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Inflammation Research

Acute inhibition of nitric oxide exacerbates airway hyperresponsiveness, eosinophilia and C–C chemokine generation in a murine model of fungal asthma

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Abstract. *Objective and Design:* This study examined he role of nitric oxide in changes in airway physiology and inflammation in a murine model of fungal allergy induced by Aspergillus fumigatus (A. fumigatus) by treatment of A. fumigatus-sensitized mice with NG-nitro-L-arginine methyl ester (L-NAME) or D-NAME (8 mg/kg; i.p.).
Materials and Methods: Female CBA/J mice received A. fumigatus antigen dissolved in incomplete Freund's adjuvant (10 mg/100 ml i.p. and s.c.) followed 2 weeks later by A. fumigatus antigens (20 mg; i.n.) and a subsequent i.t. challenge 4 days later. Airway physiology and inflammation were examined (24 to 72 h) following i.t. challenge.
Results: L-NAME-treated mice had lower lung nitrite levels 24 h after A. fumigatus challenge, but higher airway hyperresponsiveness and inflammation compared to D-NAME controls. Airway inflammation in the L-NAME treatment group (72 h) was characterized by a greater bronchoalveolar lavage (BAL), peribronchial eosinophilia and augmented levels of CC chemokines compared to controls.
Conclusions: These findings suggest that nitric oxide is an important modulator of airway hyperresponsiveness, inflammation and C–C chemokine generation during allergic airway responses to A. fumigatus.

Key words: Aspergillus fumigatus – Nitric Oxide – Eosinophil – Asthma – Chemokines

Introduction

Numerous clinical studies have demonstrated that exhaled nitric oxide is a non-invasive marker of airway inflammation associated with asthma and other allergic airway diseases [1–4]. Exhaled nitric oxide is derived predominately from endothelial (NOS III) and neural (NOS I) sources in normal airways [5], while the increased concentration of nitric oxide in asthmatic airways appears to be a consequence of inducible NOS (NOS II) expression [6]. The majority of clinical and experimental studies suggest that changes in nitric oxide levels in exhaled air reflects the inflammatory activity in asthma [3], and is influenced by anti-inflammatory therapy. It has also been suggested that specific NOS II inhibitors may represent a viable treatment option for asthma and other allergic diseases [6]. However, dissenting findings from clinical and experimental studies suggest that nitric oxide lacks a clear deleterious role and may even exert important modulatory effects during allergic airway disease. For example, gas-exchange impairment in asthma did not appear to be related to nitric oxide concentrations in exhaled air, nor did ventilation-perfusion disturbances appear to correlate to increased nitric oxide synthesis in the airways of asthmatics [7]. In addition, acute inhibition of nitric oxide has been shown to exacerbate allergen-induced airway hyperreactivity in a guinea pig model of asthma [8]. Thus, further investigation into the role of nitric oxide during allergic airway disease is warranted in light of these controversial findings.

Chemokines are unique cytokines that promote the recruitment of leukocytes to sites of acute and chronic inflammation [9]. The C–C chemokine subfamily, characterized by the conserved juxtaposition of two cysteine residues in their NH2-terminal end, have recently emerged as important mediators of allergic airway inflammation [10]. During asthmatic attacks, C–C chemokines such as monocyte chemotactic peptide-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1α), RANTES (regulated upon activation in normal T cells expressed and secreted) and eotaxin have been found to be transiently increased in the bronchoalveolar lavage (BAL) [11–14]. Experimental models of allergic airway inflammation have further revealed distinct roles for MCP-1, MIP-1α and RANTES in the trafficking of T lymphocytes, monocytes and eosinophils around the airways, thereby affecting the severity of airway inflammation and hyperresponsiveness [15–19]. Other experimental studies support a role for the C–C chemokine eotaxin in the

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selective recruitment and activation of eosinophils during allergic airway responses [20–22]. While C–C chemokines represent a major target during allergic airway responses, little is presently known about the endogenous regulation of these mediators during allergic airway disease.

Thus, we examined the effect of nitric oxide on the allergic airway response to the ubiquitous fungal organism *Aspergillus fumigatus* (*A. fumigatus*). Inhalation of fungal spores by individuals previously sensitized to *A. fumigatus* antigens results in an allergic disease with similar characteristics to asthma. This disease is characterized by a Th2-type cytokine response with varying severity of bronchial hyperresponsiveness, eosinophilia, and elevations in immunoglobulin E (IgE) [23–26]. We have recently developed an experimental model of fungal-induced asthma and have previously reported that the C–C chemokines MCP-1, eotaxin and C10 are significantly elevated in the lungs of mice experiencing pulmonary allergic responses to soluble *A. fumigatus* antigens [27]. We have also discovered that the immunoneutralization of C10 significantly attenuates the airway inflammation and hyperresponsiveness associated with experimental showing that chemokines are relevant participants in *A. fumigatus*-induced allergic airway disease [27]. In the present study, we demonstrate that nitric oxide is also relevant to experimental fungal asthma since acute inhibition of nitric oxide exacerbated the airway hyperresponsiveness, eosinophilic and lymphocytic inflammation, and C–C chemokine generation associated with this model of allergic airway disease.

Materials and methods

Murine model of fungal asthma

Specific-pathogen free (SPF), female CBA/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were kept in a SPF facility for the duration of these experiments. Prior approval for mouse usage in these studies was obtained from University Laboratory Animal Medicine facility at the University of Michigan Medical School. Sensitization of mice to soluble *A. fumigatus* antigens was achieved as previously described [16–28]. Briefly, a total of forty mice received a total of 10 µg of *A. fumigatus* crude antigen (Greer Laboratories, Lenoir, NC) dissolved in 0.2 ml of incomplete Freund's adjuvant (Sigma Chemical Company, St. Louis, MO). All mice were subsequently sensitized to this *A. fumigatus* preparation via 0.1 ml intraperitoneal and subcutaneous injections, and two weeks later each mouse received a total of 20 µg of *A. fumigatus* antigens dissolved in normal saline via the intranasal route. Four days after the intranasal challenge, sensitized mice were challenged with 20 µg of *A. fumigatus* antigen dissolved in normal saline via the intratracheal route. Non-sensitized mice were used as controls and received normal saline alone via the same routes and over the same time periods.

Acute pharmacological inhibition of nitric oxide

We have previously used L-NAME to non-selectively inhibit nitric oxide synthesis in a number of in vivo models [29–31]. D-NAME is a structural enantiomer of L-NAME that lacks nitric oxide synthase inhibitory actions [32] and was administered to control mice in this study. L-NAME was administered to 20 mice at a dose of 8 mg/kg [31] via an intraperitoneal injection immediately following the intratracheal *A. fumigatus* or normal saline challenge. An equal number of *A. fumigatus* sensitized and challenged mice received a similar dose of D-NAME at the same time.

Nitrite and nitrate assay

Previous clinical studies have demonstrated that nitrite and nitrate levels may be a more useful measure of nitric oxide production due to the fact that exhaled may not reflect total nitric oxide production [33]. Nitrite and nitrate are stable end products of nitric oxide metabolism and were measured in the present study using a technique supplied by Oxford Biomedical Research (Oxford, MI). Briefly, 50 µl of supernatants from bronchoalveolar lavage (BAL) samples were deproteinized with $ZnSO_4$ for 15 min at room temperature. Deproteinated supernatants were subsequently transferred to microcentrifuge tubes containing cadmium beads for the quantitative conversion of nitrate to nitrite. Cadmium-treated supernatants (50 µl) were finally transferred to a microtiter plate, and 100 µl of Griess reagent containing equal parts of 1% sulfanilamide in 25% (vol/vol) phosphoric acid and 0.01 N-1-napthylethylenediamine dihydrochloride in distilled water was added for 10 min. Microtiter plates were read at 540 nm in a microtiter plate reader. Nitrite and nitrate concentrations were calculated from a standard curve using sodium nitrite, and the sensitivity of this assay consistently approached 1 µM.

Determination of systemic IgE

Sera from non-sensitized and *A. fumigatus*-sensitized mice were analyzed for total IgE. *A. fumigatus*-sensitized mice were also monitored for changes in total IgE after receiving an intratracheal *A. fumigatus* challenge. Complimentary capture and detection antibody pairs for mouse IgE were obtained from PharMingen (San Diego, CA) and the IgE ELISA was performed according to the manufacturer's directions. Duplicate sera samples were diluted 1:100 and IgE levels in each were calculated from optical density readings at 492 nm, and IgE concentrations were calculated from a standard curve generated using recombinant IgE (5–2000 pg/ml).

Measurement of bronchial hyperresponsiveness

Immediately prior to and at 24, 48, and 72 h after the intratracheal *A. fumigatus* challenge, bronchial hyperresponsiveness in *A. fumigatus*-sensitized mice was assessed in a Buxco plethysmograph (Buxco, Troy, NY) specifically designed for the low tidal volumes of mice [17]. Sodium pentobarbital (Butler Co., Columbus, OH; 0.04 mg/g of mouse body weight) was used to anesthetize mice prior to their intubation for ventilation with a Harvard pump ventilator (Harvard Apparatus, Reno, NV). The following ventilation parameters were employed for each mouse: tidal volume = 0.25 ml, breathing frequency = 120/min, and positive end-expiratory pressure $\cong$3 cm H_2O. Within the sealed plethysmograph mouse chamber, transpulmonary pressure (i.e. Δ tracheal pressure – Δ mouse chamber pressure) and inspiratory volume or flow were monitored online with an adjacent computer. Airway resistance was continuously calculated online via specialized computer software (Buxco, Troy, NY), and was determined by the division of the transpulmonary pressure by the change in inspiratory volume. Following a baseline period in the Buxco apparatus, all mice received a dose of 10 µg of methacholine by tail vein injection, and airway responsiveness to this non-selective bronchoconstrictor was again calculated online. Because non-sensitized mice typically exhibited minor (i.e. less than a 1.5-fold) increases above the baseline airway resistance response following the injection of 10 µg of methacholine, this dose of methacholine was subsequently used to reveal changes in airway hyperresponsiveness in both fungal asthma models. At the conclusion of the assessment of airway responsiveness, a bronchoalveolar lavage (BAL) was performed with 1 ml of normal saline. Approximately 500 µl of blood from each mouse was also transferred to a microcentrifuge tube, and sera were obtained

after the sample was centrifuged at 15000 rpm for 10 min. Whole lungs were subsequently dissected from each mouse and snap frozen in liquid N_2, or prepared for histological analysis.

BAL and lung histological analysis

Neutrophils, macrophages, eosinophils, and lymphocytes were quantified in BAL samples obtained by i.t. instillation of 1 ml saline (BAL recovery was consistent between groups) applied to coded microscope slides using a cytospin (Shandon Scientific, Runcorn, UK). Identification of each cell type in the cytospins was facilitated by Wright-Giemsa differential stain, and average number of each cell type per animal was determined in 8 high-powered fields (HPF; 1000 X) on every slide.

Whole lungs from normal (i.e. non-sensitized) and *A. fumigatus*-sensitized prior to and after *A. fumigatus* challenge were fully inflated by the intratracheal perfusion with 4% paraformaldehyde. Lungs were then dissected and placed in fresh paraformaldehyde for 24 h. Routine histological techniques were used to paraffin-embed this tissue, and 5 µm sections of whole lung were counterstained with Mayer's hematoxylin (Mayer & Myles Laboratories, Coopersburg, PA). Inflammatory infiltrates and other histological changes were examined around blood vessels and airways using light microscopy at a magnification of 1000X (high-powered field; hpf). Eosinophils were counted in a minimum of 20 randomly selected hpf using a multiple step-analysis of coded whole lung histologic sections. Eosinophils in juxtaposition to the airway were counted since the eosinophilic inflammation in this fungal-induced asthma model was exclusively associated with bronchioles or larger airways.

Chemokine and cytokine ELISA analysis

Murine MCP-1, eotaxin and C10 protein levels were determined in 50 µl of BAL supernatants using a standardized sandwich ELISA technique previously described in detail [34]. Briefly, Nunc-immuno ELISA plates (MaxiSorp™) were coated with the appropriate capture antibody (R&D Systems, Minneapolis, MN) at a dilution of 1–5 µg/ml of coating buffer (in M: 0.6 NaCl; 0.26 H_3BO_4; 0.08 NaOH; pH 9.6) overnight at 4°C. The unbound capture antibody was washed away and each plate was blocked with 2% BSA-PBS for 90 min at 37°C. Each ELISA plate was then washed with PBS tween 20 (0.05%; vol/vol), and 50 µl samples either undiluted or diluted 1:10 were added to duplicate wells and incubated for 1 h at 37°C. Following the incubation period, the ELISA plates were then thoroughly washed and the appropriate biotinylated polyclonal rabbit antibody (3.5 µg/ml) was added. After washing the plates 30 min later, streptavidin-peroxidase (Bio-Rad Laboratories, Richmond, CA) was added to each well for 30 min, and each plate was thoroughly washed again. Chromagen substrate (Bio-Rad Laboratories) was added and optical readings at 492 nm were obtained using an ELISA plate scanner. Recombinant murine cytokines and chemokines were used to generate the standard curves from which the concentrations present in the samples were derived. The limit of ELISA detection for each cytokine and chemokine was consistently above 50 pg/ml. Each ELISA was screened to ensure the specificity of each antibody used.

Data statistical analysis

All results are expressed as mean ± standard error of the mean (SE). Treatment groups were compared by analysis of variance (ANOVA). Dunnett's test for multiple comparisons was then used to determine statistical significance between the control and experimental groups at each time point after *A. fumigatus* challenge; $P < 0.05$ was considered statistically significant.

Results

Acute pharmacological inhibition of nitric oxide with L-NAME during experimental fungal asthma

BAL samples from normal mice contained approximately 1.3 ± 0.5 µM of nitrite and these levels were similar to BAL nitrite levels measured in *A. fumigatus*-sensitized immediately prior to *A. fumigatus* intratracheal challenge (nitrite levels within the fluid used for lavage were below the level of detection with this assay). At 24 h after the intratracheal antigen challenge, mice that were treated with D-NAME exhibited significantly higher ($P \leq 0.05$; 7.7 ± 2.2 µM) BAL levels of nitrites compared with normal mice (Fig. 1). At the same time, nitrite levels in BAL samples from L-NAME-treated mice were 3.7 ± 0.1 µM, or approximately 50% lower than the levels measured in the untreated and D-NAME-treated mice. The levels of nitrite measured in the L-NAME-treated group were not different from levels measured in non-sensitized (normal) mice. However, BAL levels of nitrites in L-NAME and D-NAME-treated mice were not statistically different at 48 and 72 h after intratracheal *A. fumigatus* antigen challenge, confirming that the pharmacological inhibition of nitric oxide synthesis was probably limited to the first 24 h after the intratracheal *A. fumigatus* challenge (data not shown).

L-NAME treatment did not affect circulating IgE levels in experimental fungal asthma

Levels of total IgE in sera were approximately 20 ng/ml in non-sensitized mice. Total IgE level in all mice with fungal-induced asthma was significantly ($p \leq 0.05$) greater than levels measured in non-sensitized mice, but differences be-

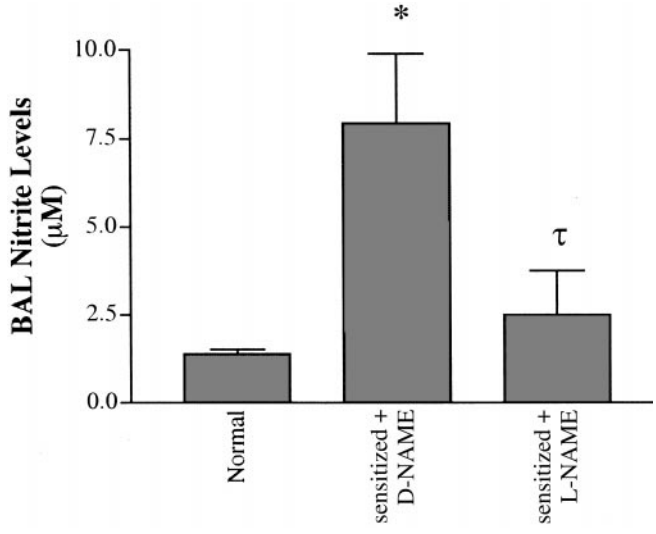

Fig. 1. Nitrite levels in bronchoalveolar lavage (BAL) from normal mice (i.e. non-sensitized) and mice sensitized to *A. fumigatus*. Sensitized mice received L-NAME or D-NAME, and BAL was collected from each ABPA group at 24 h after intratracheal *A. fumigatus* challenge. Values represent mean ± SE of 3–5 mice per treatment group. * $P \leq 0.05$ compared with normal mice. τ $P \leq 0.05$ compared with D-NAME-treated *A. fumigatus*-sensitized mice.

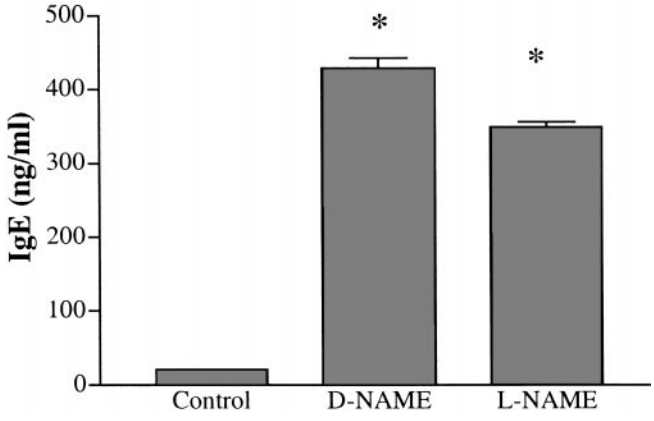

Fig. 2. Sera IgE levels in normal mice (i.e. non-sensitized) and mice with A. fumigatus-induced asthma. Allergic mice received L-NAME or D-NAME, and sera were collected from each ABPA group at 24 h after intratracheal *A. fumigatus* challenge. Total IgE was measured using a specific ELISA (see Materials and Methods). Values represent mean SE of 3–5 mice per treatment group. * $P \leq 0.05$ compared with the control in normal mice.

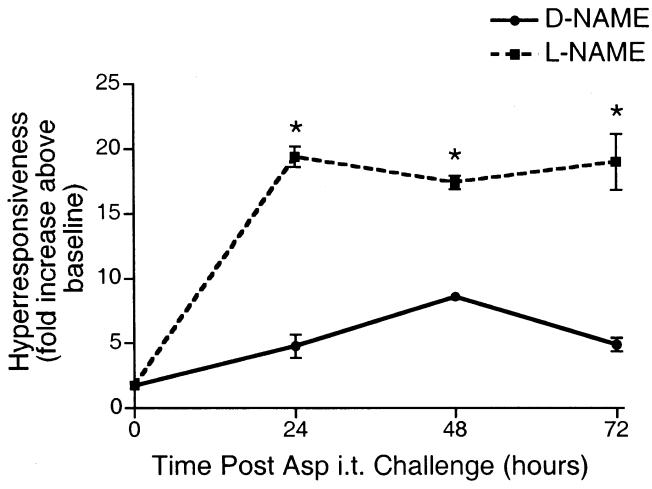

Fig. 3. Airway hyperresponsiveness in *A. fumigatus*-sensitized mice prior to and after *A. fumigatus* (Asp) intratracheal (i.t.) challenge, and L-NAME or D-NAME treatment. Airway hyperresponsiveness or resistance in all *A. fumigatus* -sensitized mice was revealed by the intravenous administration of 10 µg of methacholine. *A. fumigatus*-sensitized mice showed an approximate 2-fold increase in airway resistance above baseline immediately prior to an intratracheal *A. fumigatus* challenge (at T = 0). However, at 24 to 72 h after *A. fumigatus* challenge, the L-NAME treated group exhibited a 15-fold increase in airway resistance above baseline, and this response was significantly greater than the airway hyperresponsiveness measured in D-NAME treated mice. Values represent mean of 3–5 mice per treatment group. * $P \leq 0.05$ compared with D-NAME treatment group at the same time points.

tween the sensitized groups were not detected (Fig. 2). These observations suggested that the acute inhibition of nitric oxide during experimental fungal asthma did not exacerbate the allergic IgE response to *A. fumigatus*.

L-NAME treatment significantly enhanced airway hyperresponsiveness in experimental fungal asthma

Airway hyperresponsiveness prior to and at 24, 48 and 72 h following the intratracheal *A. fumigatus* challenge was revealed by the intravenous delivery of 10 µg of β-methacholine, a smooth muscle spasmogen. Immediately prior to the *A. fumigatus* challenge, sensitized mice given intravenous methacholine exhibited an approximate 2-fold increase in airway resistance above that measured before methacholine administration (Fig. 3). Airway resistance was significantly enhanced above the baseline response in non-sensitized mice. However, L-NAME-treated mice exhibited a 15-fold increase in airway hyperresponsiveness, whereas similarly challenged D-NAME-treated mice showed a 5-fold increase in airway responsiveness to methacholine (Fig. 3). Furthermore, at the 48 and 72 h time points after *A. fumigatus* challenge, airway responses to methacholine stimulation in L-NAME-treated mice remained approximately 3-fold greater than the airway responses measured in their D-NAME counterparts. Thus, the acute inhibition of nitric oxide synthesis with L-NAME augmented and prolonged the airway hyperresponsiveness associated with experimental fungal-induced asthma.

Acute inhibition of nitric oxide prolongs BAL and peribronchial eosinophilia in experimental fungal-induced asthma

Leukocyte counts in cytospins from non-sensitized (or normal), and *A. fumigatus*-sensitized and challenged mice are shown in Figure 4. Twenty-four hours after the intratracheal introduction of *A. fumigatus* into sensitized mice, BAL samples contained eosinophils, lymphocytes, neutrophils and macrophages while BAL from normal mice only contained macrophages (Fig. 4 A). No significant differences in leukocyte counts were detected among the sensitized groups at 24 h after *A. fumigatus* challenge. At the 72 h time point, all four types of leukocytes were again present in the sensitized groups, but the numbers of eosinophils and lymphocytes were significantly increased in the L-NAME-treated group compared with the D-NAME treated group (Fig. 4 B). Similar leukocyte counts were present in BAL samples from the untreated and D-NAME-treated groups. Thus, these data suggested that the acute inhibition of nitric oxide synthesis was associated with the persistent accumulation of eosinophils and lymphocytes in the BAL of *A. fumigatus*-sensitized mice.

Although few eosinophils were localized around the airways of *A. fumigatus*-sensitized mice immediately prior to the *A. fumigatus* challenge (not shown), a profound accumulation of eosinophils was apparent in both sensitized groups at 24 h after the *A. fumigatus* challenge (Fig. 5). However, the profound airway eosinophilia in the L-NAME-treated group persisted out to 72 h after *A. fumigatus* challenge, unlike the other two sensitized groups (Fig. 5). These data suggested that the acute inhibition of nitric oxide synthesis markedly affected the recruitment and persistence of eosinophils around allergic airways.

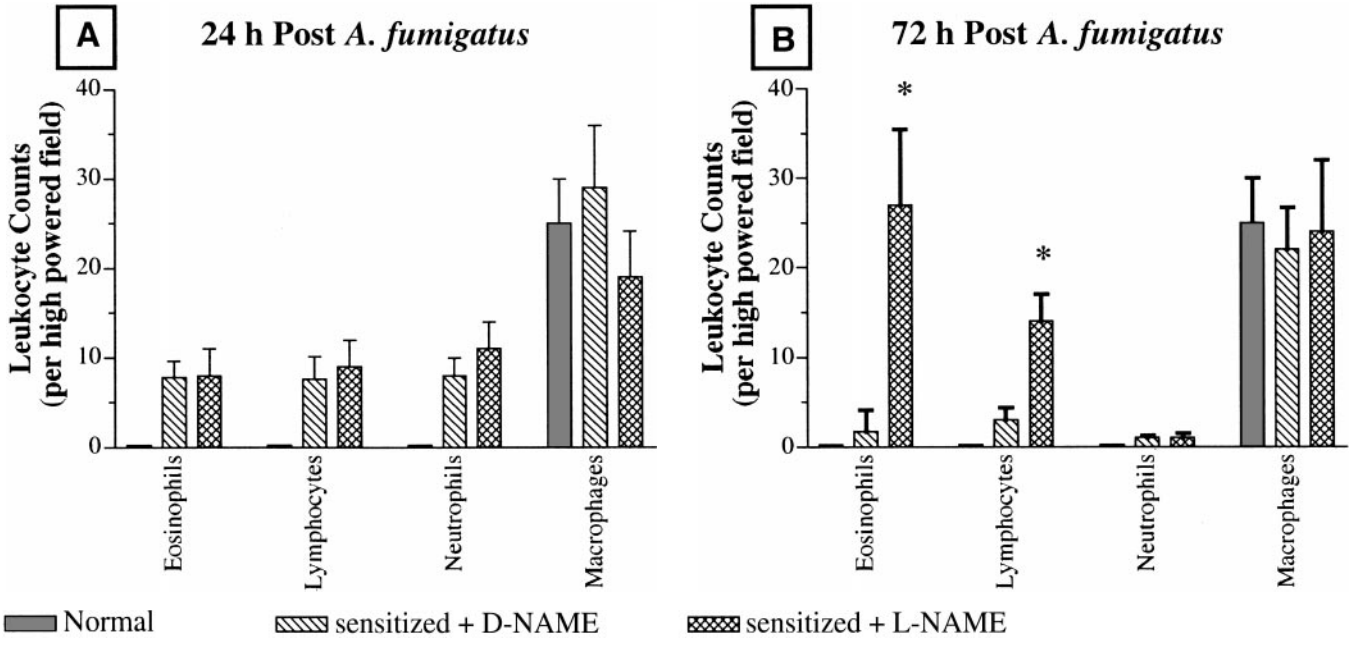

Fig. 4. Leukocyte counts in bronchoalveolar lavage (BAL) from non-sensitized (or normal) and mice with *A. fumigatus*-induced asthma treated with L-NAME or D-NAME. Eosinophils, macrophages, lymphocytes and neutrophils per animal were counted in a minimum of 8 high powered fields (HPF) from 3–5 mice from both treatment groups. Only macrophages were observed BAL samples from normal mice, but significantly increased numbers of eosinophils, lymphocytes and neutrophils were present in both treatment groups at 24 h after *A. fumigatus* challenge (Panel A). However, at 72 h after *A. fumigatus* challenge, eosinophils and lymphocytes remained significantly elevated in the L-NAME-treated group compared with the D-NAME group (Panel B). * $P \leq 0.05$ compared with the D-NAME-treated mice with *A. fumigatus*-induced asthma at 72 h after *A. fumigatus* challenge.

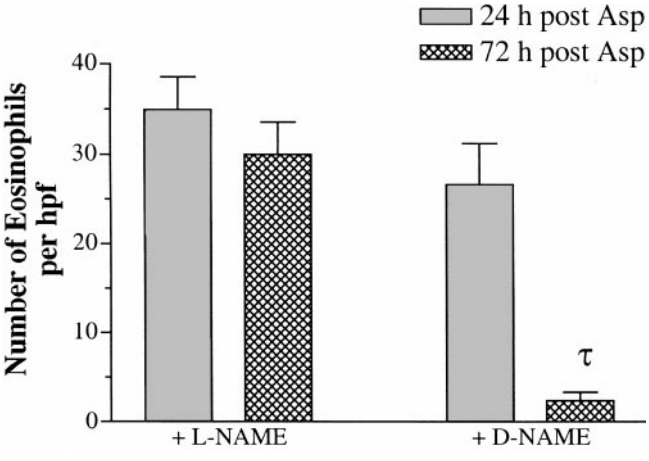

Fig. 5. Quantification of peribronchial eosinophils in histological sections from the lungs of untreated, L-NAME- and D-NAME-treated mice with fungal-induced asthma. Eosinophils were counted in a minimum of 25 high powered fields (hpf) from multiple tissue sections in all three *A. fumigatus*-induced asthmatic groups. Although eosinophils were never observed in histological sections from the lungs of normal mice, profoundly increased numbers of eosinophils were present in both ABPA groups at 24 h after *A. fumigatus* challenge. However, at 72 h after *A. fumigatus* challenge, peribronchial eosinophilia in the L-NAME-treated group was significantly greater compared with the other two allergic groups. $\tau P \leq 0.05$ compared with D-NAME-treated mice with *A. fumigatus*-induced asthma at 72 h after *A. fumigatus* challenge.

L-NAME treatment significantly increased pulmonary CC chemokine levels in allergic mice

Previous studies suggested that the increased pulmonary expression of MCP-1, eotaxin and C10 are associated with the development and progression of experimental allergic responses to *A. fumigatus* antigens [27]. Also, from these previous studies it was observed that C10 has a prominent role in the development of experimental fungal-induced asthma. In the present study, all three chemokines were detected in BAL samples from *A. fumigatus* sensitized mice prior to the intratracheal challenge with *A. fumigatus*. In contrast, eotaxin and C10 were the only two chemokines detected in BAL samples from non-sensitized mice (Fig. 6B, C; dashed line). The acute inhibition of nitric oxide synthesis with L-NAME significantly ($p \leq 0.05$) increased the levels of MCP-1 (Fig. 6A), eotaxin (Fig. 6B) and C10 (Fig. 6C) above levels of these chemokines in BAL samples from D-NAME-treated mice. Only MCP-1 was significantly elevated at 48 h after *A. fumigatus* challenge. All three CC chemokines were either below the level of detection or at baseline levels in both NAME treatment groups at 72 h after the intratracheal *A. fumigatus* challenge (Fig. 6A)

Discussion

Allergic responses to *A. Fumigatus* conidia in asthmatics is a common phenomena. *A. Fumigatus* is a major indoor and outdoor mould that can promote respiratory problems and

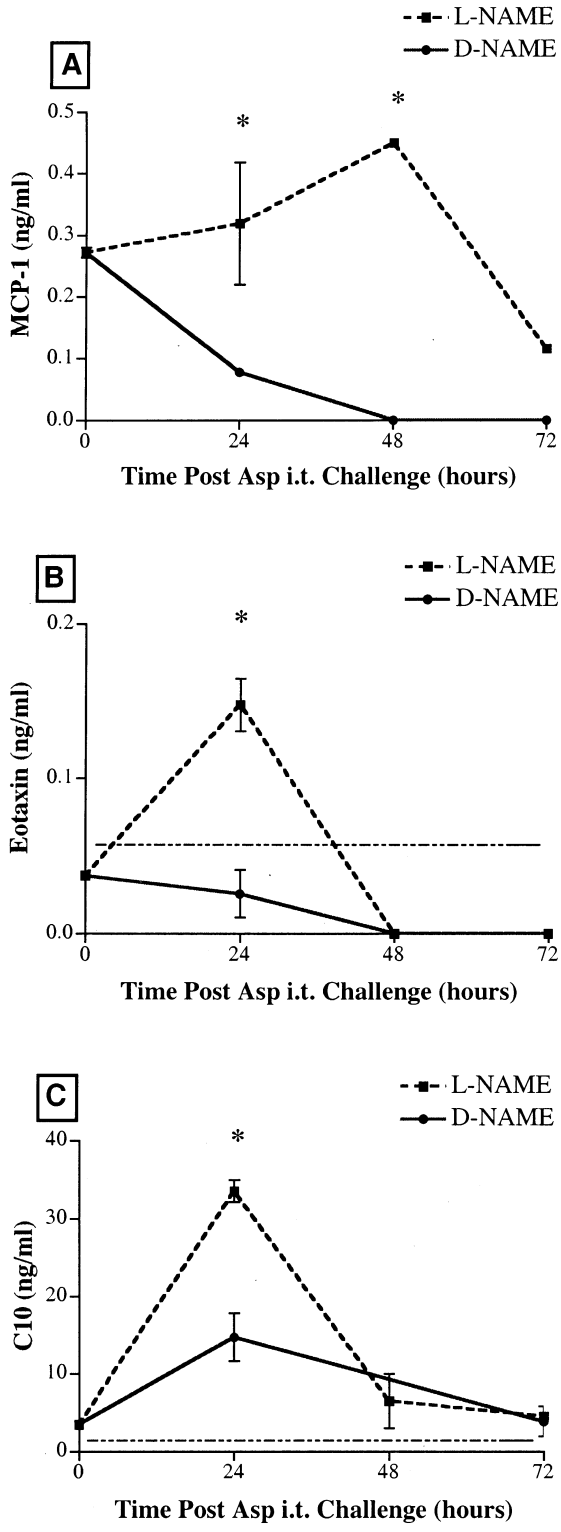

Fig. 6. MCP-1 (A), eotaxin (B), and C10 (C) protein levels in BAL samples immediately prior to and at various times after an intratracheal *A. fumigatus*-challenge, and L-NAME or D-NAME treatment in *A. fumigatus*-sensitized mice. The acute inhibition of nitric oxide with L-NAME significantly increased immunoreactive levels of MCP-1, eotaxin and C10 above levels measured in the D-NAME controls. Values represent mean ± SE of 3–5 mice per treatment group. Dashed lines denote mean levels of each CC chemokine in BAL from normal mice (Eotaxin = 0.50 ± 0.01 ng/ml; C10 = 0.7 ± 0.01; no MCP-1). *$P \leq 0.05$ compared with D-NAME treatment group at the same time point.

can increase the risk of symptomatic allergic disease in asthmatics and patients with cystic fibrosis [35, 23, 24]. Symptoms, similar to those in asthma, include recurrent episodes of wheezing, mucus production, inflammatory infiltrates around the airways, elevated IgE, and increased Th2-type cytokines such as IL-4, IL-5 and IL-10 [36]. In contrast to patients with the clinical syndrome allergic bronchopulmonary aspergillosis, or ABPA, however, chronic colonization of the airways by *A. fumigatus* does not occur. Murine models have been developed that exhibit many of the features of clinical ABPA, however little is known about the immune mechanisms that regulate *A. fumigatus*-induced asthma [37–41]. Furthermore, previous studies have not addressed the role of nitric oxide in this disease. In the present study, *A. fumigatus*-sensitized and -challenged mice treated with L-NAME exhibited significantly greater airway hyperresponsiveness and eosinophilic inflammation than their D-NAME-treated counterparts. The augmented airway physiology and eosinophilic inflammation after L-NAME treatment were also associated with significantly greater levels of potent chemoattractant C–C chemokines such as MCP-1, eotaxin and C10 in the BAL. Thus, the present findings suggest that nitric oxide is an important modulator of many facets of allergic disease induced by *A. fumigatus*. Recent evidence from a study conducted by De Sanctis et al. [42] using mice with targeted deletions of the three known isoforms of NOS (NOS I, II, and III), identified the NOS I isoform to be important in protection from airway hyperresponsiveness in an ovalbumin model of allergic asthma. It is possible that distinct NOS isoforms are involved in the observations reported in this present study and this area of study is currently being investigated.

The kinetics of leukocyte accumulation around airways during clinical asthma [10, 12] and experimental allergic airway inflammation [15] correlates with the increased pulmonary expression of C–C chemokines. Cellular sources of C–C chemokines during airway inflammation include pulmonary immune and non-immune cells such as alveolar macrophages, airway epithelial cells, lung (myo) fibroblasts, and smooth muscle cells [15]. Immunoneutralization studies in experimental allergic airway models have shown that MCP-1 is a potent T cell recruitment factor [15–19], whereas eotaxin [20–43], RANTES [16], and MIP-1α [16] promote the accumulation of eosinophils around allergic airways. Recent studies in this laboratory have suggested that specific C–C chemokines are also important participants during the development and progression of allergic airway responses to *A. fumigatus* [27]. The immunoneutralization of C10 revealed that this novel IL-4-inducible chemokine [44] had a significant effect on the airway reactivity and eosinophil and lymphocyte recruitment during experimental fungal-induced asthma [27]. In the present study, C10 was the most prominent C-C chemokine detected in the BAL following *A. fumigatus* challenge and D-NAME treatment. However, C10 levels in the D-NAME treatment group were significantly (i.e. 3-fold) lower than levels of this chemokine in BAL samples from allergic mice treated with L-NAME. While it is possible that increased C10 levels following L-NAME treatment accounted for the augmented airway hyperresponsiveness and eosinophil and lymphocyte recruitment in the allergic airways, MCP-1 and eotaxin were also significantly elevated in this group. Recent studies in this laboratory have

shown that MCP-1 mediates allergic airway inflammation, and more importantly, MCP-1 directly induces airway hyperresponsiveness in the absence of underlying allergic disease [45]. The effects of eotaxin in murine models of allergic airway disease are well documented [19, 20], and this chemokine is one of the most potent eosinophil chemoattractants yet described [46]. Thus, in the context of reduced nitrogen oxide levels in the lung, the C–C chemokines MCP-1, eotaxin and C10 were significantly increased during airway allergic responses to *A. fumigatus*, and these changes may have accounted for the exacerbated airway hyperresponsiveness and inflammation in this model of fungal-induced asthma. The mechanism of increased chemokine production following reduced NO levels in the lung may be a reflection of increased NF-κB activation. NO has recently been shown to increase IκB activation, a NF-κB inhibitory factor, and hence decrease NF-κB activity [47].

Given that a large number of C–C chemokines appear to work in a coordinated manner [17, 19] and exhibit a differential pattern of expression [20] during allergic airway responses, targeting a single chemokine may not provide a clear therapeutic effect in the more complex clinical allergic airway diseases. Therefore, one strategy for collectively regulating the generation of several C–C chemokines during pulmonary allergic responses may involve the regulation of endogenous nitric oxide synthesis. Abundant evidence presently exists demonstrating that nitric oxide inhibits C–C chemokine in a number of cells during diverse inflammatory reactions. In vitro experiments have shown that nitric oxide inhibits MCP-1 generation by the vascular endothelium [48–49], by peritoneal macrophages [31], and MIP-1α synthesis by alveolar macrophages [50]. In addition, the suppressive effect of induced nitric oxide on MCP-1 and C10 generation was observed at day 4 [30] and day 8 [51] in a Th-1 type pulmonary granuloma model. Interestingly, nitric oxide has also been shown to regulate the synthesis of C-X-C chemokines during acute inflammatory responses in the peritoneal cavity [52]. Thus, the results from the present study appear to coincide with previous observations that nitric oxide regulates the generation of CC chemokines such as MCP-1, eotaxin and C10.

In conclusion, the results from the present study demonstrate that endogenous nitric oxide generation has a major effect on the generation of allergic airway responses to *A. fumigatus*. The acute inhibition of nitric oxide using a pharmacological inhibitor aggravated airway inflammation and hyperresponsiveness, and these responses were associated with significantly enhanced C–C chemokine levels in the airways. Thus, the discovery that nitric oxide modulates the generation of a number of CC chemokines that could potentially contribute to the inflammatory events during clinical allergic airway disease provides impetus to further investigate the best strategy for regulating nitric oxide production in clinical asthmatic and allergic airway diseases.

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