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Different mechanisms underlie the effects of acute and long-term inhibition of nitric oxide synthases in antigen-induced pulmonary eosinophil recruitment in BALB/C mice

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ABSTRACT

Nitric oxide synthase (NOS) inhibitors are largely used to evaluate the NO contribution to pulmonary allergy, but contrasting data have been reported. In this study, pharmacological, biochemical and pharmacokinetic assays were performed to compare the effects of acute and long-term treatment of BALB/C mice with the non-selective NOS inhibitor L-NAME in ovalbumin (OVA)-challenged mice. Acute L-NAME treatment (50 mg/kg, gavage) significantly reduced the eosinophil number in bronchoalveolar lavage fluid (BALF). The inducible NOS (iNOS) inhibitor aminoguanidine (20 mg/kg/day in the drinking water) also significantly reduced the eosinophil number in BALF. In contrast, 3-week L-NAME treatment (50 and 150 mg/kg/day in the drinking water) significantly increased the pulmonary eosinophil influx. The constitutive NOS (cNOS) activity in brain and lungs was reduced by both acute and 3-week L-NAME treatments. The pulmonary iNOS activity was reduced by acute L-NAME (or aminoguanidine), but unaffected by 3-week L-NAME treatment. Acute L-NAME (or aminoguanidine) treatment was more efficient to reduce the NO_X^- levels compared with 3-week L-NAME treatment. The pharmacokinetic study revealed that L-NAME is not bioavailable when given orally. After acute L-NAME intake, serum concentrations of the metabolite N^{ω}-nitro-L-arginine decreased from 30 min to 24 h. In the 3-week L-NAME treatment, the N^{ω}-nitro-L-arginine concentration was close to the detection limit. In conclusion, 3-week treatment with L-NAME yields low serum N^{ω}-nitro-L-arginine concentrations, causing preferential inhibition of cNOS activity. Therefore, eosinophil influx potentiation by 3-week L-NAME treatment may reflect removal of protective cNOS-derived NO, with no interference on the ongoing inflammation due to iNOS-derived NO.

1. Introduction

Selective accumulation of eosinophils into the airways has become a central concept of the asthma pathology [1,2]. Eosinophils synthesize and release proinflammatory substances, including the preformed cytotoxic granular basic proteins (major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin) and *de novo* synthesized products (eicosanoids, platelet-activating factor, oxygen metabolite, cytokines, and neuropeptides) [3]. The mechanisms leading to mobilization of mature eosinophils from bone marrow to peripheral blood, and the sequence of events from their activation to recruitment into inflammatory tissue sites remain to be completely understood, but IL-5 and eotaxins have been clearly involved in the selective regulation of eosinophil trafficking [4,5].

Nitric oxide (NO) has been reported to modulate the pulmonary eosinophilia and airway hyperresponsiveness in allergic animal models [6]. However, contrasting findings have been reported, which may depends on several factors such as dose and type of the NO synthase (NOS) inhibitor used (selective or non-selective), route of allergen challenge (intratracheal, aerosol or instillation) and animal species employed (guinea-pig, rat or mouse), as well as if the work deals with gene deletion for the inducible NOS (iNOS)

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[7–16]. The use of NO-donor compounds in human purified eosinophils also gives contrasting data compared with the NO synthase inhibitors [17,18]. Therefore, additional studies are still required to elucidate the modulatory role of NO in the eosinophil functions.

The major pathophysiological alterations due to NO deficiency are achieved with long-term NO blockade [19], but few studies have been carried out to evaluate the influence of prolonged NO deficiency in allergic disease models. We have previously shown that long-term NO blockade with the non-selective NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) suppresses the pulmonary eosinophil infiltration in non-allergic and ovalbumin (OVA)-challenged rats, and increases the eosinophil number in bone marrow [20–22]. However, experimental models of allergy in rats present great methodological limitations, restricting the investigation progress. Therefore, we have moved to mice to further investigate the modulatory role of NO in the eosinophil migration in vivo, carrying out pharmacological, biochemical and pharmacokinetic studies to compare the effects of acute and long-term treatment with NO synthase inhibitors in BALB/C mice previously sensitized with ovalbumin (OVA). Generally, we examined the counts of leukocytes (focusing on the eosinophils) in bronchoalveolar lavage (BAL) fluid, lung parenchyma, peripheral blood and bone marrow after acute (50 mg/kg, given by gavage) or long-term L-NAME administration (50 and 150 mg/kg/day, given for 3 weeks in the drinking water). Aminoguanidine (20 mg/kg/day, given for 3 weeks in the drinking water) was used as a control for a selective iNOS inhibition. The counts of leukocytes in bronchoalveolar lavage (BAL) fluid, lung parenchyma, peripheral blood and bone marrow were evaluated. We have also evaluated the activities of constitutive (cNOS) and inducible NOS (iNOS) in brain and lungs, as well as the levels of IgE, eotaxin and NO_x^- in serum and/or BAL fluid in all groups. A pharmacokinetic study was also carried out in order to quantify the serum levels of L-NAME and its metabolite N^{ω}-nitro-L-arginine after acute and long-term L-NAME intake.

2. Materials and methods

2.1. Drugs and reagents

L-[$^{2,3,4,5-3}$H] arginine monohydrochloride was purchased from GE Healthcare (U.K.). All other products were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

2.2. Animals

The experimental protocols were approved by the Ethical Principles in Animal Research adopted by the Brazilian College for Animal Experimentation (COBEA), and followed the Guide for the Care and Use of Laboratory Animals. Male BALB/C mice (initial weight 25–30 g) were provided by Central animal House Services (CEMIB) of State University of Campinas (UNICAMP). The animals were housed 5/cage on a 12-h light–dark cycle, and fed with a standard chow diet with water ad libitum.

2.3. Sensitization procedure and ovalbumin (OVA) challenge

Mice were actively sensitized with a subcutaneous injection (0.4 mL) of 100 μg OVA mixed with 1.6 mg $Al(OH)_3$ in 0.9% NaCl (day 0). One week later (day 7), mice received a second subcutaneous injection of 100 μg OVA (0.4 mL). Non-sensitized mice received only subcutaneous injection of $Al(OH)_3$ (0.4 mL). On days 14 and 15, mice were intranasally challenged with OVA (10 μg/50 μL) twice a day, thus resulting in 4 challenges (on day 14, 1st challenge occurred at time zero and 2nd challenge at 6 h later; on day 15, 3rd challenge occurred at time zero and 4th challenge at 6 h later). On day 16 (48 h after the first challenge), mice were anaesthetized with halothane and exsanguinated, after which a sample of peripheral blood was collected from the abdominal vena cava. The bronchoalveolar lavage (BAL) was performed and the femur was isolated to obtain the bone marrow. Separate experimental procedures were carried out to obtain brain and lungs for the measurements of constitutive and inducible NOS activities, as well as for morphological study, as detailed below.

2.4. Experimental protocols

In acute treatment, mice received a single dose of L-NAME (50 mg/kg, gavage) at 30 min prior to the first OVA challenge (day 14).

In the long-term L-NAME treatments, mice received L-NAME (50 and 150 mg/kg/day) or aminoguanidine (20 mg/kg/day) dissolved in the drinking water for 3 weeks. Treatments started 1 week prior to first OVA sensitization (day 0) and continued for another 2 weeks up to day 16 (for details see the schematic representation at Fig. 1). Untreated (control) animals received tap water alone. The doses chosen for the long-term L-NAME treatment (50 and 150 mg/kg/day) were based on our prior experiences in giving daily L-NAME to rats [23,24]. The doses chosen for aminoguanidine (20 mg/kg/day) and acute L-NAME (50 mg/kg) were based on previous studies in mice [8,25,26].

2.5. Bronchoalveolar lavage (BAL) fluid

As mentioned above, BAL was performed 48 h after the first challenge, a time by which a massive pulmonary eosinophil is seen. The mice were anaesthetized with halothane, and the trachea of animals was exposed and cannulated with a polyethylene tube connected to a syringe. The lungs were washed by flushing phosphate-buffered saline (PBS). The PBS was instilled through the tracheal cannula in 5 aliquots of 300 μL. The fluid recovered after each instillation was combined and centrifuged (500 × *g* for 10 min at 4 °C). The cell pellet was resuspended in 200 μL of PBS, and total (Neubauer) and differential (Diff-Quick stain) cell counts were done. A minimum of 300 cells was counted and classified as eosinophils, neutrophils and mononuclear cells based on normal morphological criteria.

2.6. Peripheral blood and bone marrow leukocytes

Blood samples were obtained from the abdominal vena cava. The total cell counts were done (Neubauer), and cytospin smears (Diff-Quick stain) were used to differential cell count. A minimum

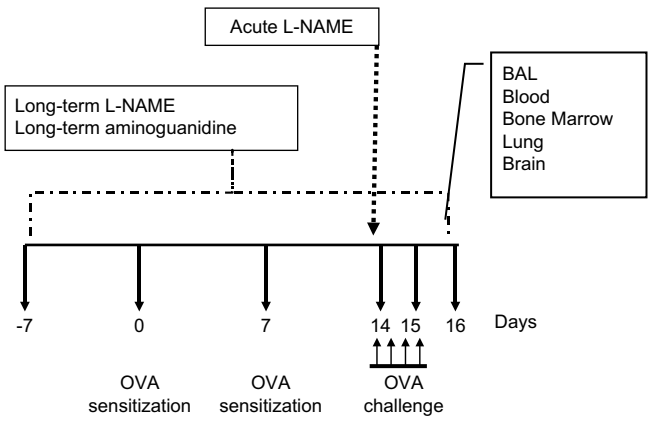

Fig. 1. Schematic representation of the experimental model.

of 300 cells was counted, and classified as eosinophils, neutrophils and mononuclear cells based on normal morphological criteria.

The femurs of mice were also removed immediately after killing and the epiphyses were cut transversely. Bone marrow cells were collected by flushing the 2 femurs with PBS, and the total (Neubauer) and differential (Diff-Quick satin) cell counts were done. A minimum of 300 cells was counted. Both bone marrow eosinophils were evaluated as the mixture of mature and immature forms, as recognized by the intensely eosinophilic granules.

2.7. Determination of NO synthase (NOS) activities

The effects L-NAME treatment on constitutive and inducible NOS activities (brain and lungs) were studied through the rate of conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline according to a previous study [27]. Animals were anesthetised with inhaled halothane, and sacrificed by cervical dislocation. The brains and lungs were rapidly removed and homogenized in 5 volumes of the cold incubation buffer (50 mM Tris–HCl buffer, pH 7.4) containing 1 mM phenylmethyl-sulphonyl fluoride (PMSF) and 1 mM L-citrulline. The homogenates were incubated for 30 min in the presence of 1 mM NADPH, 2 mM $CaCl_2$ and 10 μM L-arginine containing 100,000 dpm of [$^{2,3,4,5\text{-}3}$H]-L-arginine monohydrochloride (Amersham, U.K.) at room temperature (25–27 °C). Each homogenate sample was also assayed in the presence of 1 mM L-NAME added exogenously to the incubation medium in order to specifically characterize the conversion as due to NOS (blank). In addition, Ca^{2+}-independent inducible NOS activity was assessed in the homogenates by excluding $CaCl_2$ from the incubation medium and adding 1 mM EGTA. Protein content in the homogenates was determined according to Bradford [28], and NOS activity was expressed as pmol of L-citrulline produced per min and per mg of protein.

2.8. Histological analysis

Lungs were removed, immersed in 10% phosphate-buffered formalin for 24 h and then kept in 70% ethanol until embedding in paraffin. Tissues were sliced (5 μm sections) and stained with hematoxilin/eosin for light microscopy examination. The eosinophil counts in lung parenchyma were performed by microscopy in a 28-μm^2 around bronchi. The values represent means of 10 bronchi analyzed at ×500 magnification.

2.9. Serum levels of total IgE

Blood samples were obtained from the abdominal vena cava and left at room temperature for 30 min. After centrifugation (10 min, 4000 × *g*), serum was collected and kept at −20 °C until analysis. Total IgE was measured with commercially available enzyme-linked immunosorbent assay (ELISA) kit (Mouse IgE ELISA), following the instructions of the manufacturer (Bethyl Laboratories, Montgomery, TX, U.S.A.).

2.10. Nitrite assay

The levels of NO_x (defined as the sum of nitrate and nitrite) in the BAL fluid were determined using a commercially available kit (Nitrate/Nitrite Colorimetric Assay Kit), following the instructions of the manufacturer (Cayman Chemical, Ann Arbor, MI, U.S.A.). This assay determines the total NO based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. After the conversion, the spectrophotometric measurement of nitrite is accomplished by using the Griess reaction. The resulting deep purple azo compound absorbs light at 540–550 nm.

2.11. Eotaxin level measurement

Eotaxin was measured in BAL fluid using commercially available enzyme-linked immunosorbent assay (ELISA) kits for mouse eotaxin, following the instructions of the manufacturer (R&D, Minneapolis, U.S.A.).

2.12. Pharmacokinetic study: measurement of N$^{\omega}$-nitro-L-arginine and L-NAME in serum

Serum from 5 mice was pooled and frozen at −80 °C until use. Serum samples were thawed at room temperature and centrifuged at 2550 *g* for 5 min at 4 °C. Waters HLB Oasis® SPE cartridges were pre-conditioned by washing first with 1 mL methanol, 2 mL of water and 3 mL of bicarbonate buffer solution (pH 9.0), under light vacuum. Each aliquot of 0.1 mL of serum and 0.5 mL of bicarbonate buffer solution (pH 9.0) was slowly drawn through individual cartridge under light vacuum. The cartridges were then washed 2 times with 1 mL of water under light vacuum, and placed into 12 × 120 mm appropriately numbered glass tubes. They were then washed with 0.8 mL of methanol, and the eluate collected into the glass tubes by applying a light positive pressure using a nitrogen flow. This process was conducted in a fume cupboard. The dry residues were reconstituted with 0.1 mL of methanol and vortex-mixed for 15 s. The solutions were then transferred to the auto-injector microvials.

The determination of L-NAME and N^{ω}-nitro-L-arginine in the mouse serum was performed by liquid chromatography with positive ion electrospray ionization (ESI) coupled to tandem mass spectrometry (QUATTRO LC, Micromass, U.K.) detection. The QUATTRO LC was set up for multiple reaction monitoring (MRM) for the following transitions: 220 > 87 and 234 > 87 for N^{ω}-nitro-L-arginine and L-NAME, respectively.

The chromatography was performed on a Sodex D-4 reversed-phase analytical column. The mobile phase consisted of methanol/water (40:60 v/v) plus 0.1% formic acid. The method had a total run time of 4.0 min and was validated for recovery, linearity, precision and accuracy. The recoveries were 61.5% (RSD 2.9%) and 38.3% (RSD 3.0%) for the 150 ng/mL standard concentration of N^{ω}-nitro-L-arginine and L-NAME, respectively. The method had a linear calibration curve over the range 10–500 ng/mL ($r^2 > 0.990836$). The limit of quantification was 10 ng/mL. The between-run precision for L-NAME was 6.9% (30 ng/mL), 15.9% (150 ng/mL) and 4.7% (300 ng/mL), whereas its between-run accuracy was 106.6%, 94.4% and 97.9% for the above-mentioned concentrations, respectively. The between-run precision for N^{ω}-nitro-L-arginine was 2.2% (30 ng/mL), 5.2% (150 ng/mL) and 1.5% (300 ng/mL), whereas its between-run accuracy was 104.7%, 100.5% and 97.3% for the above-mentioned concentrations, respectively.

2.13. Statistical analysis

Data are presented as the means ± S.E.M. and were analyzed by analysis of variance (ANOVA) for multiple comparisons followed by Tukey's test, or unpaired Student's *t*-test when appropriate. A value of $p < 0.05$ was taken as significant.

3. Results

Table 1 summarizes the experimental protocols employed in this study for non-sensitized and OVA-sensitized mice, treated or not with either L-NAME or aminoguanidine.

Table 1
Experimental protocols carried out in non-sensitized and ovalbumin (OVA)-sensitized BALB/C mice.

Groups	Treatment	Sensitization	Measurements
1	None	Non-sensitized	Cell count in BAL, PB and BM Lung histology cNOS and iNOS activity IgE and eotaxin levels
2	None	OVA-sensitized	
3	Long-term L-NAME (50 mg/kg per day, 3 weeks)	Non-sensitized	BAL counts
4	Long-term L-NAME (50 mg/kg per day, 3 weeks)	OVA-sensitized	
5	Long-term L-NAME (150 mg/kg per day, 3 weeks)	Non-sensitized	Cell count in BAL, PB and BM Lung histology cNOS and iNOS activity IgE and eotaxin levels
6	Long-term L-NAME (150 mg/kg per day, 3 weeks)	OVA-sensitized	
7	Acute L-NAME (50 mg/kg, gavage)	Non-sensitized	Cell count in BAL, PB and BM cNOS and iNOS activity
8	Acute L-NAME (50 mg/kg, gavage)	OVA-sensitized	
9	Long-term aminoguanidine (20 mg/kg per day, 3 weeks)	Non-sensitized	Cell count in BAL, PB and BM Lung histology cNOS and iNOS activity IgE and eotaxin levels
10	Long-term aminoguanidine (20 mg/kg per day, 3 weeks)	OVA-sensitized	

BAL, bronchoalveolar lavage; PB, peripheral blood; BM, bone marrow; cNOS; constitutive nitric oxide synthase; iNOS, inducible nitric oxide synthase; IgE, immunoglobulin E.

3.1. BAL fluid and lung histology

The counts of eosinophils in BAL at 48 h post-OVA challenge in BALB/C mice were significantly higher ($0.1 \pm 0.01 \times 10^6$ cells/BAL) compared with non-sensitized animals instilled intranasally with OVA ($0.01 \pm 0.01 \times 10^6$ cells/BAL; $p < 0.0001$).

Long-term treatment of animals with L-NAME for 3 weeks at 50 and 150 mg/kg/day dose-dependently increased ($p < 0.001$) the eosinophil number in BAL fluid of OVA-challenged mice compared with untreated animals ($n = 7$–14; Fig. 2). For further experiments using long-term L-NAME treatment, the dose of 150 mg/kg/day was routinely used.

The increased eosinophil number in BAL fluid in mice treated with L-NAME for 3 weeks was confirmed by the histology. The eosinophil number in peribronchiolar parenchyma at 48 h post-OVA challenge was higher in the L-NAME group compared with untreated mice (27.0 ± 5.1 and 18.2 ± 1.6 eosinophils/bronchi; $n = 4$–5). Eosinophils in lungs from non-sensitized mice (either untreated or treated with L-NAME) were nearly absent.

In contrast to long-term treatment, acute L-NAME treatment (50 mg/kg), given by gavage 30 min prior to first OVA challenge, largely reduced the eosinophil infiltration, compared with untreated challenged mice ($n = 7$–14; Fig. 2). Treatment with aminoguanidine (20 mg/kg/day; 3 weeks; $n = 7$) also nearly abolished the eosinophil infiltration in BAL fluid in OVA-challenged animals (Fig. 2).

None of the above-mentioned treatments affected the eosinophil number in non-sensitized mice ($n = 7$–14 in each group; not shown).

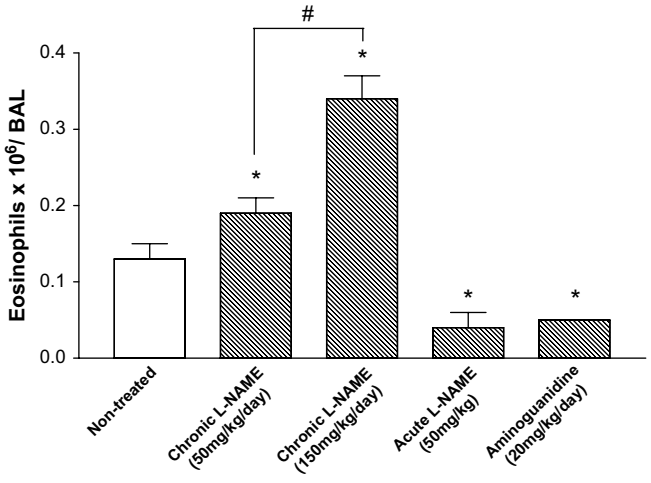

Fig. 2. The effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine in the eosinophil number in bronchoalveolar lavage (BAL) fluid from ovalbumin (OVA)-sensitized mice after 48 h following intranasal instillation of OVA. L-NAME was given either for 3 weeks (50 and 150 mg/kg/day in the drinking water) or acutely (50 mg/kg, gavage, 30 min prior the first challenge). Aminoguanidine (AG) was given at 20 mg/kg/day in the drinking water for 3 weeks (for details, see Section 2). Each column represents the mean ± S.E.M. for $n = 7$–14. $^{*}p < 0.05$ compared with non-treated group; $^{\#}p < 0.05$ comparison between long-term L-NAME treatments.

3.2. Peripheral blood and bone marrow

The counts of eosinophils in both peripheral blood and bone marrow at 48 h post-OVA challenge in BALB/C mice were significantly higher ($p < 0.05$) compared with non-sensitized animals instilled with OVA (Fig. 3A, B).

Long-term treatment of animals with L-NAME (150 mg/kg/day) increased by about of 50% ($p < 0.05$) the number of peripheral eosinophils in OVA-challenged mice in comparison with untreated mice ($n = 14$; Fig. 3A). In contrast, acute L-NAME ($n = 7$) and aminoguanidine ($n = 7$) significantly reduced the blood eosinophil counts (Fig. 3A). The number of eosinophils in peripheral blood of non-sensitized mice was unchanged in all groups (Fig. 3A).

With regard to the bone marrow (Fig. 3B), long-term treatment of animals with L-NAME ($n = 14$) had no effect in the eosinophil

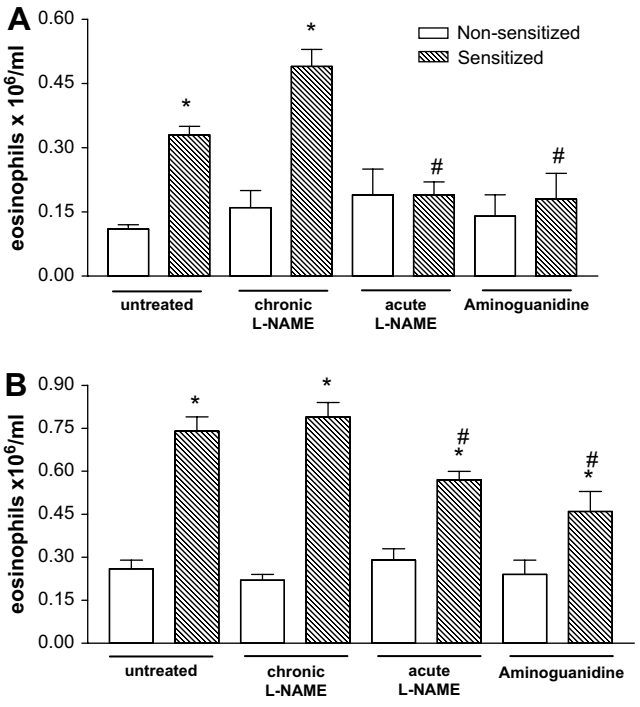

Fig. 3. The effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine in the eosinophil number in peripheral blood (panel A) and bone marrow (panel B) at 48 h following intranasal instillation of ovalbumin (OVA) in sensitized and non-sensitized mice. L-NAME was given either for 3 weeks (150 mg/kg/day in the drinking water) or acutely (50 mg/kg, gavage, 30 min prior the first challenge), whereas aminoguanidine was given at 20 mg/kg/day in the drinking water for 3 weeks (for details, see Section 2). Each column represents the mean ± S.E.M. for $n = 7$–14. $^{*}p < 0.05$ compared with the respective non-sensitized group; $^{\#}p < 0.05$ compared with respective long-term L-NAME group.

counts, whereas acute L-NAME ($n = 7$) and aminoguanidine ($n = 14$) significantly reduced bone marrow eosinophil number by about of 24% and 38%, respectively, compared with untreated OVA-challenged mice. The number of eosinophils in bone marrow of non-sensitized mice was unchanged in all groups (Fig. 3B).

3.3. Brain NOS activity

The brain Ca^{2+}-dependent NOS (bNOS) activity in untreated mice did not differ significantly between non-sensitized and OVA-sensitized mice ($n = 5$; Fig. 4). Long-term treatment with L-NAME (150 mg/kg/day) nearly abolished the brain Ca^{2+}-dependent NOS activity in both non-sensitized and OVA-sensitized mice. Treatment with aminoguanidine (20 mg/kg/day) had no effect in the brain Ca^{2+}-dependent NOS activity in any studied group (Fig. 4).

In separate experiments, the bNOS activity was measured in mice treated acutely with L-NAME (50 mg/kg). This treatment significantly reduced the bNOS activity (5.8 ± 0.4 in untreated and 3.6 ± 0.2 pmol/min/mg protein in L-NAME-treated mice; $p < 0.05$). The Ca^{2+}-independent NOS (iNOS) activity in brain was nearly absent in all groups ($n = 5$–7).

3.4. Lung NOS activity

The Ca^{2+}-independent NOS (iNOS) activity in the lung homogenates increased by about of 35-fold ($p < 0.001$) in the OVA-sensitized animal compared with non-sensitized mice ($n = 5$; Fig. 5). Long-term treatment with L-NAME (150 mg/kg/day) had no significant effect in the lung iNOS activity, whereas aminoguanidine (20 mg/kg/day) nearly abolished the increased iNOS activity seen in the lungs of OVA-sensitized mice (Fig. 5). In separate experiments, the lung iNOS activity was measured in mice treated acutely with L-NAME (50 mg/kg). This treatment significantly reduced both the iNOS activity in the lungs of OVA-challenged mice (1.2 ± 0.2 in untreated and 0.7 ± 0.05 pmol/min/mg protein in L-NAME-treated mice; $p < 0.05$). Long-term and acute L-NAME treatments, as well as aminoguanidine did not significantly affect the basal iNOS activity in the lungs of non-sensitized mice (Fig. 5).

The Ca^{2+}-dependent NOS (cNOS) activity in lungs of non-sensitized and OVA-sensitized mice was also evaluated in all groups. Pulmonary cNOS activity was detected in the lung of non-challenged mice (0.67 ± 0.26 pmol/min/mg protein; $n = 5$), whereas in the lung of OVA-challenged mice it was virtually undetectable ($n = 5$). In the lungs of non-challenged mice, the cNOS activity was nearly abolished by either long-term or acute treatment with L-NAME ($n = 5$), but unaffected by aminoguanidine treatment ($n = 5$).

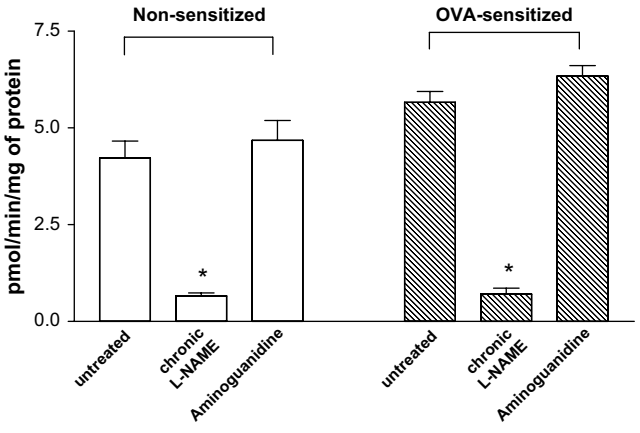

Fig. 4. The effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine in the brain nitric oxide synthase (NOS) activity. The brains were collected from non-sensitized and ovalbumin (OVA)-sensitized mice at 48 h following intranasal instillation of OVA. Aminoguanidine (20 mg/kg/day) and L-NAME (150 mg/kg/day) were given in the drinking water for 3 weeks (for details, see Section 2). Each column represents the mean ± S.E.M. for $n = 5$–7 each. $^*p < 0.05$ compared with the respective untreated group.

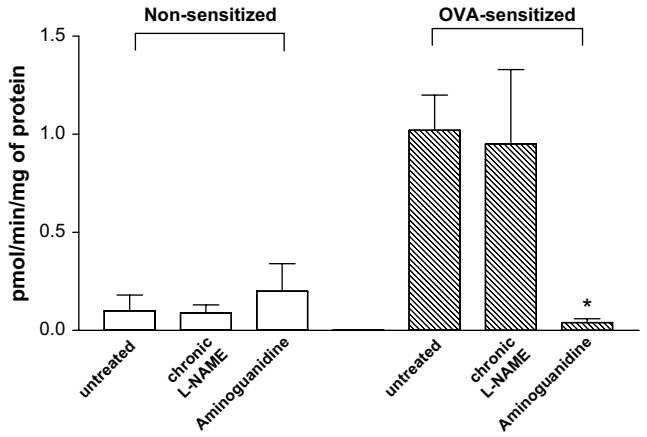

Fig. 5. The effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine in the pulmonary inducible nitric oxide synthase (NOS) activity. The lungs were collected from non-sensitized and ovalbumin (OVA)-sensitized mice at 48 h following intranasal instillation of OVA. Aminoguanidine (20 mg/kg/day) and L-NAME (150 mg/kg/day) were given in the drinking water for 3 weeks (for details, see Section 2). Each column represents the mean ± S.E.M. for $n = 5$ each. $^*p < 0.05$ compared with the respective untreated group.

3.5. Levels of IgE, eotaxin and NO_x

The serum IgE levels were significantly increased in OVA-sensitized mice compared with non-sensitized animals, as expected ($p < 0.001$). However, neither long-term L-NAME nor aminoguanidine affected the IgE levels in non-sensitized and OVA-sensitized animals (Fig. 6A).

The levels of eotaxin increased significantly in BAL fluid of OVA-challenged mice in comparison with non-sensitized animals. Long-term treatment with L-NAME (150 mg/kg/day, 3 weeks) did not significantly affect the increased eotaxin levels seen in BAL fluid of OVA-challenged mice. In contrast, aminoguanidine treatment nearly abolished the increased levels of eotaxin in BAL fluid of OVA-challenged mice, bringing the eotaxin levels back to levels observed in non-sensitized animals (Fig. 6B).

The NO_x levels in BAL fluid of OVA-challenged mice (untreated animals) were significantly higher (8.8 ± 0.8 μM; $p < 0.05$) in comparison with the non-sensitized animals (5.4 ± 0.6 μM). The increased NO_x^- levels were reduced by $37.5 \pm 6.1\%$, $68.4 \pm 7.3\%$ and $74.9 \pm 6.1\%$ in long-term L-NAME, acute L-NAME and aminoguanidine groups, respectively ($n = 5$–14). The reduction of NO_x^- levels by aminoguanidine and acute L-NAME was higher ($p < 0.05$) compared with long-term L-NAME group. These treatments did not affect the NO_x^- levels in BAL fluid of non-sensitized mice (not shown).

3.6. Pharmacokinetic study: measurement of L-NAME and N^{ω}-nitro-L-arginine in serum

The levels of L-NAME itself and its metabolite N^{ω}-nitro-L-arginine in serum pooled from 5 mice receiving either long-term (150 mg/kg/day during 3 weeks) or acute (50 mg/kg, gavage; 30 min–96 h thereafter) intake of L-NAME were determined by mass spectrometry (see Section 2).

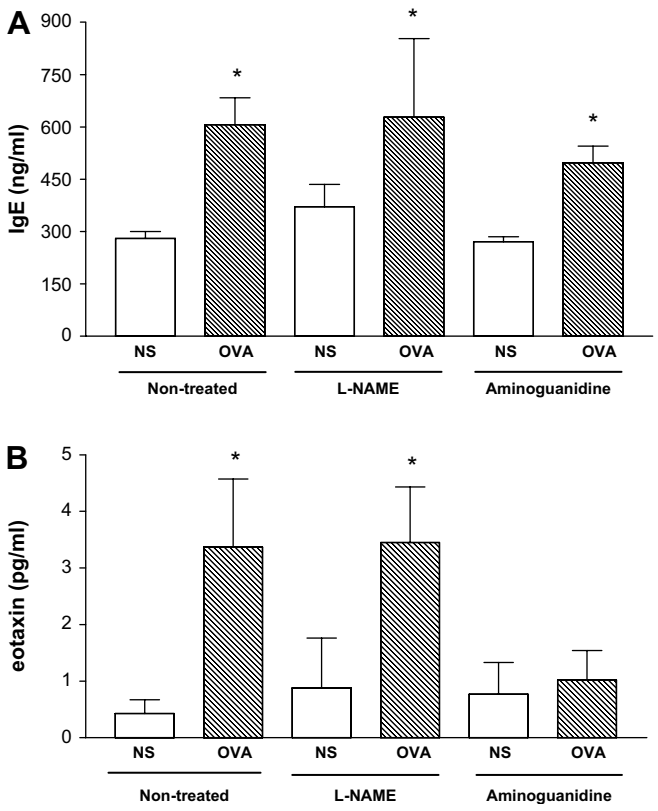

Fig. 6. The effects of long-term treatments with N^{ω}-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine on levels of serum IgE (panel A) and eotaxin in bronchoalveolar lavage (BAL) fluid (panel B). Serum and BAL fluid were collected from non-sensitized (NS) and ovalbumin-sensitized (OVA) mice at 48 h following intranasal instillation of OVA. L-NAME was given for 3 weeks (150 mg/kg/day in the drinking water), whereas aminoguanidine was given at 20 mg/kg/day in the drinking water for 3 weeks (for details, see Section 2). Each column represents the mean ± S.E.M. for $n = 5$–10. $^{*}p < 0.05$ compared with the respective non-sensitized group.

No detectable concentrations of L-NAME itself in serum were found in any studied group, most likely due to first-pass esterase activity.

In the acute treatment group, the serum levels of the metabolite N^{ω}-nitro-L-arginine time-dependently decreased from 30 min to 24 h after L-NAME intake (72.0, 53.9, 31.8, 38.6 and 32.1 ng/mL for 0.5, 1, 2, 3 and 24 h, respectively). At 96 h, no detectable levels of N^{ω}-nitro-L-arginine were found in serum.

In the chronic treatment group, the serum level of N^{ω}-nitro-L-arginine (16.2 ng/mL) was maintained close to the limit of detection (10 ng/mL).

4. Discussion

The present study carried out in BALB/C mice showed that the non-selective NOS inhibitor L-NAME can either inhibit or potentiate the eosinophil influx in BAL fluid of OVA-challenged animals, depending if this inhibitor was given acutely or during 3 weeks.

Nitric oxide plays a pivotal role in the eosinophil infiltration in airways of actively sensitized animals. Treatment of mice, guinea-pigs and rats with L-NAME has been shown to significantly reduce the eosinophil number in BAL fluid and lung tissue [8,9,21], but regarding the NOS isotype that accounts for the NO-mediated eosinophil infiltration into the airways of immunized animals, the literature is still controversial [29,30]. A previous study showed that the increased pulmonary eosinophil number, seen after exposure to aerosolized OVA in sensitized mice, was markedly reduced in iNOS-deficient animals [10]. Increased iNOS immunostaining and Ca^{2+}-independent NOS activity in lung homogenates were also observed after OVA challenge in mice [11]. Similarly, the selective iNOS inhibitors 1400 W [34] and aminoguanidine [16] reduced the eosinophil number in BAL fluid of OVA-challenged mice. The iNOS inhibitor SC-51 also attenuated the allergen-induced increase in major basic protein (MBP) eosinophil content in Brown-Norway rats challenged with OVA [14]. In contrast to all these studies, OVA challenge to sensitized mice neither affected the iNOS protein expression (or iNOS mRNA) in lung nor the nitrite levels in BAL fluid [8]. The iNOS inhibitor 1400 W also failed to affect the eosinophil influx into the airway of OVA-sensitized animals [8,12,15]. Moreover, the OVA-induced airway eosinophil recruitment was not modified in iNOS-deficient mice in comparison with wild-type animals [13,16]. In our present study, L-NAME, given acutely to mice, significantly reduced the eosinophil number in BAL fluid of OVA-challenged animals, which was accompanied by significant reductions in both pulmonary iNOS and cNOS activities, as well as by marked reduction in the NO_x levels in BAL fluid. Additionally, treatment of mice with the selective iNOS inhibitor aminoguanidine nearly abolished the airway eosinophil influx in BAL fluid of OVA-challenged animals. This was accompanied by a marked reduction in NO_x levels in BAL fluid along with a virtually abrogation of the enhanced pulmonary iNOS activity (without affecting the cNOS activity). Our data are therefore consistent with previous reports that iNOS-derived NO is largely involved in the modulation of allergic airway eosinophil influx in allergen-challenged BALB/C mice. It was intriguing, however, that L-NAME, given for 3 weeks, rather potentiated the pulmonary eosinophil influx of OVA-challenged mice. Considering that the long-term L-NAME treatment (i) failed to affect the enhanced iNOS activity in the lung homogenates of OVA-challenged mice, (ii) caused a small reduction of NO_x in BAL fluid and (iii) markedly reduced the cNOS activity in brain and lungs, it is likely that potentiation of eosinophil influx by long-term L-NAME is a consequence of removal of the protective cNOS-derived NO, maintaining intact the pathological iNOS-derived NO. Thus, our data are indicative that L-NAME, given for 3 weeks to BALB/C mice, acts to preferentially inhibit the cNOS isoform. Of interest, in an experimental model of chronic pulmonary allergic inflammation in guinea-pigs, prolonged administration of L-NAME failed to affect the eosinophil peroxidase-positive (EPO^{+}) cells around distal airways, whereas acute L-NAME treatment caused a significant reduction [31]. In our study, the cNOS activity was undetectable in the lungs of OVA-challenged mice. However, we believe this rather reflects a methodological limitation to detect cNOS activity in conditions where the iNOS is highly expressed, producing NO at large amounts and in a prolonged way, which could mask the cNOS activity measurements.

Eosinophils are derived in the bone marrow from myeloid precursors in response to cytokine activation, and following appropriate stimulus they are released into the circulation and recruited to tissues in allergic individuals [32,33]. In our study, we detected an increased number of eosinophils in the peripheral blood of OVA-challenged mice. The number of bone marrow eosinophils was also significantly increased at 48 h after the antigen challenge, which is believed to reflect an ongoing differentiation of inflammatory cells in the bone marrow in response to OVA. This is in agreement with previous studies in OVA-sensitized mice [34] and asthmatic subjects exposed to antigen inhalation [33]. In our study, a reduction in the eosinophil counts in both bone marrow and peripheral blood was observed in mice treated acutely with L-NAME (or with aminoguanidine), which parallels the attenuation of eosinophil infiltration into the lungs of OVA-challenged animals. As opposed, an increase in the eosinophil counts in peripheral blood was observed in OVA-challenged mice treated with L-NAME for 3 weeks that is also consistent with the higher

eosinophil influx into the lungs of these animals. The lack of effect of long-term L-NAME treatment in modifying the bone marrow eosinophil number is unclear, but may reflect an accelerated cell emigration from bone marrow to circulating blood to achieve the lungs at 48 h post-OVA challenge. A complete time-course response would be required to elucidate this hypothesis.

In allergic asthma, the antigen specific immunoglobulins of IgE isotypes are involved in inflammatory reaction in the airways. Sensitization of different animal species with OVA stimulates an allergic inflammation mediated by IgE [8,21,35]. In our study, elevated serum IgE levels were detected in OVA-challenged mice compared with non-sensitized animals, as expected. However, the increased IgE levels in OVA-challenged animals were unchanged by long-term L-NAME treatment, excluding the possibility that potentiation of eosinophil influx in BAL fluid reflects mechanisms involving cell accumulation via anaphylactic immunoglobulin-mediated responses.

The subfamily of CC-chemokines, including eotaxin (CCL11), eotaxin-2 (CCL24) and eotaxin-3 (CCL26) plays important roles in attracting eosinophils into inflammatory sites [36–38] via the receptor CCR-3 [39]. Accordingly, increased eotaxin levels have been detected in the serum, plasma and/or sputum of asthmatic patients and antigen-challenged animals [40–43]. In our study, high levels of eotaxin were detected in BAL fluid of OVA-challenged mice compared with non-sensitized animals, and that was suppressed by aminoguanidine treatment. However, chronic L-NAME treatment failed to affect the increased eotaxin levels in OVA-challenged mice, indicating that potentiation of eosinophil influx in BAL fluid does not reflect exaggerated production of this cytokine.

Our findings that 3-week administration of L-NAME to BALB/C mice causes a preferential inhibition of cNOS activity with no significant effects in the iNOS activity led us to hypothesize that such differences could be related to the pharmacokinetic profile of such compound. It is surprising, however, that no study exists evaluating L-NAME bioavailability after acute or long-term administration. Therefore, serum concentrations of L-NAME and its metabolite N^{ω}-nitro-L-arginine were measured in both groups. Our data showed that L-NAME is not detected in serum in any studied group indicating that the methyl ester moiety of such compound is efficiently removed during first-pass metabolism, yielding N^{ω}-nitro-L-arginine to systemic circulation. This indicates that L-NAME per se is not bioavailable when given *per os*, at least in BALB/C mice. On the other hand, N^{ω}-nitro-L-arginine could be detected in the mice serum after L-NAME intake, indicating that this metabolite accounts for the NOS inhibition in vivo when L-NAME is given *per os* either acutely or for 3 weeks. The serum levels of N^{ω}-nitro-L-arginine in mice receiving acutely L-NAME (50 mg/kg, gavage) achieved the concentration of 72.0 ng/mL at 30 min, decaying by a half at 2 h post-administration, maintaining such levels up to 24 h. In contrast, the serum levels of N^{ω}-nitro-L-arginine in mice treated with L-NAME (150 mg/kg/day) for 3 weeks in the drinking water achieved much lower concentrations (16.2 ng/mL), close to the detection limit (10 ng/mL). A previous study compared the inhibitory effects of both L-NAME and N^{G}-nitro-L-arginine in the cNOS activity in rat isolated hearts and brain, and quantified the levels of both of these substances in physiological buffers, plasma and blood [44]. They found that increased coronary perfusion pressure by L-NAME paralleled increased levels of N^{G}-nitro-L-arginine in the coronary effluent. In brain, L-NAME was 50-fold less potent than N^{G}-nitro-L-arginine to inhibit the cNOS activity, but the inhibitory effect of L-NAME approached that of N^{G}-nitro-L-arginine upon neutral or alkaline pH. These authors suggested that L-NAME lacks NOS inhibitory activity unless hydrolyzed to N^{G}-nitro-L-arginine [44]. It is likely therefore that low and sustained concentrations of N^{ω}-nitro-L-arginine in systemic circulation act to preferentially inhibit the cNOS isoform activity, whereas higher concentrations of this metabolite in serum are required to inhibit both NOS isoforms. Since long-term treatment with L-NAME reduces the eosinophil infiltration in rats [21], has no effects in guinea-pigs [31,45] and potentiates in BALB/C mice (present study), it is likely that the L-NAME metabolism greatly differs between animal species. Therefore, caution should be taken when interpreting data because contrasting data may be related to the pharmacokinetic of this NOS inhibitor.

In conclusion, daily administration of L-NAME for 3 weeks inhibits preferentially the cNOS activity without affecting significantly the pulmonary iNOS activity, causing consequently an enhancement of OVA-induced pulmonary eosinophil influx in the sensitized mice. As opposed, acute L-NAME administration inhibits both NOS isoforms leading to an inhibition of OVA-induced pulmonary eosinophil influx. These functional differences between acute and long-term treatments with L-NAME are likely to reflect the pharmacokinetic profile of such compound.

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