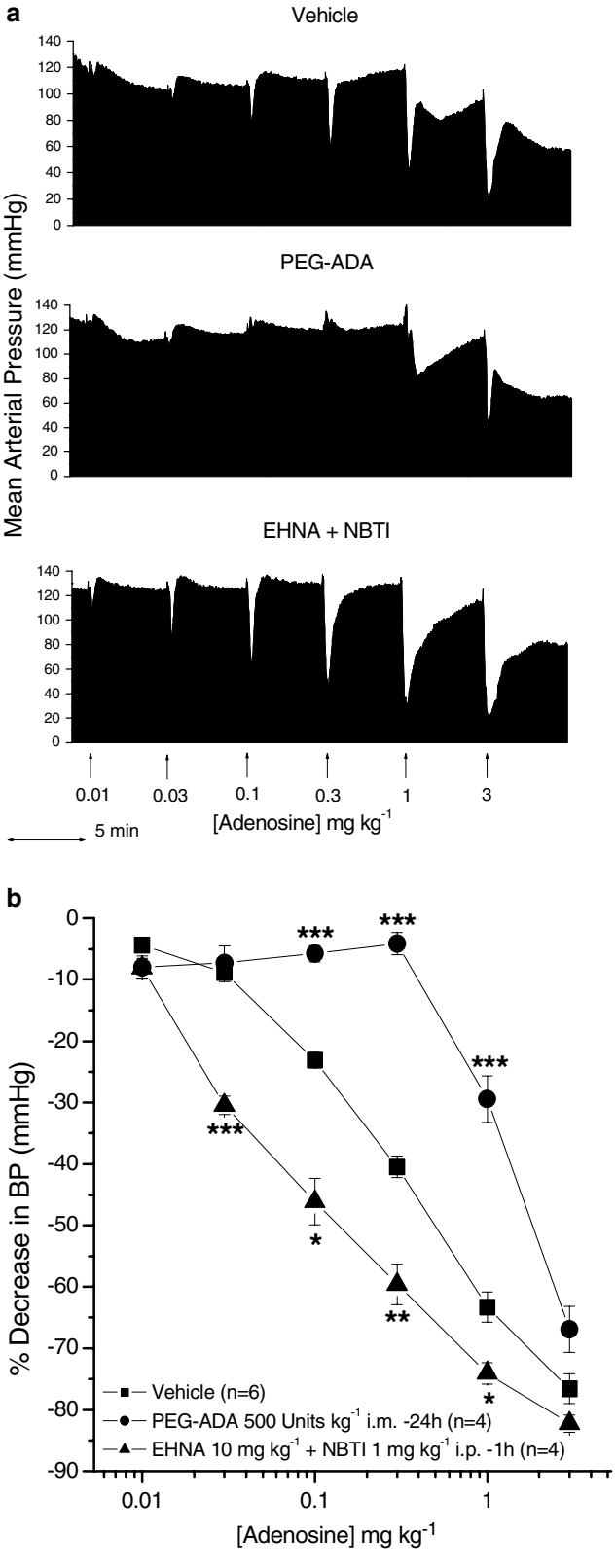

Figure 1 BP effects of adenosine given intravenously to anaesthetised BN rats: effects of PEG-ADA and EHNA/NBTI pretreatment. The data show the effects of adenosine (0.01–3 mg kg^{-1} i.v.) on MABP of anaesthetised actively sensitised BN rats following pretreatment with PEG-ADA (500 U kg^{-1} i.m. −24 h), EHNA (10 mg kg^{-1} i.p. −1 h) plus NBTI (1 mg kg^{-1} i.p. −1 h) or the respective vehicle(s). (a) Representative experimental tracings showing the effect of PEG-ADA and EHNA/NBTI in comparison to vehicle. (b) Mean changes in BP following pretreatment with PEG-ADA and EHNA/NBTI. Results are expressed as means ± s.e.m. of the number (*n*) of animals in parentheses. *$P<0.05$, **$P<0.01$, ***$P<0.001$ indicate significant differences between PEG-ADA or EHNA/NBTI treatment and vehicle. MABP values immediately prior to the adenosine sequence were for vehicle, 117 ± 6 mmHg ($n=6$); for PEG-ADA, 132 ± 3 mmHg ($n=4$) and for EHNA/NBTI-treated animals, 112 ± 8 mmHg ($n=4$).

Early response to allergen

Intravenous OA The results are presented in Figure 3. OA given i.v. induced pronounced, dose-related bronchoconstrictor responses, which were not significantly altered by PEG-ADA or EHNA/NBTI pretreatment. OA also induced dose-related falls in BP and increases in HR. In general, these were not markedly different in the three experimental groups, although small but significant effects on the tachycardia were evident following NBTI/EHNA (Figure 3).

Intratracheal OA OA was less potent and efficacious in inducing bronchoconstriction when given by the i.t. route than when given i.v. As was the case with OA given i.v., the bronchoconstrictor responses were not altered by PEG-ADA or EHNA/NBTI pretreatment (data not illustrated).

In considering the responses to 5-HT and methacholine, the data from the two series were pooled as they were generated in an identical manner. Neither the bronchoconstrictor nor the cardiovascular responses to 5-HT and methacholine were altered by pretreatment with PEG-ADA or EHNA/NBTI (Figure 3).

Late response to allergen

Challenge with OA led to an inflammatory response in the airways of sensitised BN rats when assessed by changes in the BAL fluid leukocyte numbers, myeloperoxidase and eosinophil peroxidase activities and protein concentration measured 24 h after challenge (Figure 4). Following pretreatment with PEG-ADA at a dose of 500 U kg^{-1}, given i.m. 24 h prior to allergen or vehicle exposure, none of the parameters of inflammation from the BAL fluid of allergen-challenged animals was significantly altered. Following pretreatment with EHNA plus NBTI at doses of 10 and 1 mg kg^{-1}, respectively, given i.p. 1 h prior to allergen or vehicle exposure, the parameters of inflammation were broadly similar, with the single exception that the number of lymphocytes present in the BAL fluid of allergen-challenged animals were slightly increased compared to the equivalent control value.

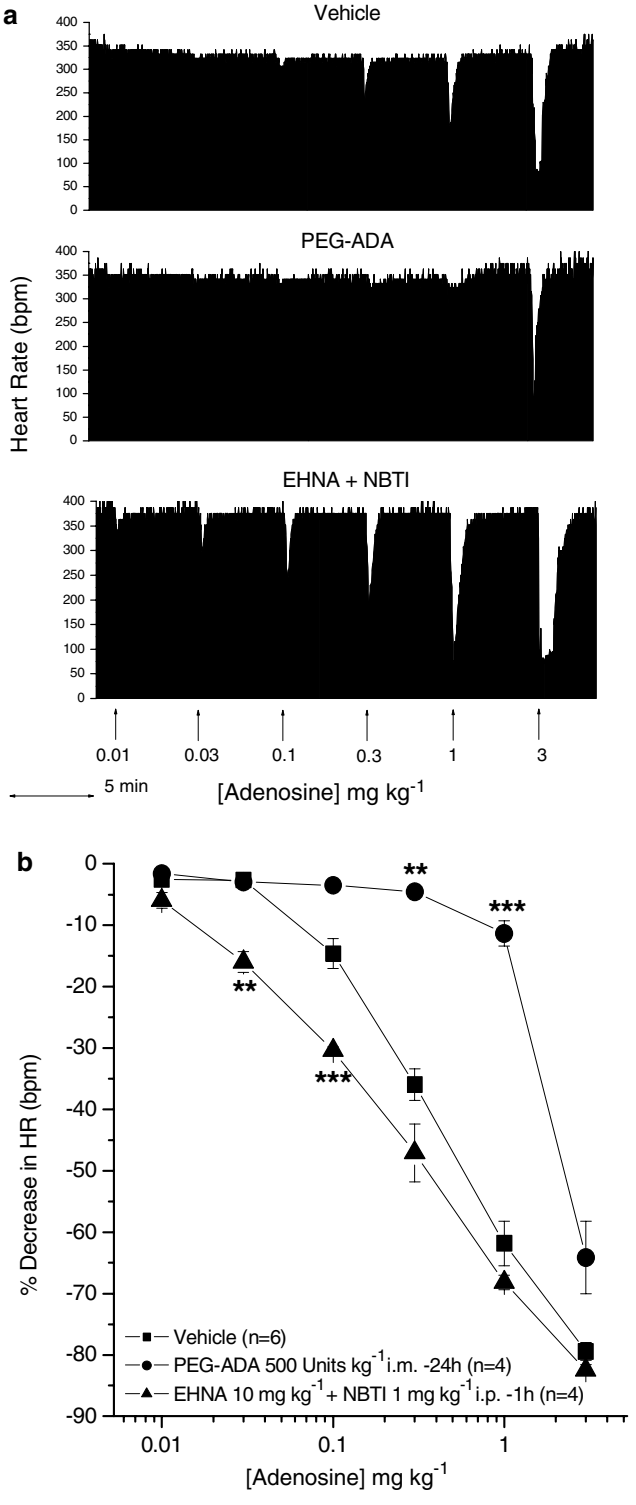

Figure 2 HR effects of adenosine given intravenously to anaesthetised BN rats: effects of PEG-ADA and EHNA/NBTI pretreatment. The data show the effects of adenosine (0.01–3 mg kg^{-1} i.v.) on HR of anaesthetised actively sensitised BN rats following pretreatment with PEG-ADA (500 U kg^{-1} i.m. −24 h), EHNA (10 mg kg^{-1} i.p. −1 h) plus NBTI (1 mg kg^{-1} i.p. −1 h) or the respective vehicle(s). (a) Representative experimental tracings showing the effect of PEG-ADA and EHNA/NBTI in comparison to vehicle. (b) Mean changes in HR following pretreatment with PEG-ADA and EHNA/NBTI. Results are expressed as means ± s.e.m. of the number (n) of animals in parentheses. *$P<0.05$, **$P<0.01$, ***$P<0.001$ indicate significant differences between PEG-ADA or EHNA/NBTI treatment and vehicle. Mean HR values immediately prior to the adenosine sequence were for vehicle, 361 ± 16 bpm ($n=6$); for PEG-ADA, 352 ± 13 bpm ($n=4$) and for EHNA/NBTI-treated animals, 355 ± 7 bpm ($n=4$).

Discussion

A variety of evidence, largely circumstantial, implicates endogenous adenosine in the airway response to allergen (see Introduction). We have attempted to provide direct evidence for or against the hypothesis by monitoring the response to allergen *in vivo* under conditions of decreased or increased availability of endogenous adenosine achieved using PEG-ADA and EHNA plus NBTI, respectively. The doses of these agents used in our study were based on literature findings which document their efficacy in increasing the availability of endogenous adenosine and the biological consequences thereof. Thus, PEG-ADA injected intramuscularly at a dose of

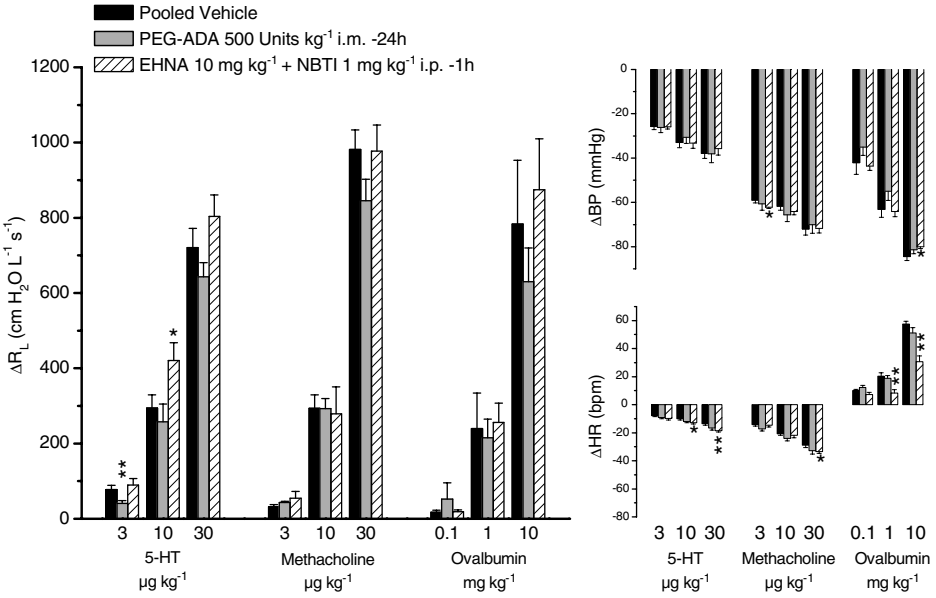

Figure 3 Effects of PEG-ADA (500 U kg^{-1} i.m. −24 h), EHNA (10 mg kg^{-1} i.p. −1 h) plus NBTI (1 mg kg^{-1} i.p. −1 h) or vehicle(s) on airway resistance (ΔR_L), BP and HR responses to bolus injections of 5-HT (3–30 μg kg^{-1} i.v.), methacholine (3–30 μg kg^{-1} i.v.) and OA (0.1–10 mg kg^{-1} i.v.) in actively sensitised BN rats. Results are expressed as means ± s.e.m. of the following numbers of animals: vehicles–OA 5, 5-HT/methacholine 11: PEG-ADA–OA 4, 5-HT/methacholine 9: EHNA/NBTI–OA 4, 5-HT/methacholine 8. *$P<0.05$, **$P<0.01$ indicate significant differences between EHNA/NBTI treatment and vehicle.

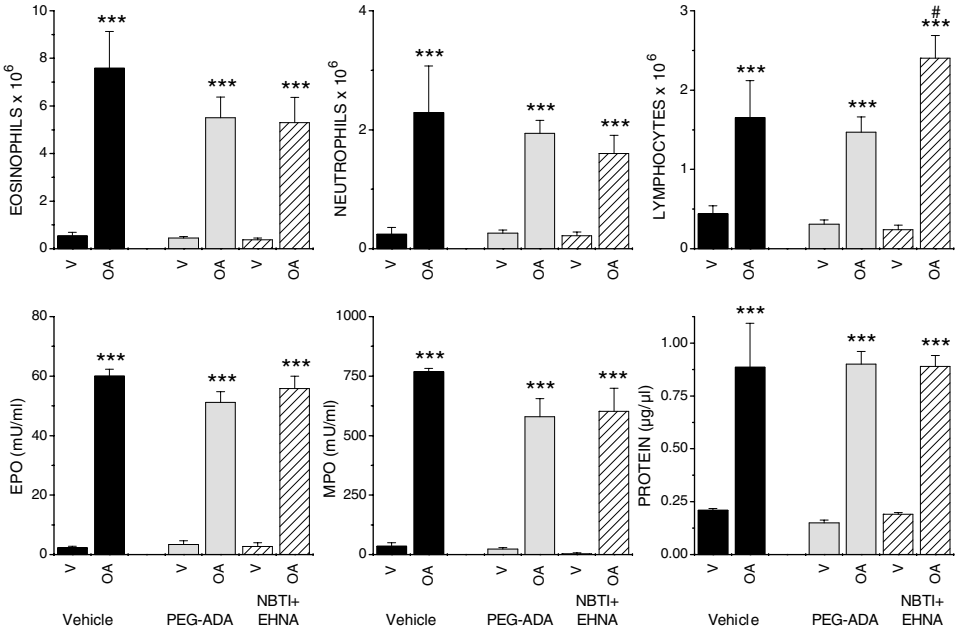

Figure 4 Inflammatory cell infiltration and activation in the lungs of BN rats induced by OA challenge: effect of pretreatment with PEG-ADA (500 U kg^{-1} i.m. −24 h) or EHNA (10 mg kg^{-1} i.p. −1 h) plus NBTI (1 mg kg^{-1} i.p. −1 h). The data (means ± s.e.m.; $n=5–20$) show the effects of PEG-ADA or EHNA/NBTI on the changes in the numbers of eosinophils, neutrophils, lymphocytes, the eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activities and the protein concentration measured in BAL fluid of actively sensitised BN rats 24 h following aerosol challenge with OA (5 mg ml^{-1} for 60 min) or vehicle (V). ***$P<0.001$ indicates significant difference between OA-challenged and vehicle-challenged animals. #$P<0.05$ indicates significant difference from the vehicle-treated animals challenged with OA.

500 U kg^{-1} per day lowered the elevated lung adenosine levels and reversed the respiratory distress and lung eosinophilia in ADA-deficient mice (Blackburn *et al*., 2000a, b; Zhong *et al*., 2001). The dose of PEG-ADA used in humans is somewhat lower than this at 15–60 U kg^{-1} per week (Hershfield, 1997). Conversely, EHNA and/or NBTI, at doses similar to those we have used, have been shown to increase endogenous adenosine levels in venous and arterial blood (Zhang & Lautt, 1994), brain (Gidday *et al*., 1996), cerebrospinal fluid (Ishikawa *et al*., 1997) and heart (Hirai & Ashraf, 1998; Manthei *et al*., 1998)

and to induce a variety of pharmacological effects (Firestein, 1996; Buolamwini, 1997; Kowaluk & Jarvis, 2000), including protection against ischaemia (Van Belle, 1993; Barankiewicz *et al*., 1997, 1998; Abd-Elfattah *et al*., 1998; Hirai & Ashraf, 1998; Peart *et al*., 2001) and decreases in arterial blood pressure in spontaneously hypertensive rats (Tofovic *et al*., 1998).

The efficacy of the pretreatment schedules was verified in our experiments by monitoring the cardiovascular response to i.v. administration of adenosine. Consistent with the literature, we observed significant blockade of the cardiovascular effects of adenosine with PEG-ADA and augmentation of the cardiovascular effects following EHNA/NBTI. The difference between responses in the treated groups indicates a marked difference in adenosine availability under the two experimental conditions. Although we have defined adenosine availability in terms of changes in responsiveness to exogenous adenosine, there is no reason to suppose that this would not extend to endogenous adenosine. As detailed above, increases or decreases of endogenous adenosine and the pharmacological consequences thereof have been documented with similar drug-treatment regimes. Moreover, in our studies, baseline blood pressures were significantly higher in animals given PEG-ADA than in the vehicle-treated controls consistent with the removal of a vasodilator tone mediated through endogenous adenosine (Ledent *et al*., 1997). On this basis, it might have been expected that BP would have been reduced following treatment with EHNA/NBTI, but this was not the case. We have no explanation for the apparent inconsistency, but recall that in the experiments of Tofovic *et al*. (1998), a fall in BP after EHNA was seen in older, spontaneously hypertensive rats, but not in younger animals or the normotensive controls.

Despite the clear evidence of a difference in adenosine availability, both the acute and late response to allergen were similar in the two treatment groups. Thus, we could find no evidence that endogenous adenosine is involved in the allergic response in the rat. This finding contrasts with the work of Nyce & Metzger (1997) who worked with a dust-mite-conditioned allergic rabbit model of human asthma. They administered an antisense oligodeoxynucleotide to deplete the number of adenosine A_1 receptors in airway smooth muscle by $\sim$75%. Animals so treated not only manifested a reduced bronchoconstrictor response to adenosine but also a 55% reduction in the bronchoconstrictor response to allergen challenge and a similar degree of reduction in bronchial hyperresponsiveness to histamine. Species and/or experimental differences may explain the discrepancy between our data and the work of Nyce & Metzger (1997).

A final comment on the cardiovascular response to OA when given acutely. This presumably reflects the cardiovascular effects of mediators such as histamine, 5-HT and possibly adenosine if the latter were involved in the acute response to allergen. In the event, the cardiovascular response to OA was unaffected by PEG-ADA or EHNA/NBTI treatment, suggesting that adenosine plays no role in the cardiovascular response to OA.

In conclusion, using PEG-ADA and EHNA plus NBTI to alter the availability of endogenous adenosine, we have been unable to provide evidence for a role of endogenous adenosine in the acute or late response to allergen in this *in vivo* model of allergic asthma.

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