ELSEVIER

European Journal of Pharmacology 358 (1998) 253–259

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Nitric oxide modulates eosinophil infiltration in antigen-induced airway inflammation in rats

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Received 26 June 1998; revised 28 July 1998; accepted 31 July 1998

Abstract

The influence of nitric oxide (NO) on eosinophil infiltration into the airways was investigated in rats actively sensitized with ovalbumin. The animals were treated chronically with the NO synthase inhibitor, N^{ω}-Nitro-L-arginine methyl ester (L-NAME; 75 μmol rat^{-1} day^{-1}), for 4 weeks. Bronchoalveolar lavage was performed at 6, 24, 48 and 72 h after intratracheal injection of ovalbumin. Intratracheal challenge of the sensitized rats with ovalbumin caused a significant increase in total leucocyte infiltration in bronchoalveolar lavage fluid both 24 and 48 h post-ovalbumin injection. Neutrophils and eosinophils peaked, respectively, at 24 h (29%) and 48 h (30%) in bronchoalveolar lavage fluid whereas the mononuclear cell did not differ significantly from the counts in non-sensitized rats at any time. At both 6 and 24 h post-ovalbumin injection, the chronic treatment of the animals with L-NAME affected neither the total nor the differential leucocyte content. However, at 48 h post-ovalbumin challenge, the total cell count was reduced by approximately 48% in the L-NAME-treated animals and this was associated with a marked inhibition (81%) of the eosinophil influx. Histological examination of the lungs from these animals (48 h post-ovalbumin challenge) also showed a prominent reduction (69.5%; $P < 0.05$) of the eosinophil infiltration in the respiratory segments. Our results demonstrate that NO plays a pivotal role in the eosinophil infiltration in airways of actively sensitized rats.

Keywords: Eosinophil; Nitric oxide (NO); Allergic inflammation; Lung

1. Introduction

Nitric oxide (NO) is enzymatically synthesized from L-arginine (Palmer et al., 1988) through the action of either constitutive (Bredt et al., 1991; Lamas et al., 1992; Janssens et al., 1992) or inducible (Xie et al., 1992) NO synthase isoforms. Nitric oxide has been described as an endogenous non-adrenergic non-cholinergic (NANC) neurotransmitter in the airways responsible for the modulation of bronchial tone in experimental animals and humans (Barnes and Belvisi, 1993; Hogman et al., 1993). Vascular tone (Alving et al., 1992) and plasma exudation (Kuo et al., 1992; Erjefalt et al., 1994) in the airways are also modulated by NO.

Asthma is a specific inflammation in the airways characterised by accumulation of mucus in the lumen of the airway, edema in bronchial mucosa and increased bronchial responsiveness. Besides activation of mast cells, macrophages and T lymphocytes in the airway mucosa, eosinophilic infiltration into the airways plays a key role in the pathogenesis of asthma (Kroegel et al., 1994). Nitric oxide modulates the asthmatic inflammation in animals (Robbins et al., 1994; Yeadon and Price, 1995) and humans (Hamid et al., 1993; Persson et al., 1994; Kharitonov et al., 1994) and has also recently been reported as an important mediator responsible for the eosinophil migration in non-allergic inflammation (Ferreira et al., 1996). In this study we investigated whether NO influences the development of the allergic reaction in the airway mucosa following antigen challenge in ovalbumin-sensitized rats. Therefore, we studied the total and differential (neu-

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0014-2999/98/$ - see front matter
PII: S0014-2999(98)00575-5

trophils, eosinophils and mononuclear cells) leucocyte counts in the bronchoalveolar lavage fluid from actively sensitized rats treated chronically (four weeks) with the NO synthesis inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME). Lung histopathology of non-sensitized and ovalbumin-sensitized rats, from both non-treated and L-NAME-treated animals was also done.

2. Materials and methods

2.1. Sensitization procedure and antigen challenge

Male Wistar rats (180–200 g) provided by the Biology Institute Laboratory Animal Center (CEMIB) of the State University of Campinas (UNICAMP) were actively sensitized by injecting subcutaneously a mixture of 200 μg of ovalbumin and 8 mg $Al(OH)_3$ (0.15 ml) prepared in saline. Non-sensitized rats received only 8 mg $Al(OH)_3$. Fourteen days later, both sensitized and non-sensitized animals were anaesthetized with chloral hydrate (300 mg/kg, i.p.) and the trachea was exposed through a midline ventral incision of approximately 0.5 cm length in the neck. With the aid of a 26.5-gauge needle, 0.4 ml of 0.25% solution of ovalbumin was injected into the airways.

To evaluate the sensitization procedure, a group of both non-sensitized ($n = 3$) and ovalbumin-sensitized animals ($n = 3$) were anaesthetized with chloral hydrate (300 mg kg^{-1}, i.p.), the trachea was removed, suspended in a cascade system (Vane, 1964) and continuously superfused (5 ml min^{-1}) with oxygenated (95% O_2 + 5% CO_2) and warmed (37°C) Krebs solution. Challenge of the trachea strips with ovalbumin (10 μg) caused a significant contraction (94 ± 34%, as compared with the sub-maximal contraction induced by 0.1 mmol KCl) of the tissues obtained from ovalbumin-sensitized rats whereas strips obtained from non-sensitized animals did not show any contractile effect.

2.2. Leucocyte number in bronchoalveolar lavage

Bronchoalveolar lavage was performed 6, 24, 48 and 72 h after ovalbumin challenge. Briefly, the animals were anaesthetised with chloral hydrate (300 mg kg^{-1}, i.p.) and exsanguinated by cutting the abdominal aorta. The trachea was exposed and cannulated with a polyethylene tube (1 mm diameter) connected to a syringe. The lungs were washed by flushing with phosphate buffered saline (PBS) solution containing heparin (20 IU ml^{-1}) and 0.03% serum albumin. The PBS buffer was instilled through the tracheal cannula as one 10-ml aliquot followed by three 5-ml aliquots. The fluid recovered after each aliquot instillation was combined and centrifuged ($1000 \times g$ for 10 min at 20°C). The cell supernatant was discarded and the cell pellet was resuspended in 2 ml of PBS buffer. Total cell counts were done with an automated cell counter (CELL-DYN 1600) while differential counts were carried out on air-dried smears stained with May–Grunwald–Giemsa. A minimum of 400 cells was counted and classified as neutrophils, eosinophils and mononuclear cells based on normal morphological criteria.

2.3. Histopathology analysis

For histopathological analysis of the lungs, the rats were anaesthetised and exsanguinated as described above. The lungs were removed and fixed in 4% paraformaldehyde–0.5% glutaraldehyde PBS buffer (0.1 M, pH 7.4). Twenty-four h after fixation, the lungs were cut into small fragments, dehydrated in an ethanol gradient (70–100%), cleared in xylol and embedded in paraffin wax. The fragments were then cut into 5-μm thick sections and stained with hematoxylin-eosin. Histological evaluation of eosinophil accumulation in the lungs was based on the number of eosinophils in ten randomly selected areas of the bronchioles. The cells were counted at X 800 magnification and expressed as the number of eosinophils per high-power field (Feder et al., 1997).

2.4. Chronic treatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME) and D-NAME

The chronic treatment with L-NAME was performed as previously described (Ribeiro et al., 1992). Briefly, male Wistar rats (80–100 g at the beginning of the study) received L-NAME dissolved in the drinking water at a concentration of 1.2 mM to give a daily intake of approximately 75 μmol rat^{-1} day^{-1} for up to four weeks. Another group of animals was given the inactive enantiomer, D-NAME (75 μmol rat^{-1} day^{-1}). Control animals received tap water alone. Systemic arterial pressure was measured weekly with a tail-cuff method (Zatz, 1990).

2.5. Experimental protocols

The animals were divided into the following groups: (1) Control group ($n = 71$): rats that received tap water alone, (2) L-NAME group ($n = 73$): rats that received L-NAME in the drinking water for four weeks, (3) D-NAME group ($n = 10$): rats that received D-NAME in the drinking water for four weeks.

Two weeks after the start of L-NAME treatment, each of these groups of animals was divided into another two groups: non-sensitized (rats that received s.c. only 8 mg $Al(OH)_3$) and ovalbumin-sensitized animals (rats that received s.c. 200 μg ovalbumin + 8 mg $Al(OH)_3$). On day 28, all the animals were intratracheally injected with ovalbumin (0.4 ml of 0.25% solution). Bronchoalveolar lavage of these animals was then performed at 6, 24, 48 and 72 h after ovalbumin instillation.

Another two group of naive (non-sensitized, non-challenged) animals, control ($n = 15$) and treated with L-NAME

($n = 15$) were used. They were intratracheally injected with PBS buffer (0.4 ml) and their airways were washed with PBS buffer (as stated above) at 6, 24, 48 and 72 h post-PBS injection. Leucocyte counts of the bronchoalveolar lavage fluid were then done. The lungs from the animals were also removed for histopathological analysis.

2.6. Drugs

N^{ω}-Nitro-L-arginine methyl ester (L-NAME), D-NAME and ovalbumin (Grade III) were obtained from Sigma (St. Louis, MO, USA). Chloral hydrate was obtained from Quimibrás Ind. Química (Rio de Janeiro, RJ, Brazil). The composition of the Krebs solution was (mM): NaCl, 118; $NaHCO_3$, 25; glucose, 5.6; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4 7H_2O$, 1.17 and $CaCl_2 6H_2O$, 2.5.

2.7. Statistical analysis

Data are presented as the means ± S.E.M. and were analysed by analysis of variance (ANOVA) for multiple comparisons followed by Duncan's test. A P value of less than 0.05 was considered to indicate significance.

Table 1
Lack of effect of N^{ω}-nitro-L-arginine methyl ester (L-NAME) on the leucocyte content in the bronchoalveolar lavage (BAL) fluid from non-sensitized rats

Cell type	Time (h)	Leucocytes ($\times 10^6$/BAL)	
		Control	L-NAME
TL	6	2.07 ± 0.4	2.89 ± 0.4
	24	3.88 ± 0.3	3.21 ± 0.7
	48	1.86 ± 0.3	2.2 ± 0.4
	72	0.8 ± 0.08	1.15 ± 0.2
NE	6	0.05 ± 0.01	0.06 ± 0.01
	24	0.38 ± 0.1	0.47 ± 0.1
	48	0.27 ± 0.1	0.23 ± 0.07
	72	0.17 ± 0.12	0.16 ± 0.11
MN	6	2.02 ± 0.4	2.83 ± 0.4
	24	3.5 ± 0.3	2.74 ± 0.6
	48	1.6 ± 0.3	1.97 ± 0.4
	72	0.63 ± 0.2	0.99 ± 0.1

TL, total leucocytes; NE, neutrophils; MN, mononuclear cells.
The results represent the mean ± S.E.M. of 5–14 rats.
The non-sensitized rats were injected subcutaneously with 0.15 ml of 8 mg $Al(OH)_3$ and two weeks later intratracheally injected with ovalbumin (0.4 ml of 0.25% solution).
L-NAME (75 μmol rat^{-1} day^{-1}) was administered in the drinking water for four weeks.
Control animals received tap water alone.

3. Results

Rats treated chronically with L-NAME (75 μmol rat^{-1} day^{-1}) for 4 weeks showed a significant increase in tail cuff pressure, whether non-sensitized (151 ± 2.0 mmHg; $P < 0.05$) or ovalbumin-sensitized (155 ± 2.4 mmHg; $P < 0.05$) compared to untreated animals (120 ± 1.9 and 117 ± 2.5 mmHg for non-sensitized and ovalbumin-sensitized animals, respectively). The body weight gain among these four groups of animals did not differ significantly (not shown).

3.1. Bronchoalveolar lavage fluid analysis: naive rats

The intratracheal injection of PBS (0.4 ml) per se both in Control and L-NAME-treated naive rats did not evoke leucocyte infiltration, as assessed at 6 (1.2 ± 0.1 and 1.1 ± 0.08 × 10^6 leucocytes/bronchoalveolar lavage, respectively; $n = 4$), 24 (0.9 ± 0.1 and 1.1 ± 0.15 × 10^6 leucocytes/bronchoalveolar lavage, respectively; $n = 4$), 48 (1.0 ± 0.1 and 1.1 ± 0.4 × 10^6 leucocytes/bronchoalveolar lavage, respectively; $n = 3$) and 72 (1.2 ± 0.06 and 0.8 ± 0.1 × 10^6 leucocytes/bronchoalveolar lavage, respectively; $n = 4$) h post-PBS injection. This was made up of > 99% mononuclear cells.

3.2. Bronchoalveolar lavage fluid analysis: non-sensitized rats

The intratracheal injection of ovalbumin (0.4 ml of 0.25% solution) into the non-sensitized rats evoked a discrete total leucocyte infiltration which peaked 24 h post-ovalbumin injection (Table 1). At this time, the infiltrate was made up of mononuclear cells (90%) and neutrophils (10%; Table 1). Eosinophils were virtually absent in the bronchoalveolar lavage from the non-sensitized rats regardless of time of evaluation. Chronic treatment of the animals with L-NAME (75 μmol rat^{-1} day^{-1}, 4 weeks) did not significantly affect the total and differential leucocyte infiltration from the non-sensitized animals (Table 1).

3.3. Bronchoalveolar lavage fluid analysis: ovalbumin-sensitized rats

Intratracheal injection of ovalbumin (0.4 ml of 0.25% solution) into the sensitized rats caused a significant increase in total leucocyte infiltration both at 24 and 48 h post-ovalbumin injection (Fig. 1) compared to non-sensitized animals (Table 1). The time-course of counts of the leucocytes present in the bronchoalveolar lavage of these animals showed that neutrophils peaked at 24 h (29%) whereas eosinophils peaked at 48 h (30%) post-ovalbumin injection. The content of mononuclear cells in bronchoalveolar lavage fluid of the ovalbumin-sensitized animals (2.7 ± 0.3, 3.7 ± 1.0 and 3.3 ± 0.6 × 10^6 cells/bronchoalveolar lavage at 6, 24 and 48 h, respectively) did not differ significantly from that of non-sensitized rats (Table 1). At 72 h post-ovalbumin injection, no significant leucocyte infiltration was detected (Fig. 1) as compared to the non-sensitized ones (Table 1).

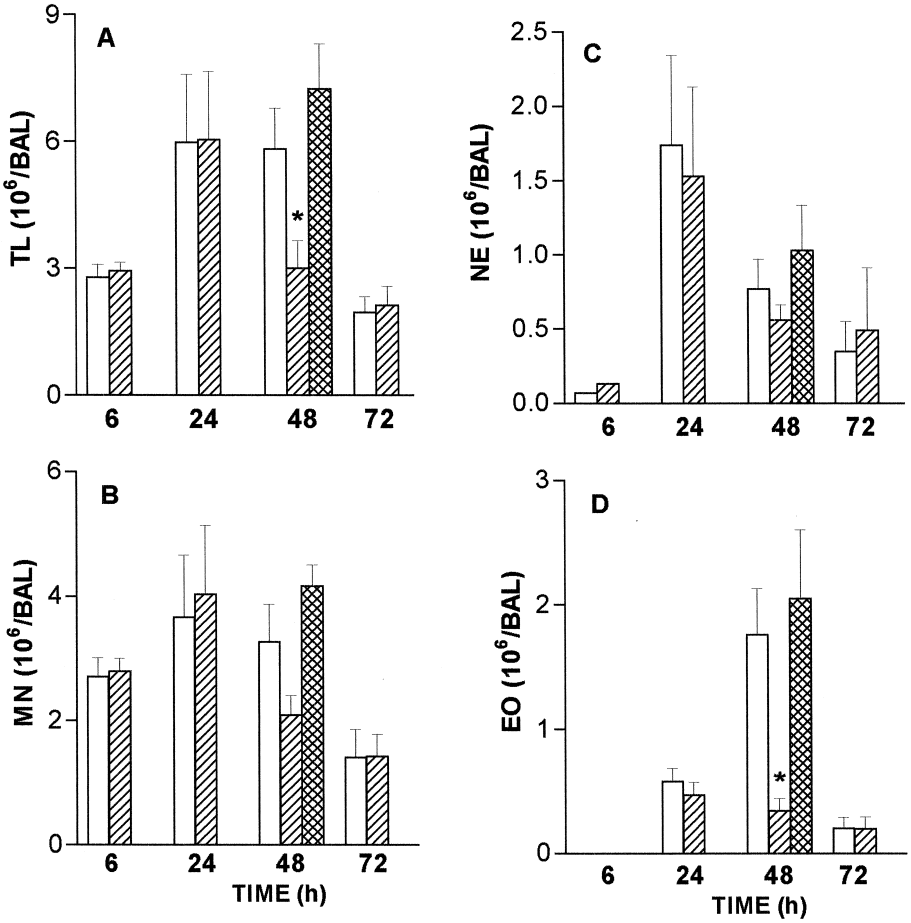

Fig. 1. The effect of chronic treatment of rats with N^{ω}-nitro-L-arginine methyl ester (L-NAME) in the total and differential leucocyte counts in bronchoalveolar lavage fluid from actively sensitized rats after 6, 24, 48 and 72 h following intratracheal injection of ovalbumin. The sensitization procedure and ovalbumin challenge as well as the chronic treatment of the animals with L-NAME are described in Section 2. Open columns represent control animals at 6 ($n = 11$), 24 ($n = 11$), 48 ($n = 13$) and 72 ($n = 4$) h whereas hatched columns represent L-NAME-treated animals at 6 ($n = 10$), 24 ($n = 13$), 48 ($n = 10$) and 72 ($n = 4$) h after intratracheal injection of ovalbumin. The cross-hatched columns represent D-NAME-treated animals 48 h ($n = 5$) after intratracheal injection of ovalbumin. Each column represents the mean ± S.E.M. $*$ $P < 0.05$ when compared to the respective control values. TL, total leucocytes; NE, neutrophil; MN, mononuclear cell; EO, eosinophil.

At both 6 and 24 h post-ovalbumin injection, chronic treatment of the animals with L-NAME (75 μmol rat^{-1} day^{-1}, 4 weeks) affected neither the total nor the differential (neutrophils, eosinophils and mononuclear cells) leucocyte content (Fig. 1). However, at 48 h post-ovalbumin challenge, the total leucocyte influx was reduced by approximately 48% ($P < 0.05$) in the L-NAME-treated animals compared to the non-treated ones (Fig. 1A). This was associated with a marked inhibition (81%; $P < 0.05$) of the recruitment of eosinophils (Fig. 1D) without a significant effect on the influx of neutrophils (Fig. 1C). L-NAME treatment had no significant effect on the content of mononuclear cells (Fig. 1B). At 72 h post-ovalbumin injection, L-NAME had no effect on either total or differential cell counts (Fig. 1).

Chronic treatment of the animals with the inactive enantiomer D-NAME, (75 μmol rat^{-1} day^{-1}, 4 weeks), had no significant effect on either total or differential airway leucocyte influx, as measured 48 h post-ovalbumin injection (Fig. 1A–D).

3.4. Histopathological analysis

Histological examination of the lungs from non-sensitized rats intratracheally injected with PBS showed normal tissue, with no significant amount of inflammatory cells throughout the pulmonary parenchyma as was observed in both non-treated and L-NAME-treated animals (Fig. 2, Panel 1). In non-sensitized rats intratracheally injected with ovalbumin, a slight infiltration of polymorphonuclear and monocytes was seen around respiratory and small vascular segments, also in those animals that received chronic L-NAME (Fig. 2, Panels 2a,b). This picture was completely changed in the ovalbumin-sensitized animals at both 24 and 48 h, when the polymorphonuclear influx became markedly prominent in the connective tissue sur-

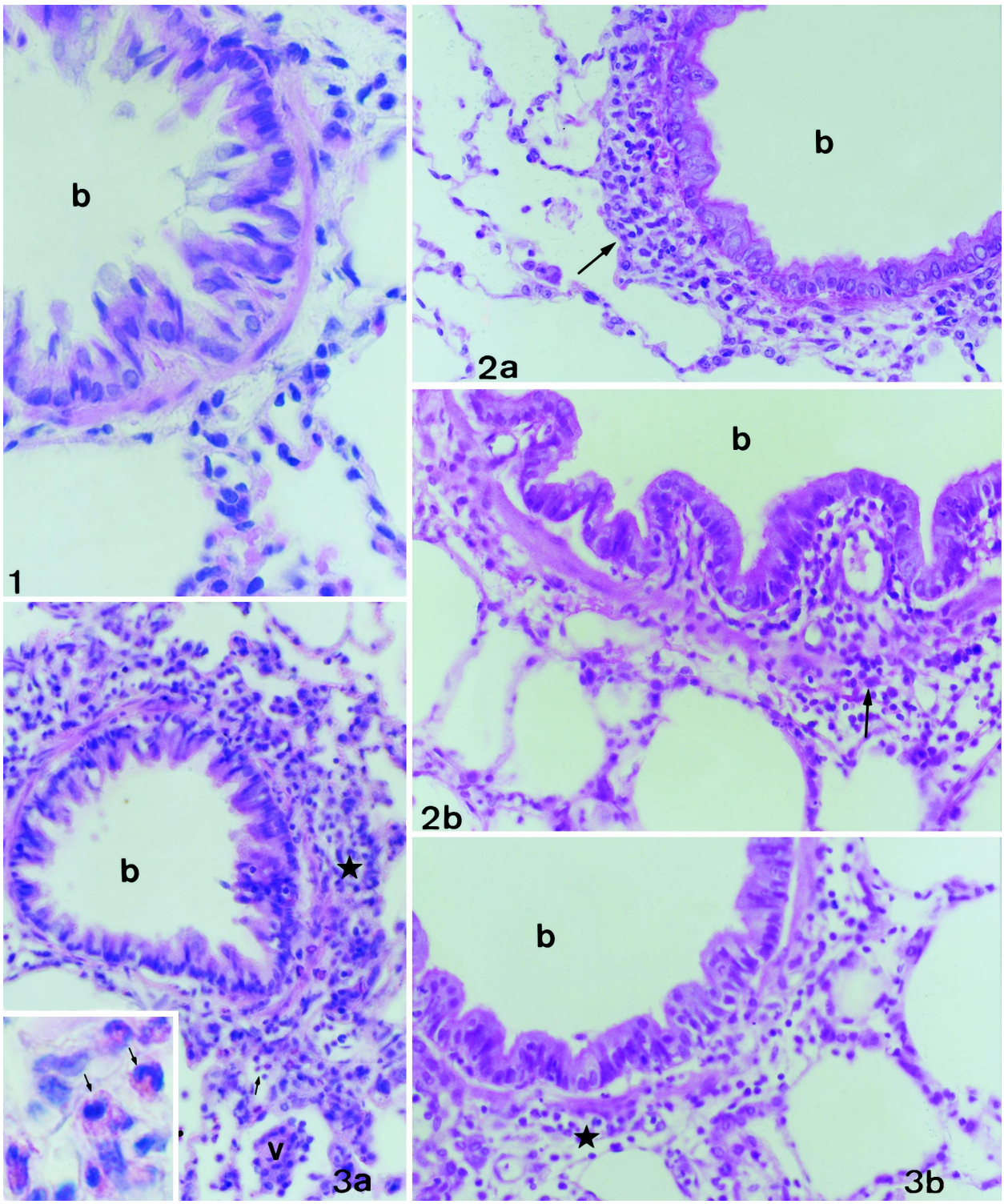

Fig. 2. Panel 1: Bronchioles (b) in lung from non-sensitized rats and intratracheally injected with PBS. The pulmonary parenchyma display a normal histological appearance in both non-treated and L-NAME-treated rats. photomicrography, HE, 512 X. Panel 2: Light photomicrographs from lungs from non-sensitized rats and intratracheally injected with ovalbumin at 48 h. A discrete polymorphonuclear infiltration (arrows) is seen around bronchioles (b) of non-treated (a) and L-NAME-treated (b) rats. HE, 256 X. Panels 3: Histological sections of ovalbumin-sensitized lungs (48 h). A large number of leucocytes (★) are seen around bronchioles (b) and vessels (v) of non-treated rats (a) in comparison with those found in L-NAME-treated rats (b). HE, 256 X. Inset: Higher-power view of the bronchiole wall showing eosinophils (arrows).

rounding the bronchial and bronchiolar segments. The polymorphonuclear infiltration was composed mainly of neutrophils (24 h; not shown) and eosinophils (48 h; Fig. 2, Panel 3a). When compared with this group, the chronically L-NAME-treated rats had a reduced leucocyte infiltration, with eosinophils no longer present in large amounts around the respiratory segments (Fig. 2, Panel 3b). The number of eosinophils observed in the peribronchial regions of sensitized rats intratracheally injected with ovalbumin was significantly reduced in L-NAME-treated animals (4.6 ± 0.3 eosinophils per high-power field) compared to control animals (15.1 ± 0.8 eosinophils per high-power field, $P < 0.05$), as evaluated 48 h post-ovalbumin injection.

4. Discussion

In this study, we clearly demonstrated that the airway eosinophil infiltration in response to intratracheal injection of ovalbumin into actively sensitized animals was markedly attenuated in rats treated chronically with L-NAME, emphasising the importance of NO for in vivo eosinophil migration in both allergic (this study) and non-allergic (Ferreira et al., 1996) inflammation.

In the present model of airway inflammation in rats, we found in bronchoalveolar lavage fluid an initial recruitment of neutrophils (at 24 h post-ovalbumin injection) followed by a delayed eosinophil influx (at 48 h), a pattern of leucocyte migration previously observed in a number of animal models during exposure to antigen (Sanjar et al., 1990; Matsumoto et al., 1994; Vianna and Garcia-Leme, 1995; Fornhem et al., 1995). The inhibition of the leucocyte influx by L-NAME treatment, as assessed by the analysis of bronchoalveolar lavage fluid, was specific for eosinophils, since at the peak of neutrophil influx (24 h) there was no significant difference between non-treated and L-NAME-treated animals. Furthermore, the content of mononuclear cells observed in both non-sensitized and ovalbumin-sensitized rats was also unchanged by L-NAME. Similarly, the lung histopathology of L-NAME-treated animals revealed a reduction of eosinophil infiltration (48 h) surrounding the respiratory segments, compared with non-treated animals. In agreement with the pleurisy model (Ferreira et al., 1996), these results suggest that is unlikely that the inhibition of eosinophil recruitment by L-NAME is secondary to a decrease in airway blood flow. Since the intradermal administration of L-NAME in guinea pig skin inhibits the local accumulation of eosinophils and neutrophils due to a reduction of the cutaneous basal blood flow (Teixeira et al., 1993), it is likely that different mechanisms underlie the leucocyte infiltration into inflammatory sites in experimental animals treated acutely or chronically with NO synthesis inhibitors.

The mechanisms involved in eosinophil recruitment into tissues and their pathophysiological role in allergic reactions are not completely understood (Kroegel et al., 1994). Peritoneal eosinophils obtained from L-NAME-treated rats fail to migrate in vitro in response to fMLP, platelet-activating factor or zymosan-activated serum (Ferreira et al., 1996). The influx of eosinophils into the bronchoalveolar lavage fluid and lung tissue of ovalbumin-challenged allergic mice is also significantly reduced by various nitric oxide inhibitors (Feder et al., 1997). Furthermore, recent studies using affinity-purified mouse monoclonal antibodies demonstrated that rat eosinophils express both type II and type III NO synthases and, also the NO-mediated eosinophil locomotion in vitro takes place through a cyclic-GMP transduction mechanism (Zanardo et al., 1997). These findings suggest strongly that inhibition of eosinophil recruitment in bronchoalveolar lavage fluid by L-NAME is most likely to be due to a direct effect on the eosinophil itself rather than an indirect effect involving other leucocyte types. Accordingly, the failure of L-NAME to affect the neutrophil influx (24 h post-ovalbumin injection) excludes the possibility that eosinophil infiltration relies on the previous accumulation of neutrophils. The $CD4^+$ T-lymphocytes seem to play a role in the antigen-induced eosinophil infiltration into the airways (Gavett et al., 1994). The cloned murine $CD4^+$ Th1 lymphocytes express inducible NO synthase (Taylor-Robinson et al., 1994) and the enhanced NO production is believed to down-regulate the proliferation of Th1 cells causing an increased proliferation of the Th2 subset thus raising the levels of interleukin-4 and interleukin-5, two cytokines with a critical role in the Immunoglobulin E expression and recruitment of eosinophils into the airways, respectively (Barnes and Liew, 1995; Abbas et al., 1996). Whether the reduction of eosinophil influx by chronic blockade of NO synthesis reflects an increased proliferation of Th1 cells (and thus interferon-γ), inhibition of the proliferation of Th2 cells and suppression of interleukin-4 and interleukin-5 production is unclear. Further studies addressed to measure the levels of cytokines (mainly interleukin-4 and interleukin-5) in the bronchoalveolar lavage fluid are necessary to clarify the contribution of $CD4^+$ lymphocytes to the NO-mediated eosinophil migration in the rats in vivo.

Eosinophilia is frequently observed in blood, bronchoalveolar lavage and lungs from patients suffering from bronchial asthma and may be associated with the appearance of inducible NO synthase. Furthermore, both adults (Alving et al., 1993; Persson et al., 1994; Kharitonov et al., 1995; Abbas et al., 1996) and children (Lundberg et al., 1996) with asthma show increased levels of NO in the exhaled air compared with that of healthy age-matched controls. The findings that glucocorticoids inhibit both the levels of NO in the exhaled air (Kharitonov et al., 1995; Lundberg et al., 1996) and the expression of the inducible NO synthase (Knowles et al., 1990) suggest that their well-known beneficial effects in asthma (Barnes, 1995) may be in part mediated by the suppression of inducible

NO synthase in eosinophils. We may, therefore speculate, that selective inducible NO synthase inhibitors would be of therapeutic value in asthma treatment.

Acknowledgements

H.H.A.F. thanks Conselho Nacional de Pesquisas e Tecnologia (CNPq).

References

Abbas, A.K., Murphy, K.M., Sher, A., 1996. Functional diversity of helper T lymphocytes. Nature 383, 787–793.

Alving, K., Fornhem, C., Weitzberg, E., Lundberg, J.M., 1992. Nitric oxide mediates cigarette smoke-induced vasodilatory response in the lung. Acta Physiol. Scand. 146, 407–408.

Alving, K., Weitzberg, E., Lundberg, J.M., 1993. Increased amounts of nitric oxide in exhaled air of asthmatics. Eur. Resp. J. 6, 1368–1370.

Barnes, P.J., 1995. Inhaled glucocorticoids for asthma. New Engl. J. Med. 332, 868–875.

Barnes, P.J., Liew, F.Y., 1995. Nitric oxide and asthmatic inflammation. Immunol. Today 16, 128–130.

Barnes, P.J., Belvisi, M.G., 1993. Nitric oxide and lung disease. Thorax 48, 1034–1043.

Bredt, D.S., Hwang, P.M., Glatt, C.E., Lowenstein, C., Reed, R.R., Snyder, S.H., 1991. Cloned and expressed nitric synthase structurally resembles cytochrome P-450 reductase. Nature 351, 714–718.

Erjefalt, J.S., Erjefalt, I., Sundler, F., Persson, C.G.A., 1994. Mucosal nitric oxide may tonically suppress airways plasma exudation. Am. J. Respir. Dis. Crit. Care Med. 150, 227–232.

Feder, L.S., Stelts, D., Chapman, R.W., Manfra, D., Crawley, Y., Jones, H., Minnicozzi, M., Fernandez, X., Paster, T., Egan, R.W., Kreutner, W., Kung, T.T., 1997. Role of nitric oxide on eosinophilic lung inflammation in allergic mice. Am. J. Respir. Cell Mol. Biol. 17, 436–442.

Ferreira, H.H.A., Medeiros, M.V., Lima, C.S.P., Flores, C.A., Sannomiya, P., Antunes, E., De Nucci, G., 1996. Inhibition of eosinophil chemotaxis by chronic blockade of nitric oxide biosynthesis. Eur. J. Pharmacol. 310, 201–207.

Fornhem, C., Kumlin, M., Lundberg, J.M., Alving, K., 1995. Allergen-induced late-phase airways obstruction in the pig: mediator release and eosinophil recruitment. Eur. Respir. J. 8, 1100–1109.

Gavett, S.H., Chen, X., Finkelman, F., Wills-Karp, M., 1994. Depletion of murine $CD4^+$ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. Am. J. Resp. Cell Mol. Biol. 10, 587–593.

Hamid, Q., Springall, D.R., Riveros-Moreno, V., 1993. Induction of nitric oxide synthase in asthma. Lancet 342, 1510–1513.

Hogman, M., Frostell, C.G., Hedenstrom, H., Hedenstierna, G., 1993. Inhalation of nitric oxide modulates adult human bronchial tone. Am. Rev. Respir. Dis. 148, 1474–1478.

Janssens, S.P., Shimouchi, A., Quertermous, T., Block, D.B., Block, K.D., 1992. Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase. J. Biol. Chem. 267, 14519–14522.

Kharitonov, S.A., Yates, D., Robbins, R.A., Logan-Sinclair, R., Shinebourne, E.A., Barnes, P.J., 1994. Increased nitric oxide in exhaled air of asthmatic patients. Lancet 343, 133–135.

Kharitonov, S.A., Yates, D.H., Barnes, P.J., 1995. Regular inhaled budesonide decreases nitric oxide concentration in the exhaled air of asthmatic patients. Am. J. Resp. Crit. Care Med. 151, A387.

Knowles, R.G., Salter, M., Brooks, S.L., Moncada, S., 1990. Anti-inflammatory glucocorticoids inhibit the induction by endotoxin of nitric oxide in the lung, liver, and aorta of the rat. Biochem. Biophys. Res. Commun. 172, 1042–1048.

Kroegel, C., Virchow, J.-C. Jr., Luttmann, W., Walker, C., Warner, J.A., 1994. Pulmonary immune cells in health and disease: the eosinophil leucocyte. Eur. Respir. J. 7, 519–543.

Kuo, H.P., Liu, S., Barnes, P.J., 1992. The effect of endogenous nitric oxide on neurogenic plasma exudation in guinea pig airways. Eur. J. Pharmacol. 221, 385–388.

Lamas, S., Marsden, P.A., Li, G.L., Tempst, P., Michel, T., 1992. Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Proc. Natl. Acad. Sci. USA 89, 6348–6352.

Lundberg, J.O.N., Nordvall, S.L., Weitzberg, E., Kollberg, H., Alving, K., 1996. Exhaled NO in pediatric asthma and cystic fibrosis. Arch. Dis. Child 75, 323–326.

Matsumoto, T., Ashida, Y., Tsukuda, R., 1994. Pharmacological modulation of immediate and late airway response and leucocyte infiltration in the guinea pig. J. Pharmacol. Exp. Ther. 269, 1236–1244.

Palmer, R.M.J., Ashton, D.S., Moncada, S., 1988. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 33, 664–666.

Persson, M.G., Zetterstrom, O., Agrenius, V., Ihre, E., Gustafsson, L.E., 1994. Single-breath nitric oxide measurements in asthmatic patients and smokers. Lancet 343, 146–147.

Ribeiro, M.O., Antunes, E., De Nucci, G., Lovisolo, S.M., Zatz, R., 1992. Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. Hypertension 20, 298–303.

Robbins, R.A., Springall, D.R., Warren, J.B., Kwon, O.J., Buttery, L.D.K., Wilson, A.J., Adcock, I.M., Riveros-Moreno, V., Moncada, S., Polak, J., Barnes, P.J., 1994. Inducible nitric oxide synthase is increased in murine lung epithelial cells by cytokine stimulation. Biochem. Biophys. Res. Commun. 198, 1027–1033.

Sanjar, S., Aoki, S., Kristersson, A., Smith, D., Morley, J., 1990. Antigen challenge induces pulmonary airway eosinophil accumulation and airway hyperreactivity in sensitised guinea pigs: the effect of anti-asthma drugs. Br. J. Pharmacol. 99, 679–686.

Taylor-Robinson, A.W., Liew, F.Y., Severn, A., Xu, D., McSorley, S.J., Garside, P., Padron, J., Phillips, R.S., 1994. Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cells. Eur. J. Immunol. 24, 980–984.

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.

Vane, J.R., 1964. The use of isolated organs for detecting active substances in the circulating blood. Br. J. Pharmacol. Chemother. 23, 360–373.

Vianna, E.O., Garcia-Leme, J., 1995. Allergen-induced airway inflammation in rats. Role of insulin. Am. J. Resp. Crit. Care Med. 151, 809–814.

Xie, Q.W., Cho, H.J., Calaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D., Ding, A., Troso, T., Nathan, C., 1992. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 256, 225–228.

Yeadon, M., Price, R., 1995. Induction of calcium-independent nitric oxide synthase by allergen challenge in sensitized rat lung in vivo. Br. J. Pharmacol. 116, 2545–2546.

Zanardo, R.C.O., Costa, E., Ferreira, H.H.A., 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.

Zatz, R., 1990. A low-cost tail-cuff method for the estimation of mean