British Journal of Pharmacology (1998) 124, 1019–1028
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Role of endogenous nitric oxide in allergen-induced airway responses in guinea-pigs

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1 Endogenous nitric oxide (NO) can be detected in exhaled air and accumulates in inflamed airways. However its physiological role has not been fully elucidated. In this study, we investigated a role for endogenous NO in allergen-induced airway responses. Sensitised guinea-pigs were treated with N^G-nitro-L-arginine methyl ester L-NAME (2.0 mM) or aminoguanidine (AG) (2.0 mM) 30 min before the allergen challenge, and 3 and 4 h after the challenge. Alternatively, L-arginine (2.4 mM) treatment was performed 30 min before, and 2 and 3 h after the challenge. In all groups, ovalbumin (OVA) challenge (2 mg ml^{-1} for 2 min) was performed, and airway responses, NO production, infiltration of inflammatory cells, plasma exudation and histological details were examined.

2 Allergen-challenged animals showed an immediate airway response (IAR) and a late airway response (LAR), which synchronised with an increase in exhaled NO. Treatment with L-NAME and AG did not affect IAR while they significantly blocked LAR (72% and 80% inhibition compared to vehicle) and production of NO (35% and 40% inhibition). On the other hand, treatment with L-arginine did not affect IAR but potentiated LAR (74% augmentation).

3 In bronchoalveolar lavage (BAL) fluid, allergen-induced increases in eosinophils were reduced by 48% for L-NAME treatment compared to vehicle, and increased by 56% for L-arginine treatment.

4 Treatment with L-NAME significantly decreased airway microvascular permeability to both Monastral blue (MB) and Evans blue (EB) dye (50.6% and 44% inhibition).

5 We conclude that allergen-induced LAR is closely associated with NO production, and that NO plays a critical role in inflammatory cell infiltration and plasma exudation in the allergic condition.

Keywords: Nitric oxide; allergic guinea-pigs; airway edema; airway inflammation; plasma exudation; N^G-nitro-L arginine methyl ester; aminoguanidine; L-arginine

Introduction

Nitric oxide (NO) was regarded merely as an air pollutant until the discovery that NO acts as an endothelium-derived relaxing factor through activation of guanylate cyclase in vascular smooth muscle (Palmer *et al*., 1987; Ignarro *et al*., 1987). Now it has been revealed that NO is involved in a variety of biological processes; host defence, immune regulation, platelet aggregation, neurotransmission and inflammation (Moncada *et al*., 1991; Moncada & Higgs, 1993). NO is catalysed by a class of enzymes known as NO synthase (NOS) and each NOS generally requires three substrates (L-arginine, nicotinamide adenine dinucleotide phosphate [NADPH], and O_2) and five cofactors (flavin adenine dinucleotide [FAD], flavin mononucleotide [FMN], calmodulin, tetrahydrobiopterin [BH_4] and heme) (Nathan & Xie, 1994). The isoform of this enzyme is classified into three groups, endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) forms (Moncada *et al*., 1997). Both eNOS and nNOS are constitutively expressed, and referred to as constitutive NOS (cNOS), which is regulated in response to intracellular Ca^{2+} calmodulin concentration, and thought to be involved in physiological regulations. On the other hand, iNOS is independent of Ca^{2+} concentration and its enzymatic activity is regulated at a transcriptional level in response to proinflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferon (IFN)-γ (Asano *et al*., 1994; Robbins *et al*., 1994). The transcriptional activity of the iNOS gene has been shown to be dependent on the transcriptional regulating factor, nuclear factor-κB (NF-κB) in its promoter region (Adcock *et al*., 1994). In human monocytes, NO triggers the activation of NF-κB (Lander, 1993).

In the lungs, eNOS is found in endothelial cells, bronchial epithelial cells, platelets, neutrophils and mast cells, and nNOS is found in nerve cells (Kobzik *et al*., 1993; Gaston *et al*., 1997). On the other hand, iNOS is also found in bronchial epithelial cells, macrophages, fibroblasts, smooth muscle cells and endothelial cells (Kobzik *et al*., 1993; Gaston *et al*., 1997). Endogenous NO in the respiratory tract can be detected in exhaled air of both humans and animals (Gustafsson *et al*., 1991; Persson & Gustafsson, 1993). In asthmatics, exhaled NO levels were elevated in asthmatics compared to normal subjects (Alving *et al*., 1993; Kharitonov *et al*., 1994) and this difference is preserved in direct sampling of exhaled NO, using a fiber-optic bronchoscope (Massaro *et al*., 1996). Although iNOS is expressed in airway epithelial cells in asthmatics but not in normals (Hamid *et al*., 1993), the cellular source of NO and its physiological role in asthma is unclear.

In this study, we investigated the role of endogenous NO in allergen-induced airway responses in guinea-pigs, using a nonselective eNOS, nNOS and iNOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (Rees *et al*., 1990), a relatively selective iNOS inhibitor; aminoguanidine (AG) (Misko *et al*., 1993), and the NO precursor, L-arginine. The effects of these reagents on airway responses, NO production, infiltration of inflammatory cells, airway microvascular permeability and histological details were examined.

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Methods

Animals

Female Hartley guinea-pigs, weighing 250 to 300 g (SLC farm, Shizuoka, Japan), were pretreated with cyclophosphamide (30 mg kg^{-1}) intraperitoneally. Two days later, animals were sensitised to 1 mg of ovalbumin (OVA), emulsified in $Al(OH)_3$ (100 mg in 1 ml sterile saline), by intraperitoneal injection. Three weeks after the primary sensitisation, 10 μg OVA and 100 mg $Al(OH)_3$ were intraperitoneally injected as a booster. Three weeks after the booster injection, these guinea-pigs were used as sensitised animals. Cyclophosphamide is an alkylating agent and has an anti-suppressor T cell effect (Turk & Parker, 1982). Cyclophosphamide pretreatment resulted in a marked increase in bronchial reactivity and IgE-like titer (Andersson, 1981; de Macedo & Mota, 1982; Uchida *et al*., 1996).

Measurement of pulmonary function

Six weeks after the primary sensitisation, guinea-pigs were placed in a body-plethysmograph chamber equipped with a mouth-nose mask. Specific airways conductance (SGaw; s^{-1} cmH_2O^{-1}) was measured according to Agrawal's methods (Agrawal, 1981). Respiratory air flow ($\dot{V}$) was determined by a pneumotachograph (TV-241T, Nihon Koden, Tokyo, Japan) and box pressure change was monitored by differential transducer (TP-601G, Nihon Koden) as $\Delta(V_1\text{-}V_2)$. SGaw was obtained by fitting the following equation during the quick transition from the expiration to inspiration (TEI) phase:

$$\text{SGaw } (s^{-1}\ cmH_2O^{-1}) = \frac{\dot{V}}{\Delta\ (V_1 - V_2)} \cdot \frac{Kp}{K} \cdot \frac{1}{P_B - P_{H_2O}}$$

where K_p and K are the calibration factors of $\dot{V}$ and $\Delta\ (V_1\text{-}V_2)$, respectively, P_B is barometric pressure and P_{H_2O} is the water vapor pressure at body temperature. Sampling was obtained at 1024 Hz by digital oscilloscope (DS-9121, Iwatsu, Tokyo, Japan) and sent to a personal computer (Macintosh Centris 660 AV, Apple, Tokyo, Japan). The estimate of SGaw over each TEI was determined by linear regression analysis of the ratio $\dot{V}$ and $\Delta\ (V_1\text{-}V_2)$. The analysis program was built with commercial software LabView 3.1.1 for Macintosh (National Instrument, Austin, U.S.A.).

Experimental protocol

Sensitised guinea-pigs were treated with either L-NAME (2.0 mM inhalation for 20 min), AG (2.0 mM inhalation for 20 min), or vehicle (phosphate buffered saline [PBS] inhalation for 20 min) at 30 min before the allergen challenge, and 3 and 4 h after the challenge, respectively. Alternatively, either L-arginine (2.4 mM inhalation for 20 min), its enantiomer D-arginine (2.4 mM inhalation for 20 min), or vehicle (PBS inhalation for 20 min) were treated at 30 min before, and 2 and 3 h after the challenge. The inhalation was performed using an ultrasonic-nebulizer (NE-U11, Omuron, Tokyo, Japan) with an airflow of 3 l min^{-1}. Additionally, exhaled NO was measured in NOS inhibitor-treated and vehicle animals.

Sensitised animals were challenged with inhalation of OVA (2 mg OVA per ml saline solution) and each SGaw value was compared with that obtained before the challenge, and the difference was defined as per cent change in SGaw. SGaw was monitored at 15-min intervals for a total of 7 h. The magnitude of LAR was quantified by calculating the area under the response curve (percentage under the baseline) (AUC) between 3 and 7 h after the challenge. AUC was calculated by a trapezoid integration.

In a separate study, sensitised animals were treated as above and bronchoalveolar lavage (BAL), histological details and the amount of plasma exudation at 6 h after the challenge were examined in PBS, L-NAME and L-arginine treated groups.

Measurement of NO production

Assay of exhaled NO levels was performed according to the method of Gustafsson (Gustafsson *et al*., 1991). Guinea-pigs breathed spontaneously NO free air at a flow rate of 200 ml min^{-1} through a nose-mouth piece. Then, exhaled air was collected every 10 min till 7 h after the allergen challenge into a 5 l sampling bag (Tedlar, E. I. Dupon De Nemours and Co., DE, U.S.A.), in which the concentration of NO (part per billion, ppb) was analysed with a chemiluminescence system (CLM-500, Shimadzu, Kyoto, Japan) at a sample gas flow of 1 liter min^{-1}. NO per minute volume (NO $\dot{V}E^{-1}$) was calculated by correcting with respiratory rate and tidal volume. The magnitude of NO production (NO $\dot{V}E^{-1}$) in both IAR (between 0 and 2 h after the challenge) and LAR (between 3 and 7 h after the challenge) was quantified by AUC as described above.

BAL

Six h after the challenge, guinea-pigs were sacrificed by exsanguination under anaesthesia with an intraperitoneal injection of 50 mg kg^{-1} pentobarbital. BAL fluid (BALF) was obtained by three repeat instillations of 10 ml sterile saline through the trachea. After counting the total cell number in BALF, preparations were centrifuged at 150 g for 10 min with a cytospin centrifuge (Cytospin 3, Shandon, Pittsburgh, U.S.A.). Then, the pellet was stained with haematoxylin-eosin (HE) for differential cell counting.

Light microscopic examinations

Light microscopic examination was performed at 6 h after the challenge. Animals were anaesthetized and exsanguinated as described above. The right intrapulmonary bronchus was removed and fixed in 10% formalin (pH 7.4). After the fixation, these sections were trimmed, embedded in paraffin, cut into 5 μm thick sections and stained with HE.

Quantification of plasma extravasation

At the same time points as BAL and light microscopic examinations, the LAR-guinea-pigs treated with L-NAME, L-arginine or vehicle were injected intravenously with a mixture containing 1.5% Monastral blue (MB) and 1.5% Evans blue (EB) dyes (30 mg kg^{-1} of each tracer in sterile saline) for the measurement of vascular permeability. Under anaesthesia described above, the chest was opened 5 min after the tracers were injected intravenously into the right femoral vein, and the vasculature was perfused with 1% paraformaldehyde in 50 mM citrate buffer (pH 3.5) for 2 min at a pressure of 120 mm Hg. The trachea, incised along the ventral midline, was cleared in toluene and mounted flat on slides in order to estimate the MB-labelled vessels in the tracheal mucosa (Sulakvelidze & McDonald, 1994). The amount of extravasated MB was measured by the point counting method (Weibel, 1979) and was expressed as the area density of MB-labelled vessels. Point counts of MB-labelled vessels were performed in 20 regions of trachea (each measuring 0.25 mm^2 in area) on the right side of

each whole mount (Sulakvelidze & McDonald, 1994). Images of histologic sections were digitalised (SCANTOUCH 210, Nikon, Tokyo, Japan) and fed into a computer (Macintosh Centris 660 AV, Apple, Tokyo, Japan). An electronically-generated grid of 50 points was superimposed onto the images and sampled using NIH Image (Version 1.3.8, provided by Dr. Wayne Rasband, National Institutes of Health) (Gatlin *et al*., 1993). In addition, EB was extracted by immersion of tracheal segments for 4 days at room temperature in 3 ml of 1% suramin sodium dissolved in methanol. Optical density (OD) of the EB in suramin extracts was measured by spectrophotometry at a wavelength of 620 nm and after this measurement the trachea was desiccated at room temperature and weighed. The concentration was expressed in nanograms dye per milligram dry tracheal weight.

Observation by electron microscopy

Following the treatment by MB and EB described above, the rest of the trachea was removed for electron microscopy (EM). Specimens were fixed in 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, and post-fixed in 1% osmium tetroxide for 1 h in 0.1 M sodium phosphate buffer (Noguchi *et al*., 1995). Then, these were dehydrated in a graded series of ethanol (50% – 100%) followed by propylene oxide, and were embedded in Epon 812 (Abbott; North Chicago, U.S.A.) for transmission EM. Ultrathin sections of 70 – 80 nm, cut with a diamond knife by Ultrotome III 8800 (LKB-Produkter AB., Bromma, Sweden), were stained with uranyl acetate and lead citrate and examined with a H-7000 EM (Hitachi, Tokyo, Japan).

Drugs and chemicals

OVA (grade VI), cyclophosphamide, L-NAME, AG, pentobarbital, MB and EB were obtained from Sigma Chemical Co., St. Louis, U.S.A. $Al(OH)_3$, haematoxylin, eosin, formalin, glutaraldehyde and osmium tetroxide were from Wako Pure Chemicals Co., Osaka, Japan. Suramin sodium was from FBA Pharmaceuticals Division, Mobay Chemical Corp., New York, U.S.A. All chemicals used were of the highest grade available. L-NAME, AG, and L-arginine were dissolved in PBS and were adjusted to pH 7.1.

Expression and analysis of results

All values were expressed as means ± s.e.mean. When data were sequential measurements, differences among groups were tested with repeated measures analysis of variance (ANOVA) at each time point. When a significant result in ANOVA was obtained, Bonferroni post test was used as a multiple comparison test. Differences were considered significant at $P<0.05$. All statistics were performed with the commercial software InStat Mac (GraphPad Software, San Diego, U.S.A.).

Results

Effects of NOS inhibitors and NO precursor on airway responses

The baseline value of SGaw did not significantly differ among the groups (0.228 ± 0.03 s^{-1} cmH_2O^{-1} for L-NAME, 0.218 ± 0.018 s^{-1} cmH_2O^{-1} for AG and 0.223 ± 0.05 s^{-1} cmH_2O^{-1} for vehicle). In IAR, there was no difference between these groups while LAR was significantly blocked by treatment with both L-NAME and AG (Figure 1). When the magnitude of airway responses was computed as AUC, treatment of L-NAME and AG significantly inhibited LAR (72% and 80% inhibition, compared to vehicle, respectively) (Figure 3a). Treatment with AG had a tendency to inhibit the LAR more than that with L-NAME, although the effect was not significant.

Figure 2 illustrates the effect of L-arginine. The baseline SGaw was not significantly different among the groups (0.210 ± 0.08 s^{-1} cmH_2O^{-1} for L-arginine, 0.214 ± 0.05 s^{-1} cmH_2O^{-1} for D-arginine and 0.220 ± 0.014 s^{-1} cmH_2O^{-1} for

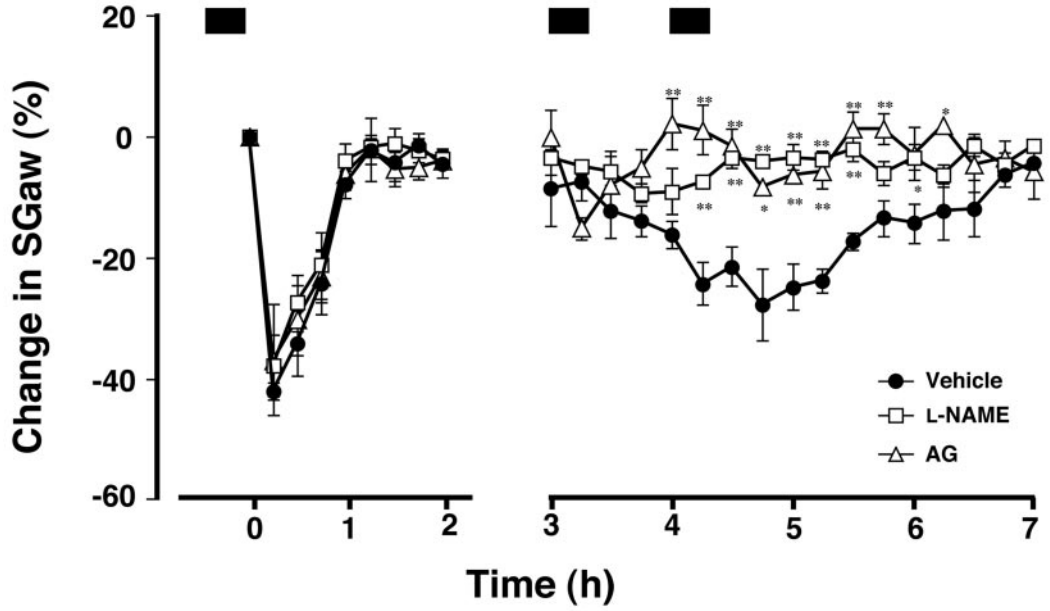

Figure 1 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (AG) on airway responses. Inhalation of 2.0 mM L-NAME for 20 min ($n=6$), 2.0 mM AG for 20 min ($n=6$) or phosphate buffered saline (PBS) as vehicle for 20 min ($n=6$) was performed at 30 min before, and 3 and 4 h after, allergen challenge as shown by black boxes. All values are expressed as means ± s.e.mean. $^*P<0.05$, $^{**}P<0.01$, compared to vehicle group (Bonferroni post test).

vehicle). Inhalation of L-arginine for 20 min at 2 and 3 h after the challenge decreased SGaw immediately and extended the duration of LAR (74% augmentation compared to vehicle). In contrast, D-arginine did not affect LAR (Figure 3b).

Exhaled NO (Figures 4 and 5)

We confirmed that baseline was significantly higher in sensitised animals than non-sensitised naive animals (104.0 ± 5.8 ppb l^{-1} min^{-1} and 70.3 ± 8.2 ppb l^{-1} min^{-1}, respectively). There was no significant difference in baseline exhaled NO level among the L-NAME, AG and PBS inhalation groups (98.6 ± 6.8 ppb l^{-1} min^{-1}, 104.0 ± 5.8 ppb l^{-1} min^{-1} and 104.0 ± 5.8 ppb l^{-1} min^{-1}, respectively). NO levels decreased shortly after L-NAME and AG inhalation and remained low compared with the vehicle control group before OVA challenge (74 and 80% decrease compared with baseline levels, respectively). As stated above, inhalation of both L-NAME and AG did not affect the baseline SGaw. No change in blood pressure or heart rate was observed among these groups (data not shown).

After allergen challenge, all of the groups showed an increase in exhaled NO in the phase of IAR. Although NO levels tended to be low following treatment with L-NAME and AG, they were not significantly decreased.

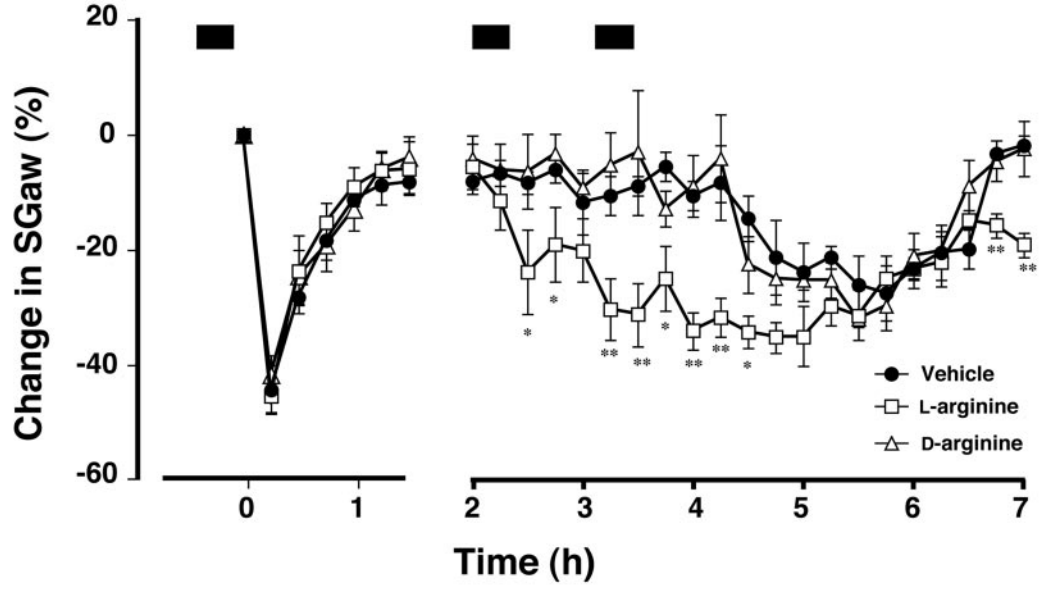

Figure 2 Effects of L-arginine and D-arginine on airway responses. Inhalation of 2.4 mM L-arginine for 20 min ($n=6$), 2.4 mM D-arginine for 20 min ($n=6$) or PBS as vehicle for 20 min ($n=6$) was performed at 30 min before, and 2 and 3 h after, allergen challenge as shown by black boxes. All values are expressed as means ± s.e.mean. $^{*}P<0.05$, $^{**}P<0.01$, compared to vehicle group (Bonferroni post test).

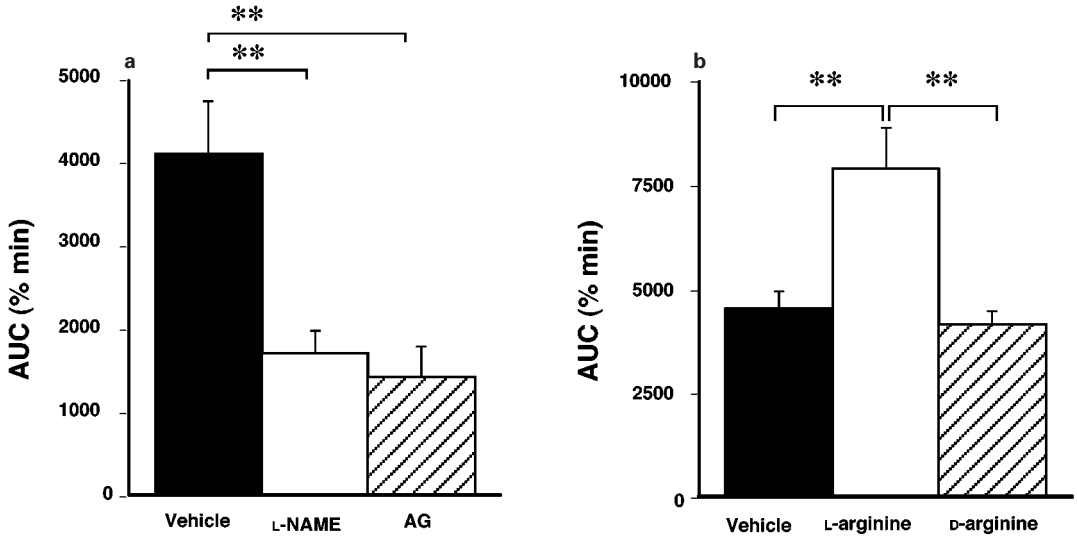

Figure 3 Magnitude of late airway response (LAR). LAR, illustrated in Figures 1 and 2, was quantified by AUC (area under the response curve) between 3 and 7 h after the challenge. (a) Inhalation of nitric oxide (NO) synthase (NOS) inhibitors significantly blocked LAR. Treatment with AG had a tendency to inhibit the LAR more than that of L-NAME, although the effect was not significant. (b) Treatment with L-arginine significantly augmented LAR, while D-arginine did not affect LAR. All values are expressed as means ± s.e.mean. $^{**}P<0.01$, compared to vehicle group (Bonferroni post test).

Following IAR, a gradually sustained increase in NO (maximal increase 230.0±21.5 ppb l^{-1} min^{-1}, 270 min after challenge) was observed 4 to 7 h after the challenge, which synchronised with the dynamics of airflow limitation, namely LAR. On the other hand, treatment with NOS inhibitors significantly inhibited NO production. In particular, treatment with AG had a tendency to inhibit NO production more than that with L-NAME (40% inhibition for AG and 35% inhibition for L-NAME, compared with vehicle).

BAL (Figure 6)

The volume of fluid recovered from BAL was not significantly different between drug- and PBS-treated animals (almost 90% recovery, data not shown). In a subpopulation of inflamma-

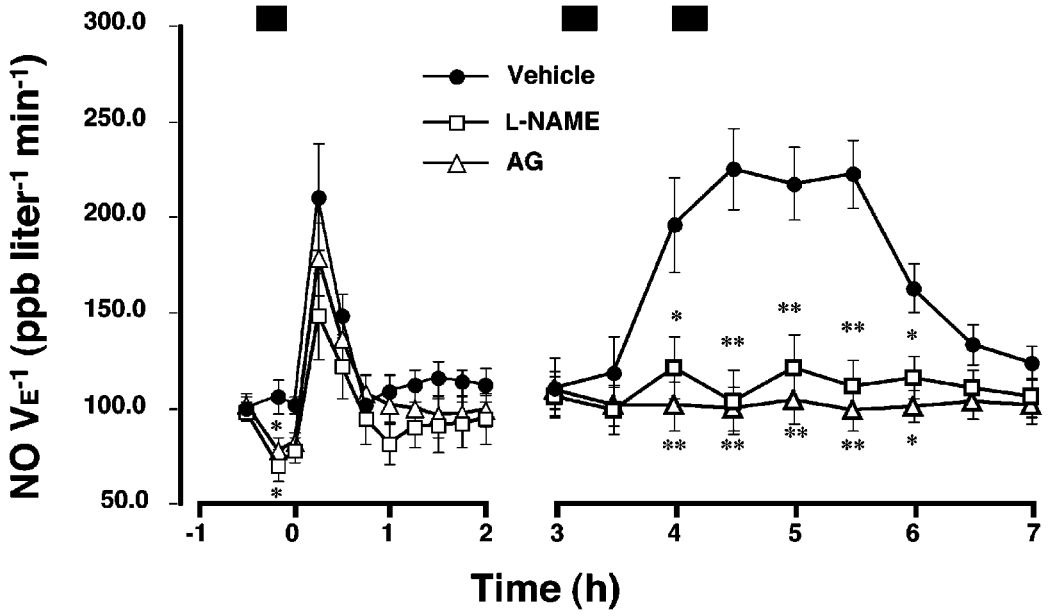

Figure 4 Effects of NOS inhibitors on NO production. Solutions of 2.0 mM L-NAME ($n=6$), 2.0 mM AG ($n=6$) or PBS as vehicle ($n=6$) were inhaled by the animals 30 min before the allergen challenge for 20 min. The treatment was repeated for 20 min at 3 and 4 h after the challenge as shown by black boxes. Before the allergen challenge, exhaled NO levels decreased shortly after L-NAME and AG inhalation and remained low compared with vehicle control group. In the immediate airway response (IAR), treatment with L-NAME had a tendency to decrease exhaled NO more than that of AG and vehicle control, although the effect was not significant. In contrast, both L-NAME and AG significantly inhibited NO production in LAR. All values are expressed as means±s.e.mean. $*P<0.05$, $**P<0.01$, compared to vehicle group (Bonferroni post test).

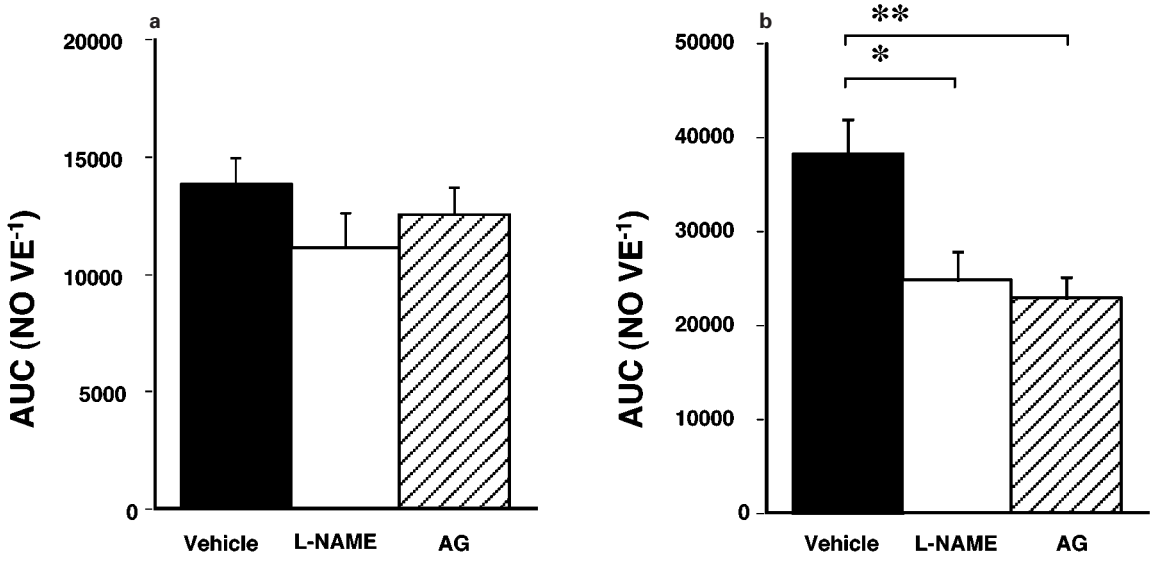

Figure 5 Magnitude of exhaled NO. The magnitude of exhaled NO illustrated in Figure 4 was quantified by AUC between 0 and 2 h after the challenge as IAR (a) and 3 to 7 h after the challenge as LAR (b). All values are expressed as means±s.e.mean. $*P<0.05$, $**P<0.01$, compared to vehicle group (Bonferroni post test).

tory cells in BALF, the number of eosinophils in BALF decreased due to inhalation of L-NAME solution (48% inhibition compared with vehicle control), while inhalation of L-arginine augmented infiltration of eosinophils (56% augmentation compared with vehicle control).

Observation by microscopy (Figure 7)

The airway submucosa in LAR showed a substantial oedema and dilatation of vessels (a). Furthermore, treatment with L-arginine potentiated these pathological alterations (b) and caused damage of endothelial cells and basement membrane (h). However, such changes were not observed in L-NAME-treated animals. The sites of increased vascular permeability are evident in Figure 7-d, e, f. MB-labelled vessels were particularly observed after treatment with L-arginine compared to PBS. In contrast, treatment with L-NAME abolished MB extravasation.

Quantitative analysis of plasma extravasation (Figures 8 and 9)

The measurement of extracted EB dye revealed that extravasation was prominent in LAR tracheae and that L-arginine further potentiated this (36% augmentation compared with vehicle). In contrast, treatment with L-NAME significantly inhibited plasma leakage in trachea (44% inhibition compared with vehicle) (Figure 8).

As quantified by the stereological pointing method, L-NAME induced 50.6% inhibition of plasma extravasation compared with vehicle, while L-arginine produced 33% augmentation compared with vehicle (Figure 9).

Discussion

In this study, we demonstrated a role for endogenous NO in allergen-induced airway responses. One feature of our model is that it has a high IgE titer as well as IgG_1 titer against OVA and shows biphasic airway responses. In addition, airway responses and exhaled NO levels can be measured without anaesthesia.

Before the allergen challenge, neither inhalation of NOS inhibitors nor L-arginine produced any alteration in SGaw or cardiovascular parameters, while exhaled NO levels were significantly decreased on treatment with NOS inhibitors. This suggests that these effects are confined to the respiratory tract and endogenous NO does not have an important role in regulating airway tone in sensitised animals. In a human study, inhalation of NOS inhibitors did not affect forced expiratory volume in one second ($FEV_{1.0}$) in either normal or asthmatic subjects (Yates *et al*., 1996).

On the other hand, we found exhaled NO levels in sensitised animals showed a 1.5-fold increase compared with naive animals, suggesting that the booster injection with alum was likely to increase NO production. The isoform of NOS responsible for NO production is unknown. Liu, *et al*. (1997) examined the alteration in iNOS mRNA in sensitised rats and reported a 3-fold upregulation of iNOS mRNA in lungs in animals sensitised to OVA alone. In this study, there was no difference between L-NAME and AG. This may reflect a difference of inhibitory potency for each NOS. However, exhaled NO in sensitised guinea-pigs might be mainly derived from iNOS in lungs.

In IAR, a rapid increase in expired NO was observed in PBS- and NOS-inhibitor treated animals, although there was no difference in the magnitude of airway response. It has been well established that endogenous NO derived from cNOS in airway epithelial cells has an important role in counteracting the bronchoconstrictor stimuli (Persson & Gustafsson, 1993; Nijkamp *et al*., 1993; Mehta *et al*., 1997). IAR is characterised as an acute bronchoconstriction of airway smooth muscle (Barnes, 1989). Therefore, from this hypothesis, NOS inhibitors should affect IAR. However, as mentioned above, iNOS is upregulated in sensitised animals to some extent, and this upregulation might affect the downregulation of a homeostatic cNOS, or impair cyclic guanosine 3′,5′-monophosphate (GMP) function.

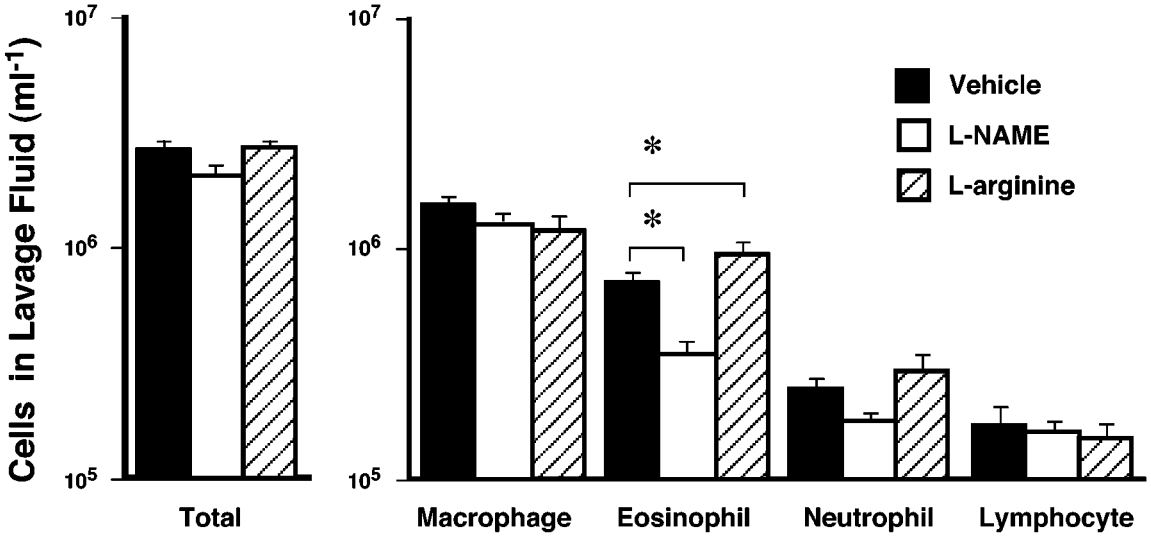

Figure 6 Total cell counts and subpopulations in bronchoalveolar lavage (BAL) fluid (BALF). Inhalation of PBS as vehicle for 20 min ($n=6$) or 2.4 mM L-arginine for 20 min ($n=6$) was performed at 30 min before, and 2 and 3 h after the challenge. Inhalation of 2.0 mM L-NAME for 20 min ($n=6$) was performed at 30 min before, and 3 and 4 h after the challenge. Animals were sacrificed at 6 h after the allergen challenge. Treatment with L-NAME significantly suppressed infiltration of eosinophils (48% inhibition compared to vehicle) while that of L-arginine augmented it (56% augmentation compared to vehicle). All values are expressed as means ± s.e.mean. $^*P<0.05$ compared to vehicle group (Bonferroni post test).

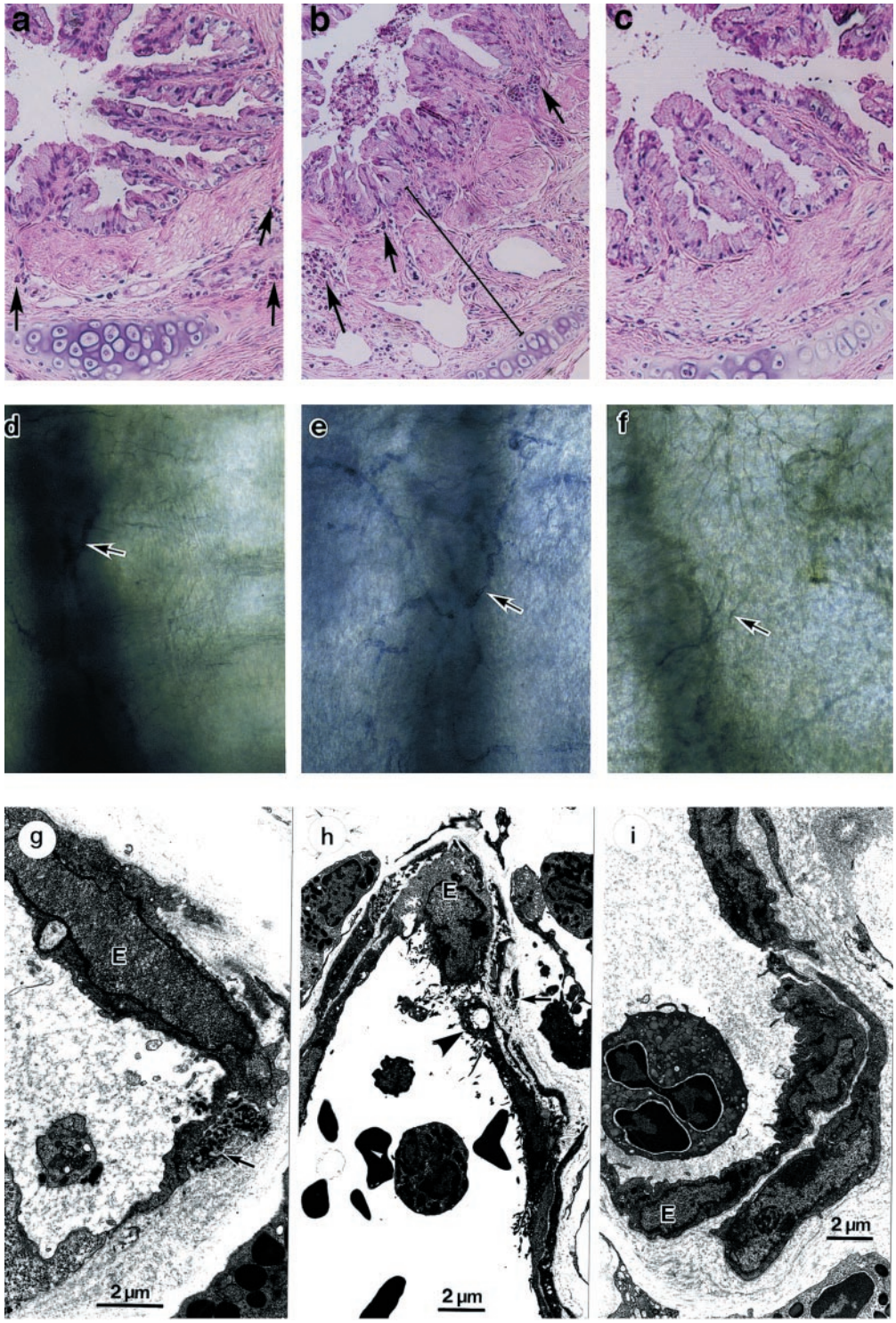

Figure 7 Histological details of airways. Inhalation of PBS as vehicle for 20 min ($n=6$) (a, d, g) and 2.4 mM L-arginine for 20 min ($n=6$) (b, e, h) were performed at 30 min before, and 2 and 3 h after allergen challenge. Inhalation of 2.0 mM L-NAME for 20 min ($n=6$) (c, f, i) was performed at 30 min before, and 3 and 4 h after the challenge. Animals were sacrificed by exsanguination under anaesthesia with an intraperitoneal injection of 50 mg kg^{-1} pentobarbital at 6 h after the challenge. (a, b, c) Light microscopic observations of right intrapulmonary bronchus stained with haematoxylin and eosin (HE). Leukocytes consisting predominantly of eosinophils are seen in large numbers between the epithelial cells and beneath the smooth muscle in vehicle and L-arginine treated animals (arrows) (a, b). In accord with leukocyte infiltration, substantial oedema (the bar indicates marked thickness of submucosa in L-arginine-treated airway) and vasodilation were seen. These phenomena were abolished by L-NAME (c). Similar results were reproduced in every animal. Original magnification: $\times 200$. (d, e, f) Histological demonstration of plasma leakage. Extravasated vessels (arrow) were labelled by Monastral Blue (MB) . Quantification of MB is illustrated in Figure 9. Original magnification: $\times 100$. (g, h, i) Electron micrographs of submucosal vessels of airways. MB dye (arrow) localises outside endothelial cells in the submucosa of the LAR-animal (g). Endothelial cells treated with L-arginine indicate damage of endothelial cells (arrow head) (h), and MB dye grains migrate outside the vessel (arrow). However, cells treated with L-NAME show no grains of MB dye around the vessel (i). Similar results were reproduced in every animals. E: endothelial cell.

Following IAR, a gradually sustained increase in NO and LAR were observed, and treatment with NOS inhibitors at 3 and 4 h after the challenge inhibited both NO production and LAR, indicating that the development of LAR could be related to production of NO. Treatment with AG tended to inhibit LAR more than that of L-NAME and this may reflect a different selectivity for iNOS. On the other hand, L-arginine inhalation potentiated LAR. This suggests that L-arginine availability is increased because of induction of iNOS *via* cytokine stimulation. It is known that an increased L-arginine transport system, namely cationicamino acid transporter (CAT) or Y^+ system, activity is observed in lipopolysaccharide (LPS)-stimulated macrophages (Sato *et al*., 1992). This system is also identified in vascular smooth muscle (Low *et al*., 1993) and there is a report of treatment for endotoxaemia by inhibition of the cytokine-inducible L-arginine transport system (Bianchi *et al*., 1995). Therefore, it is possible that L-arginine is the limiting factor for NO production in this model.

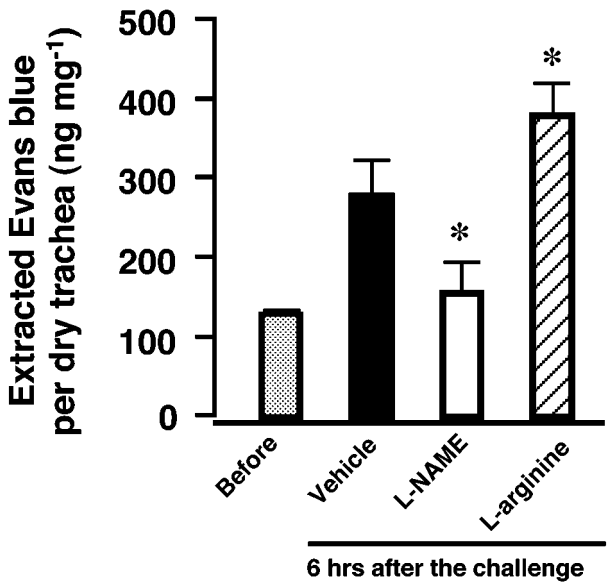

Figure 8 Quantification of Evans Blue dye. Inhalation of PBS as vehicle for 20 min (n = 6) or 2.4 mM L-arginine for 20 min ($n = 6$) were performed at 30 min before, and 2 and 3 h after the challenge. Inhalation of 2.0 mM L-NAME for 20 min ($n = 6$) was also performed at 30 min before, and 3 and 4 h after the challenge. Animals were sacrificed at 6 h after the allergen challenge. Histograms showing the effect of L-arginine and L-NAME on vascular permeability in the LAR-trachea. The content of extracted EB in the L-arginine treatment group increased significantly, while that in the L-NAME treatment group decreased significantly, compared to vehicle. All values are expressed as means ± s.e.mean. $^*P < 0.05$ compared to vehicle group (Bonferroni post test).

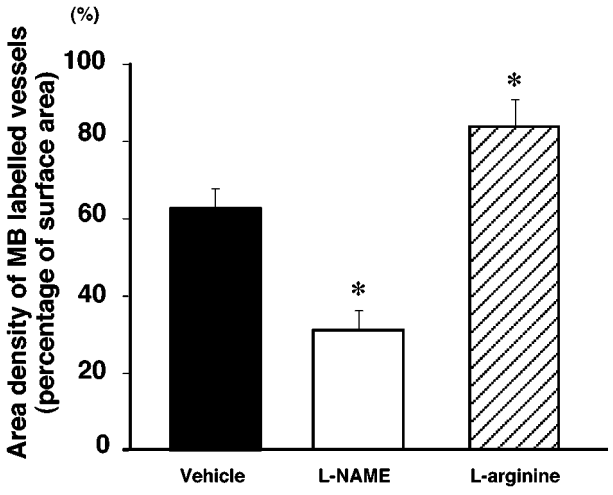

Figure 9 Quantification of Monastral blue (MB) dye. Quantification of MB dye (Figure 7d,e,f) was performed by a stereological counting method. In accord with extracted EB dye (Figure 8), L-NAME treatment significantly inhibited the degree of extravasation of MB, while L-arginine treatment potentiated. Inhalations of PBS as vehicle for 20 min ($n = 6$) or 2.4 mM L-arginine for 20 min ($n = 6$) were performed at 30 min before, and 2 and 3 h after the challenge. Inhalation of 2.0 mM L-NAME for 20 min ($n = 6$) was also performed at 30 min before, and 3 and 4 h after the challenge. Animals were sacrificed at 6 h after the allergen challenge. All values are expressed as means ± s.e.mean. $^*P < 0.05$ compared to vehicle group (Bonferroni post test).

The mechanism of LAR was further examined by histological studies. The sections from the intrapulmonary bronchi showed a marked vasodilation and oedema in the submucosa in LAR-guinea-pigs and this was augmented by L-arginine inhalation, while completely abolished by L-NAME. These observations indicate that NO production in LAR is associated with bronchial circulation and oedema formation in airways.

In accord with histological findings, both tracers were inhibited by L-NAME and augmented by L-arginine. We chose relatively central airways in this study because there is sparse vascularity in peripheral airways and it is hard to evaluate exudation accurately with these tracers due to lack of resolving power.

Plasma exudation is thought to be due to hydrostatic mechanisms, endothelial contraction and subsequent enlargement of the gaps in postcapillary venules (McDonald, 1994). EB, known to bind to albumin, reflects the extravasation, and it can be influenced by local blood flow (Rogers *et al*., 1989), suggesting that NO in LAR increased bronchial blood flow. This is supported by the report that NO is a potent vasodilator in bronchial circulation (Alving *et al*., 1992). In guinea-pig skin, several kinds of inflammatory mediators induced leukocyte accumulation and oedema formation inhibited by L-NAME and this mechanism is reported to be due to the decrease in local blood flow by L-NAME (Teixeira *et al*., 1993). On the other hand, MB dye has a size of 5 to 30 nm and is trapped only at the basal lumina of the leakage site (McDonald, 1988). There are several reasons why endothelial contraction or endothelial damage occur at the leakage site. In this study, endothelial damage was seen in L-arginine-treated animals by EM, indicating that toxic radicals were produced at the leakage sites. However, in guinea-pigs, allergen-induced plasma leakage is reported to involve mainly leukotrienes (LT) and partly histamine (Evans *et al*., 1988). LTD_4-inhalation induced plasma leakage is significantly inhibited by infusion of L-NAME (Miura *et al*., 1996), suggesting that mechanisms other than decreased local blood flow or endothelial damage are involved in the inhibition of allergen-induced plasma leakage by L-NAME. The role of NO in vascular permeability is controversial and conflicting results have been reported. However, most studies that found endogenous NO had a protective role against vascular leakage, such as inhibition of neutrophil adherence to endothelial cells (Kubes *et al*., 1991), were performed under basal conditions. In contrast, the report that NO mediates microvascular permeability was performed in allergic conditions. The difference between these reports seems to lie in whether the allergic condition was used.

With regard to toxic radicals, it is reported that when NO and superoxide are produced at the same time, they combine to form peroxynitrite (Huie & Padmaja, 1993). The reaction rate

for the formation of peroxynitrite is reported to be $6.7 \pm 0.9 \times 10^9$ M^{-1} S^{-1}, which is approximately three times faster than the scavenging of superoxide with copper, zinc superoxide dismutase at physiological ionic strength (Huie & Padmaja, 1993). Peroxynitrite is a strong oxidant and causes lipid peroxidation and DNA strand breaks (Royall *et al*., 1997). This production of peroxynitrite also raises the possibility of impairment of nonadrenergic noncholinergic (iNANC) nerve in airways (Miura *et al*., 1997).

BALF data showed eosinophil influx into the airway was inhibited by L-NAME and augmented by L-arginine. This suggests that NO is involved in eosinophilic inflammation in airways. It is unclear at which step of inflammation NO is involved: for example, rolling and firm adhesion, transendothelial migration or chemotaxis (Butcher, 1991). In this study, L-NAME was given by inhalation, indicating that migration is a predominant action of NO in the allergic condition. This is supported by the report that *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or LTB_4 induced-eosinophil migration is significantly inhibited by L-NAME and this is mimicked by cyclic guanosine 3′,5′-monophosphate (GMP) inhibitor and reversed by dibutyryl GMP in rat peritoneal eosinophils (Zanardo *et al*., 1997). However, it can not be excluded that NO is involved in adhesion and chemotaxis of eosinophils because it has been reported that eosinophils have iNOS (Pozo *et al*., 1997) and NO inhibits apoptosis of eosinophils (Beauvais *et al*., 1995). Moreover, eosinophils are reported to produce a large amount of superoxide in LAR (Sugiyama *et al*., 1995) and it is possible that eosinophils produce peroxynitrite and worsen airway inflammation. Consequently, the mechanisms by which NOS inhibitors suppress eosinophilic inflammation appear to be complicated.

It is known that airflow limitation consists of several components, i.e. direct or neuronal smooth muscle contraction, microvascular permeability and mucus hypersecretion (James *et al*., 1989). A theoretical analysis shows that a moderate increase in airway wall thickness has little effect on baseline resistance (Hogg *et al*., 1987), but can produce a marked increase in maximal airway narrowing *via* smooth muscle contraction (James *et al*., 1989). In this experiment, we found marked submucosal oedema and bronchoconstriction in airways, suggesting that submucosal oedema has an important role in airflow limitation.

Which cells of the lung are responsible for the expired NO is unknown. Airway epithelial cells (Hamid *et al*., 1993), alveolar macrophage and eosinophil are candidates for NO production. On the other hand, the NO pathway through ligation of a low-affinity IgE receptor, CD23, has been observed in human monocytes (Paul-Eugene *et al*., 1995) and eosinophils (Arock *et al*., 1994), and this pathway is thought to be important for iNOS induction (Dugas *et al*., 1995). As mentioned above, our model has a high IgE titer similar to the Brown-Norway rat model which was successful in detecting upregulation of iNOS after allergen challenge (Liu *et al*., 1997; Renzi *et al*., 1997). Thus, we speculate that IgE has an important role in induction of iNOS.

In summary, our results demonstrate that allergen-induced airflow limitation is closely associated with NO production in the airways. This airflow limitation was blocked by NOS inhibitors, and potentiated by L-arginine inhalation. The mechanisms by which NOS inhibitors blocked LAR are considered to be inhibition of plasma exudation, eosinophilic inflammation and production of toxic radicals. Endogenous NO plays a critical role in the physiological changes of LAR.

Part of this work was supported by a Grant-in-Aid for Scientific Research of the Ministry of Education, Science, Sports and Culture in Japan and a grant from Nippon Glaxo Ltd., Tokyo, Japan.

References

ADCOCK, I.M., BROWN, C.R., KWON, O. & BARNES, P.J. (1994). Oxidative stress induces NF-κB DNA binding and inducible NOS mRNA in human epithelial cells. *Biochem. Biophys. Res. Commun.,* **199,** 1518 – 1524.

AGRAWAL, K.P. (1981). Specific airways conductance in guinea-pigs: normal values and histamine induced fall. *Respir. Physiol.,* **43,** 23 – 30.

ALVING, K., FORNHEM, C., WEITZBERG, E. & LUNDBERG, J.M. (1992). Nitric oxide mediates cigarette smoke-induced vasodilatory responses in the lung. *Acta. Physiol. Scand.,* **146,** 407 – 408.

ALVING, K., WEITZBERG, E. & LUNDBERG, J.M. (1993). Increased amount of nitric oxide in exhaled air of asthmatics. *Eur. Respir. J.,* **6,** 1368 – 1370.

ANDERSSON, P. (1981). Antigen-induced bronchial anaphylaxis in actively sensitised guinea-pigs. The effect of booster injection and cyclophosphamide treatment. *Int. Arch. Allergy Appl. Immunol.,* **64,** 249 – 258.

AROCK, M., LE GOFF, L., BECHEREL, P.A., DUGAS, B., DEBRE, P. & MOSSALAYI, M.D. (1994). Involvement of Fcε RII/CD23 and L-arginine dependent pathway in IgE-mediated activation of human eosinophils. *Biochem. Biophys. Res. Commun.,* **203,** 265 – 271.

ASANO, K., CHEE, C.B., GASTON, B., LILLY, C.M., GERARD, C., DRAZEN, J.M. & STAMLER, J.S. (1994). Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 10089 – 10093.

BARNES, P.J. (1989). New concepts in the pathogenesis of bronchial hyperresponsiveness and asthma. *J Allergy Clin Immunol,* **83,** 1013 – 1026.

BEAUVAIS, F., MICHEL, L. & DUBERTRET, L. (1995). The nitric oxide donors, azide and hydroxylamine, inhibit the programmed cell death of cytokine-deprived human eosinophils. *FEBS Lett.,* **361,** 229-232.

BIANCHI, M., ULRICH, P., BLOOM, O., MEISTRELL, M.R., ZIMMERMAN, G.A., SCHMIDTMAYEROVA, H., BUKRINSKY, M., DONNELLEY, T., BUCALA, R., SHERRY, B. & *et al.* (1995). An inhibitor of macrophage arginine transport and nitric oxide production (CNI-1493) prevents acute inflammation and endotoxin lethality. *Mol. Med.,* **1,** 254 – 266.

BUTCHER, E.C. (1991). Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell,* **67,** 1033 – 1036.

DE MACEDO, M.S. & MOTA, I. (1982). Antigenic competition in IgE antibody production. II. Effect of cyclophosphamide. *Immunology,* **47,** 67 – 74.

DUGAS, B., MOSSALAYI, M.D., DAMAIS, C. & KOLB, J.P. (1995). Nitric oxide production by human monocytes: evidence for a role of CD23. *Immunol. Today,* **16,** 574 – 580.

EVANS, T.W., ROGERS, D.F., AURSUDKIJ, B., CHUNG, K.F. & BARNES, P.J. (1988). Inflammatory mediators involved in antigen-induced airway microvascular leakage in guinea-pigs. *Am. Rev. Respir. Dis.,* **138,** 395 – 399.

GASTON, B., KOBZIK, L. & STAMLER, S. (1997). Distribution of Nitric Oxide Synthase in the Lung. In *Nitric Oxide and the Lung*. ed. Zapol, W.M. & Bloch, K.D. pp. 75 – 86. New York: Marcel Dekker.

GATLIN, C.L., SCHABERG, E.S., JORDAN, W.H., KUYATT, B.L. & SMITH, W.C. (1993). Point counting on the Macintosh. A semiautomated image analysis technique. *Anal. Quant. Cytol. Histol.,* **15,** 345 – 350.

GUSTAFSSON, L.E., LEONE, A.M., PERSSON, M.G., WIKLUND, N.P. & MONCADA, S. (1991). Endogenous nitric oxide is present in the exhaled air of rabbits, guinea-pigs and humans. *Biochem. Biophys. Res. Commun.,* **181,** 852 – 857.

HAMID, Q., SPRINGALL, D.R., RIVEROS, M.V., CHANEZ, P., HOWARTH, P., REDINGTON, A., BOUSQUET, J., GODARD, P., HOLGATE, S. & POLAK, J.M. (1993). Induction of nitric oxide synthase in asthma. *Lancet.,* **342,** 1510 – 1513.

HOGG, J.C., PARE, P.D. & MORENO, R. (1987). The effect of submucosal edema on airways resistance. *Am. Rev. Respir. Dis.,* **135,** S54 – 56.

HUIE, R.E. & PADMAJA, S. (1993). The reaction of nitric oxide with superoxide. *Free Radic. Res. Commun.,* **18,** 195 – 199.

IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E. & CHAUDHURI, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.,* **84,** 9265 – 9269.

JAMES, A.L., PARE, P.D. & HOGG, J.C. (1989). The mechanics of airway narrowing in asthma. *Am. Rev. Respir. Dis.,* **139,** 242 – 246.

KHARITONOV, S.A., YATES, D., ROBBINS, R.A., LOGAN, S.R., SHINEBOURNE, E.A. & BARNES, P.J. (1994). Increased nitric oxide in exhaled air of asthmatic patients. *Lancet,* **343,** 133 – 135.

KOBZIK, L., BREDT, D.S., LOWENSTEIN, C.J., DRAZEN, J., GASTON, B., SUGARBAKER, D. & STAMLER, J.S. (1993). Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. *Am. J. Respir. Cell Mol. Biol.,* **9,** 371 – 377.

KUBES, P., SUZUKI, M. & GRANGER, D.N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 4651 – 4655.

LANDER, H.M., SEHAJPAL, P., LEVINE, D.M. & NOVOGRODSKY, A. (1993). Activation of human peripheral blood mononuclear cells by nitric oxide-generating compounds. *J. Immunol.,* **150,** 1509 – 1516.

LIU, S.F., HADDAD, E.B., ADCOCK, I., SALMON, M., KOTO, H., GILBEY, T., BARNES, P.J. & CHUNG, K.F. (1997). Inducible nitric oxide synthase after sensitization and allergen challenge of Brown Norway rat lung. *Br. J. Pharmacol.,* **121,** 1241 – 1246.

LOW, B.C., ROSS, I.K. & GRIGOR, M.R. (1993). Characterization of system L and system Y^+ amino acid transport activity in cultured vascular smooth muscle cells. *J. Cell Physiol.,* **156,** 626 – 634.

MASSARO, A.F., MEHTA, S., LILLY, C.M., KOBZIK, L., REILLY, J.J. & DRAZEN, J.M. (1996). Elevated nitric oxide concentrations in isolated lower airway gas of asthmatic subjects. *Am. J. Respir. Crit. Care Med.,* **153,** 1510 – 1514.

McDONALD, D.M. (1988). Neurogenic inflammation in the rat trachea. I. Changes in venules, leukocytes and epithelial cells. *J. Neurocytol.,* **17,** 583 – 603.

McDONALD, D.M. (1994). Endothelial gaps and permeability of venules in rat tracheas exposed to inflammatory stimuli. *Am. J. Physiol.,* **266,** L61 – L83.

MEHTA, S., LILLY, C.M., ROLLENHAGEN, J.E., HALEY, K.J., ASANO, K. & DRAZEN, J.M. (1997). Acute and chronic effects of allergic airway inflammation on pulmonary nitric oxide production. *Am. J. Physiol.,* **272,** L124 – L131.

MISKO, T.P., MOORE, W.M., KASTEN, T.P., NICKOLS, G.A., CORBETT, J.A., TILTON, R.G., MCDANIEL, M.L., WILLIAMSON, J.R. & CURRIE, M.G. (1993). Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur. J. Pharmacol.,* **233,** 119 – 125.

MIURA, M., ICHINOSE, M., KAGEYAMA, N., TOMAKI, M., TAKAHASHI, T., ISHIKAWA, J., OHUCHI, Y., OYAKE, T., ENDOH, N. & SHIRATO, K. (1996). Endogenous nitric oxide modifies antigen-induced microvascular leakage in sensitised guinea-pig airways. *J. Allergy Clin. Immunol.,* **98,** 144 – 151.

MIURA, M., YAMAUCHI, H., ICHINOSE, M., OHUCHI, Y., KAGEYAMA, N., TOMAKI, M., ENDOH, N. & SHIRATO, K. (1997). Impairment of neural nitric oxide-mediated relaxation after antigen exposure in guinea-pig airways in vitro. *Am. J. Respir. Crit. Care Med.,* **156,** 217 – 222.

MONCADA, S. & HIGGS, E.A. (1993). The L-arginine-nitric oxide pathway. *N. Engl. J. Med.,* **329,** 2002 – 2012.

MONCADA, S., HIGGS, E.A. & FURCHGOTT, R. (1997). International Union of Pharmacology Nomenclature in Nitric Oxide Research. *Pharmacol. Rev.,* **49,** 137 – 142.

MONCADA, S., PALMER, R.M. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.,* **43,** 109 – 142.

NATHAN, C. & XIE, Q.W. (1994). Regulation of biosynthesis of nitric oxide. *J. Biol. Chem.,* **269,** 13725 – 13728.

NIJKAMP, F.P., VAN, D.-L.-.H. & FOLKERTS, G. (1993). Nitric oxide synthesis inhibitors induce airway hyperresponsiveness in the guinea-pig in vivo and in vitro. Role of the epithelium. *Am. Rev. Respir. Dis.,* **148,** 727 – 734.

NOGUCHI, Y., UCHIDA, Y., ENDO, T., NINOMIYA, H., NOMURA, A., SAKAMOTO, T., GOTO, Y., HARAOKA, S., SHIMOKAMA, T., WATANABE, T. & HASEGAWA, S. (1995). The induction of cell differentiation and polarity of tracheal epithelium cultured on the amniotic membrane. *Biochem. Biophys. Res. Commun.,* **210,** 302 – 309.

PALMER, R.M., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature,* **327,** 524 – 526.

PAUL-EUGENE, N., KOLB, J.P., SARFATI, M., AROCK, M., OUAAZ, F., DEBRE, P., MOSSALAYI, D.M. & DUGAS, B. (1995). Ligation of CD23 activates soluble guanylate cyclase in human monocytes via an L-arginine-dependent mechanism. *J. Leukoc. Biol.,* **57,** 160 – 167.

PERSSON, M.G. & GUSTAFSSON, L.E. (1993). Allergen-induced airway obstruction in guinea-pigs is associated with changes in nitric oxide levels in exhaled air. *Acta. Physiol. Scand.,* **149,** 461 – 466.

POZO, V.D., ARRUDA-CHAVES, E.D., ANDRES, B.D., CARDABA, B., LOPEZ-FARRE, A., GALLARDO, S., CORTEGANO, I., VIDARTE, L., JURADO, A., SASTRE, J., PALOMINO, P. & LAHOZ, C. (1997). Eosinophils transcribe and translate messenger RNA for inducible nitric oxide. *J. Immunol.,* **158,** 859 – 864.

REES, D.D., PALMER, R.M., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and *in vivo*. *Br. J. Pharmacol.,* **101,** 746 – 752.

RENZI, P.M., SEBASTIAO, N., AL ASSAAD, A.S., GIAID, A. & HAMID, Q. (1997). Inducible nitric oxide synthase mRNA and immunoreactivity in the lungs of rats eight hours after antigen challenge. *Am. J. Respir. Cell Mol. Biol.,* **17,** 36 – 40.

ROBBINS, R.A., BARNES, P.J., SPRINGALL, D.R., WARREN, J.B., KWON, O.J., BUTTERY, L.D., WILSON, A.J., GELLER, D.A. & POLAK, J.M. (1994). Expression of inducible nitric oxide in human lung epithelial cells. *Biochem. Biophys. Res. Commun.,* **203,** 209 – 218.

ROGERS, D.F., BOSCHETTO, P. & BARNES, P.J. (1989). Plasma exudation. Correlation between Evans blue dye and radiolabelled albumin in guinea-pig airways in vivo. *J. Pharmacol. Methods.,* **21,** 309 – 315.

ROYALL, J., KOOY, N. & BECKMAN, J. (1997). Peoxynitrite and Other Nitric Oxide- Derived Oxidants. In *Nitric Oxide and the Lung*. ed. Zapol, W.M. & Bloch, K.D. pp. 223 – 246. New York: Marcel Dekker.

SATO, H., FUJIWARA, M. & BANNAI, S. (1992). Effect of lipopolysaccharide on transport and metabolism of arginine in mouse peritoneal macrophages. *J. Leukoc. Biol.,* **52,** 161 – 164.

SUGIYAMA, H., EDA, R., OKADA, C., HOPP, R.J., BEWTRA, A.K. & TOWNLEY, R.G. (1995). Eosinophil accumulation and activation in antigen-induced late asthmatic response in guinea-pigs. *J. Asthma.,* **32,** 37 – 45.

SULAKVELIDZE, I. & MCDONALD, D.M. (1994). Anti-edema action of formoterol in rat trachea does not depend on capsaicin-sensitive sensory nerves. *Am. J. Respir. Crit. Care Med.,* **149,** 232 – 238.

TEIXEIRA, M.M., WILLIAMS, T.J. & HELLEWELL, P.G. (1993). Role of prostaglandins and nitric oxide in acute inflammatory reactions in guinea-pig skin. *Br. J. Pharmacol.,* **110,** 1515 – 1521.

TURK, J.L. & PARKER, D. (1982). Effect of cyclophosphamide on immunological control mechanisms. *Immunol. Rev.,* **65,** 99 – 113.

UCHIDA, Y., JUN, T., NINOMIYA, H., OHSE, H., HASEGAWA, S., NOMURA, A., SAKAMOTO, T., SARDESSAI, M.S. & HIRATA, F. (1996). Involvement of endothelins in immediate and late asthmatic responses of guinea-pigs. *J. Pharmacol. Exp. Ther.,* **277,** 1622 – 1629.

WEIBEL, E. (1979). Stereological Methods. In Practical Methods for Biological Morphometry. pp. 101 – 159. London: Academic Press Inc.

YATES, D.H., KHARITONOV, S.A., THOMAS, P.S. & BARNES, P.J. (1996). Endogenous nitric oxide is decreased in asthmatic patients by an inhibitor of inducible nitric oxide synthase. *Am. J. Respir. Crit. Care Med.,* **154,** 247 – 250.

ZANARDO, R.C., COSTA, E., FERREIRA, H.H., ANTUNES, E., MARTINS, A.R., MURAD, F. & DE NUCCI, G. (1997). Pharmacological and immunohistochemical evidence for a functional nitric oxide synthase system in rat peritoneal eosinophils. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 14111 – 14114.

(Received July 14, 1997
Revised March 6, 1998
Accepted April 16, 1998)