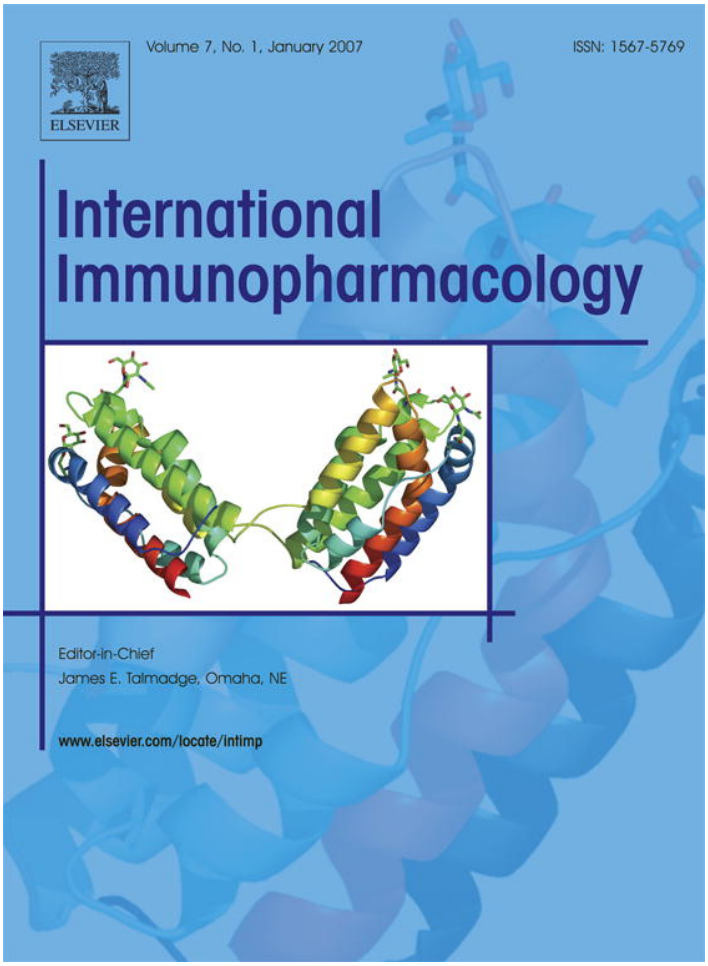

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Production of nitric oxide by airways neutrophils in the initial phase of murine asthma

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Abstract

In experimental models of asthma, nitric oxide (NO) is produced and contributes to the physiopathology of the disease. Neutrophil is the first cell to infiltrate the lung in response to antigen stimulation, it has the capacity to produce NO but a clear demonstration that neutrophils contribute to NO production in asthma is lacking. This was the aim of the present study. At weekly intervals C57Bl/6 mice were sensitized twice with ovalbumin–alumen and challenged twice with ovalbumin aerosol. The peak of neutrophil infiltration in the bronchoalveolar lavage fluid (BALF) was 12 h after challenge, when neutrophils constituted 70% of the cell population and eosinophils only 1.5%. BALF cell preparations were stained with a NO-sensitive fluorescent dye (DAF-2) and with a nucleus marker (DAPI). Most DAF-2 stained cells could be identified as polymorphonuclear leukocytes, by the co-localization of both DAF-2 and DAPI staining. Cells from animals treated with L-NAME, were not stained for DAF-2 confirming the specificity of DAF-2 staining for NO. Moreover, the peak expression of inducible nitric oxide synthase (NOS2), in BALF cells and lung homogenates, was coincident with the peak of BALF neutrophil influx. NOS2 protein expression (arbitrary units) was detected 6 h after challenge (17.8±9.1 in BALF cells; 47.5±7.7 in lung homogenates), peak expression was at 12 h (54.5±8.7 and 133.7±10), decreasing thereafter, being no longer detected after 24 h. Thus, the neutrophils infiltrating the lung in the initial phase of murine asthma are producing NO via NOS2.

Keywords: Asthma; Inducible nitric oxide synthase; Neutrophils; Nitric oxide

1. Introduction

Asthma is a complex syndrome with many clinical phenotypes in both adults and children. Its major characteristics include a variable degree of airflow obstruction, bronchial hyperreactivity, and airway inflammation [1]. The pathological process is linked to chronic inflammation, which is responsible for bronchial hyperresponsiveness following the infiltration and accumulation of inflammatory cells and the remodeling of the airways [2]. These cells, as well as mast cells and lung resident macrophages, release a series of mediators and cytokines that are responsible for induction and maintenance of the airways inflammation [1].

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Nitric oxide (NO) seems to have an important role in asthma, not only in the induction of the Th2 immune response but also in amplifying and perpetuating the Th2-mediated inflammatory response. It has been speculated that the large amount of NO generated in the asthmatic airways may result in the suppression of Th1 cells and a concomitant reduction of interferon-γ (IFN-γ), leading to the proliferation of Th2 cells [4]. Synthesis of NO is catalyzed by NO synthases (NOS). Constitutive NOS (endothelial/NOS1 and neuronal/NOS3) are both calcium-dependent and generates NO within seconds [5,6]. On the other hand, inducible NOS (iNOS/NOS2) is calcium-independent, requires cytokines and/or lipopolysaccharide to be expressed and maximal induction occurs after few hours [7,8].

It has been demonstrated that pulmonary NOS2 expression is up-regulated in the lungs of asthmatics [9,11,12] but the role of NO as a pro- or anti-inflammatory mediator in asthma is still unresolved. Experimental models of asthma in mice have shown that acute inhibition of NOS2 activity either suppresses [13] or exacerbates airway inflammation [14]. Controversial data are also found in studies with $NOS2^{-/-}$ mice. Xiong et al., [15] showed that $NOS2^{-/-}$ animals presented a diminished airway inflammation, while others clearly showed that airway inflammation is fully expressed [16–18].

Regarding the cell responsible for NO production in the lung from asthmatics, the existing data are also controversial. Pulmonary vascular endothelial cells are an important source of NO in the lung and it was suggested that the localized production of NO in endothelium is responsible for the extravasation of eosinophils from the circulation into the lung tissue [22]. Mast cells, eosinophils and neutrophils also have the capacity to synthesize NO [19,20]. The neutrophil is the first cell to infiltrate the airways in response to antigen stimulation but demonstration that neutrophils infiltrating the lung in the initial phase of asthma are producing NO is lacking. In the present study we addressed this question and, by using a NO fluorescent marker and western blot analysis of NOS2 expression we were able to demonstrate that BALF neutrophils are producing NO and that the peak of NOS2 expression in BALF cells and in lung homogenates occurs in parallel with the peak of neutrophils infiltration into the airways.

2. Materials and methods

2.1. Animals

C57Bl/6 mice, male, weighing 20–25 g, 6–8 weeks old, from our own animal facilities were housed in a room with 12 h light–dark cycle with water and food *ad libitum*. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Ethical Committee for Animal Research of the Institute of Biomedical Science/USP.

2.2. Immunization protocol

The procedure used was previously described by Gama Landgraf et al. (2003). Briefly, mice were sensitized on days 0 and 7 by intraperitoneal injection of a mixture containing 50 μg of ovalbumin and 1 mg of Al $(OH)_3$ in saline (a total volume of 0.2 ml). At 14th and 21st days after the first immunization the animal were challenged by exposure to an aerosol of ovalbumin (2.5% w/v) generated by an ultrasonic nebulizer (ICEL US-800, SP, Brazil) delivering particles of 0.5–10 μm diameter at approximately 0.75 cc/min for 20 min. The control group consisted of animals immunized as described and challenged with phosphate buffered saline (PBS, pH 7.4 at 4 °C). Groups of animals received N^{ω}-nitro-L-arginine methyl ester (L-NAME, 30 mg/kg, i.p.), 30 min before each challenge.

2.3. Cell counts in bronchoalveolar lavage fluid (BALF)

The animals were killed by injection of ketamine/xylazine (50 μl of a 100 mg/ml solution, i.p.) at different times after exposure to the second aerosol challenge. A tracheal cannula was inserted via a midcervical incision and the airways were lavaged twice with 1 ml of PBS. The BALF was centrifuged at 170 ×*g* for 10 min at 4 °C, the supernatant was removed, and the cell pellet was resuspended in 0.5 ml of RPMI medium. One volume of a solution containing 0.5% crystal violet, dissolved in 30% acetic acid, was added to nine volumes of the cell suspension. The total number of cells was determined with the aid of a hematocytometer. Differential cell counts were performed after cytocentrifugation and staining with hematoxylin-eosin (Hema 3).

2.4. Detection of NO production in BALF cells

BALF cells (0.5×10^6/ml) taken 12 h after challenge, were placed on glass coverslips and after 15 min incubation at 37 °C, 5% CO_2; coverslips were washed with RPMI to remove non-adherent cells. This incubation time was chosen because of the preferential adherence of neutrophils compared to mononuclear cells. Neutrophils constituted more than 90% of the cells in the coverslips measured by hematoxylin/eosin staining (Hema 3). To demonstrate that the BALF neutrophils were producing NO we used the NO-sensitive dye, 4, 5-diaminofluorescein diacetate (DAF-2) [23]. Adherent cells were incubated at 37 °C with 12.5 μM DAF-2 in 0.1 M phosphate buffer (pH 7.4) containing 0.45 μM $CaCl_2$. After 2 h, digital images were collected on a Nikon E1000 microscope equipped for epifluorescence (excitation at 485 nm; emission 538 nm). The images were analyzed using the Image software (NIH, USA) by measuring the mean optical density

of the fluorescence observed in the cells in relation to the background staining. This fluorescence ratio was evaluated in at least five locations in each image and in at least two preparations from each animal.

That further demonstrate that the NO producing cells were neutrophils, cells were incubated with DAPI (4′, 6-diamidino-2-phenyindole) diacetate which is used to stain the cell nucleus (Matsumoto et al., 1981). The cells adhered to the coverslips were incubated at 37 °C with 10 μM DAPI in 0.1 M phosphate buffer (pH 7.4). After 2 h, digital images were collected on a Nikon E1000 microscope equipped for epifluorescence (excitation at 359 nm; emission 461 nm). Neutrophils were identified by the characteristic polymorphic nucleus.

2.5. Evaluation of NOS2 expression in BALF cells and lung homogenates

After collection of BALF, the whole lung was perfused via pulmonary artery with cold PBS and homogenized in 2 ml of ice-cold lyses buffer (10% Nonodiet P40, 150 mM NaCl, 10 mM Tris HCl pH 7.6, 2 mM PMSF, 5 μM Leupeptin) by 5 passes though a Potter/glass motorized homogenizer at medium speed. After centrifugation (50,000 ×*g*, 45 min, 4 °C), the protein content of each supernatant was determined using the BCA method (BCA™ Protein Assay Kit, Pierce). Aliquots of 1 ml, containing the same amount of protein, were mixed with 50 μl of an ADP-Sepharose 4B suspension (1:1 w/v, in the same medium). After thorough mixing for 1 h at 4 °C, the samples were centrifuged (10,000 ×*g*, 10 min, 4 °C), the pellet was boiled in Laemmli buffer (100 mM DTT, 0.1% bromophenol blue) for 4 min and submitted to SDS-PAGE.

2.6. SDS-PAGE for NOS2

For SDS-PAGE, aliquots containing the same amount of protein were submitted to electrophoresis on a 10% polyacrylamide gel. The proteins were electrophoretically transferred onto nitrocellulose membranes, and blocked with 5% nonfat dried milk in Tris buffered saline-tween (TBS-T, Tris HCl 10 mM, NaCl 150 mM, tween 20 0.1%) at room temperature for 2 h. After washing with TBS-T, a solution of antibody solution was applied overnight at 4 °C (mouse monoclonal IgG against NOS2 at dilution of 1:2000). Membranes were washed with TBS-T and a secondary antibody solution (anti-rabbit IgG antibody conjugated to horseradish peroxidase) at a dilution of 1:10,000 was applied for 1 h at room temperature. The immunocomplexed peroxidase-labeled antibodies were visualized by an ECL chemiluminescence's kit following manufacturer's instruction and exposed to a photographic film (Kodak, Brazil). Densitometry analysis of the bands was

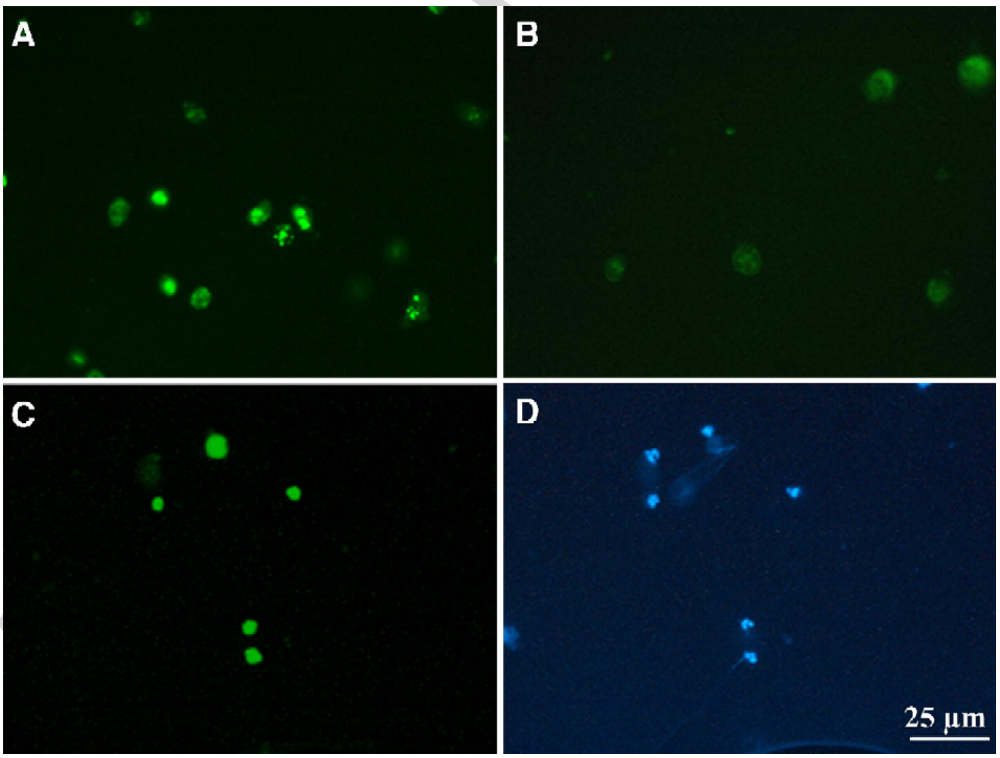

Fig. 1. *Digital images of BALF cells 12 h after induction of allergic lung inflammation*. Panel A: BALF cells from sensitized mice that received two antigen aerosol challenges showing high levels of fluorescence to DAF-2. Panel B: BALF cells from sensitized mice treated with the NOS inhibitor (L-NAME, 30 μg/kg) 30 min before each challenge, showing reduced fluorescence to DAF-2. Panels C and D: After staining adherent cells with DAPI, a nucleus marker, an intense blue staining could be seen in cells with multisegmented nucleus which is characteristic of polymorphonuclear cells. Most DAF-2 stained cells could be identified as polymorphonuclear leukocytes, by the co-localization of both DAF-2 and DAPI staining.

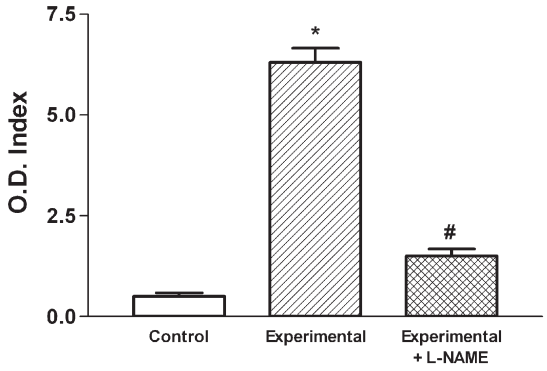

Fig. 2. *Intensity of DAF fluorescence in BALF cells*. Cell preparation as in Fig. 1 were analyzed for intensity of DAF-fluorescence by measuring the mean optical density of the fluorescence in the cells in relation to background staining. Fluorescence index was evaluated in at least five locations in each image and in at least two preparations from each animal. Values are expressed as mean ±S.E.M. and are representative of 4 animals. *$p<0.001$ in comparison with the control group and $^{\#}p<0.001$ in comparison with the immunized group.

performed using a laser scanner and processed by AlphaEaseFc™ software. The results were expressed as arbitrary units.

2.7. Drugs and reagents

Ovalbumin (Grade III), streptomycin, penicillin, L-glutamine, glycine, L-NAME and peroxidase-labeled monoclonal anti-rabbit IgG were all purchased from Sigma (USA). TRIS, sodium dodecyl sulfate, dithiothreytol, glycerol, bromophenol blue, tween 20, 4-(2-aminoethyl)-benzenesulfonyl fluoride, leupeptin, RPMI 1640 and HEPES were all from GIBCO (USA). Rabbit antiserum to NOS2 were from Cayman Chem. Co. Nitrocellulose membranes, Rainbow™ protein molecular weight markers, and the ECL Plus chemiluminescence kits were all purchased from Amersham-Pharmacia (USA); the Hema 3 was from Biochemical Sciences Inc. (USA) and aluminum hydroxide gel (Rehydragel) from Reheis Inc. (USA).

2.8. Statistical analysis

The number of experiments is indicated in the figures. Results are presented as mean ±S.E.M. Statistical differences (*$p<0.05$ and ***$p<0.001$) between mean values were determined by one-way ANOVA or Student's t test for non-paired samples followed by Bonferroni post-test.

3. Results

3.1. Production of NO by BALF neutrophils

BALF cells collected 12 h after challenge, were incubated for 15 min at 37 °C in glass coverslips when neutrophils constituted more than 90% of the cells and these cell preparations were incubated with DAF-2, a NO-sensitive fluorescent marker. Fig. 1 shows BALF cell preparations with high levels of fluorescence (Fig. 1A). Cells from mice pre-treated with L-NAME before antigen challenges show reduced fluorescence (Fig. 1B). After staining the cells with DAPI, a marker of the nucleus, it could be noted that the fluorescence was localized on cells showing multisegmented nucleus, a characteristic of polymorphonuclear leukocytes. Most DAF-2 stained cells could be identified as polymorphonuclear cells by DAPI staining of multisegmented nuclei (Fig. 1C and D).

Fig. 2 shows the image analysis of BALF cells stained with the NO-sensitive marker DAF-2. Fluorescence index was very low in cells from the control group (0.5±0.2) whereas it increased significantly in cells obtained from OVA challenged animals (6.3±0.8). Fig. 2 also shows that treatment of the animals with L-NAME, an inhibitor of NO synthases, before the antigen challenges of immunized mice, reduced fluorescence index by 76%.

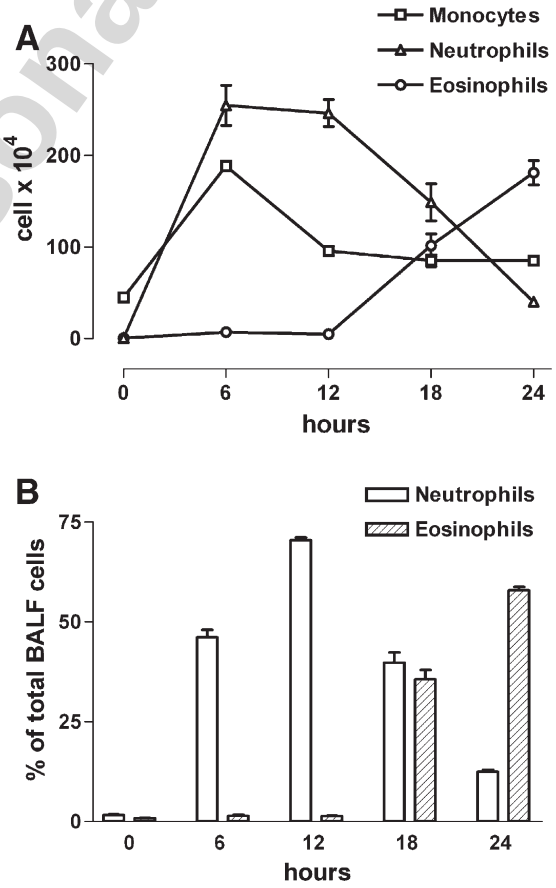

Fig. 3. *Time course of neutrophil and eosinophil infiltration into airways*. C57Bl/6 mice were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post-immunization. BALF was performed 6, 12, 18 and 24 h after the second challenge. Neutrophils and eosinophils were counted in cytocentrifuge preparation of BALF stained with hematoxylin/eosin. (A) Number of cells $\times 10^4$ and (B) percentage of neutrophils and eosinophils. Results are the mean ±S.E.M. of 8–10 animals.

3.2. Time course of BALF neutrophil infiltration

Mice immunized with ovalbumin were submitted to two ovalbumin aerosol challenges and a bronchoalveolar lavage was performed 6, 12, 18 and 24 h after the second aerosol challenge. Total cell count in the control group (immunized mice submitted to saline aerosol) was less than 10^4 cells/ml at all times examined. Six hours after the ovalbumin challenge, there was an increase in the total number of cells (4.49×10^6 cells/ml) which persisted elevated until 24 h.

Differential counts in BALF cells stained with hematoxylin-eosin showed that in the control group the number of neutrophils and eosinophils is very low (less than 1×10^4 cells/ml). In the OVA challenged lungs there is a marked increase of BALF neutrophils at 6 h ($254.5 \pm 48.6 \times 10^4$ cells/ml), 12 h ($246 \pm 44.6 \times 10^4$ cells/ml), 18 h (148.8 ± 45, 4×10^4 cells/ml) and 24 h ($40.2 \pm 9.7 \times 10^4$ cells/ml). Until the 12th hour, eosinophil count was very low, increasing only after 18 h ($101.6 \pm 27.6 \times 10^4$ cells/ml) and 24 h ($181 \pm 39.9 \times 10^4$ cells/ml) (Fig. 3A). After 12 h of the OVA challenge, the percentage of neutrophils in BALF was 70% whereas the percentage of eosinophil was 1.5%. An inverse relationship was observed 24 h after the challenge when eosinophils constituted 58% and neutrophils 12% of BALF cell population (Fig. 3B).

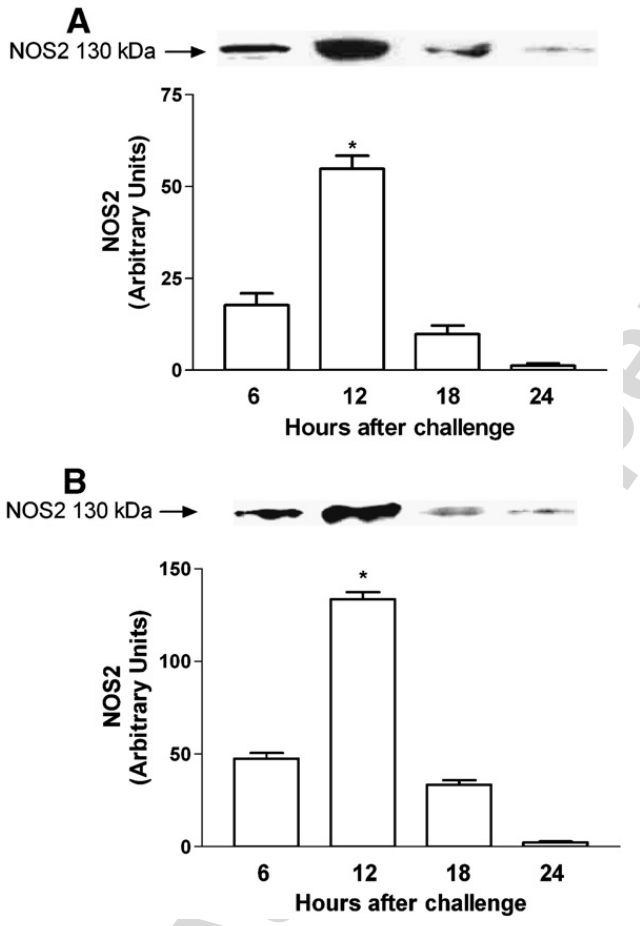

Fig. 4. *Time course of NOS2 protein expression in BALF cells and lung homogenates.* C57Bl/6 mice were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post-immunization. Lung tissue was processed 6, 12, 18 and 24 h after the second challenge. Western blot analysis showed NOS2 is detectable as early as 6 h after challenge both in BALF cells (A) and in lung homogenates (B). There was a peak after 12 h, a decrease after 18 h and at the 24 h NOS2 was no longer detected. Results are the mean ± S.E.M. of 8–10 animals. *$p < 0.01$ in comparison with the 6 h group.

3.3. Time course of lung NOS2 protein expression

Fig. 4 shows the time course of NOS2 protein expression in BALF cells and in lung homogenates from OVA challenged animals. Western blot analysis of BALF cells showed that NOS2 is detected 6 h after challenge (17.8 ± 9,1 arbitrary units). This value increased at 12 h (54.5 ± 8.7 arbitrary units) and decreased thereafter (9.8 ± 6.6 arbitrary units at 18 h and less than 1.3 ± 1.2 arbitrary units at 24 h). Similar results were obtained with lung homogenates where NOS2 was also detected at 6 h after challenge (47.5 ± 7.7 arbitrary units), reached a peak after 12 h (133.7 ± 10 arbitrary units), decreased on the 18th h (33.6 ± 6.6 arbitrary units), and was undetectable after 24 h (less than 2.5 ± 1.2 arbitrary units).

4. Discussion

The model used here to study allergic lung inflammation consisted of immunization with ovalbumin using aluminum hydroxide as adjuvant followed, 21 days later, by two ovalbumin aerosol challenges, one week apart. This model presented many of the characteristic features of allergic asthma, such as eosinophil and lymphocyte infiltration, mucus production and airway hyperreactivity [24,25].

Our results obtained with fluorescent markers showed that in the initial phase of murine asthma (12 h after antigen aerosol challenge) neutrophils infiltrating the BALF are producing NO.

We also observed that in this model of lung inflammation there is an increase in the number of neutrophils in BALF 12 h after the second antigen challenge. At 24 h, the number of neutrophils diminished, while the number of eosinophils increased. Simultaneous analysis of NOS2-enzyme expression by Western blot showed that the enzyme was detectable as early as 6 h after challenge, reached a peak after 12 h, decreased on the 18th hour, and was undetectable at the 24th hour in both BALF cells and lung homogenates. Although in lung homogenates it is impossible to discriminate which cells are expressing NOS2, since several cell types are potentially able to express it, the fact that lung homogenates expression of NOS2 correlates with BALF expression and BALF neutrophils infiltration, allows the

speculation that neutrophils infiltrating the peribronchovascular area of lung parenchyma are the major NOS2 expressing cells in the lung homogenates at this time.

In allergic inflammation, eosinophils are predominant cells in the late phase. Some authors have shown that neutrophils are also observed in bronchial biopsies and BALF from asthmatics, but in relatively low numbers [30,31]. However, eosinophils are considered the effector cells in asthma by the observations that the eosinophil-derived granule products promote denudation of airway epithelium, destruction of epithelial morphology, increased micro vascular permeability, edema and hyperreactivity. Although neutrophil granules also possess a wide array of substances potentially tissue damaging, the contribution of neutrophils to asthma features remains to be determined. When adequately stimulated both cell types are able to produce NO. The role of NO in asthma, however, is still controversial. We have recently shown [18] that inhibition of NOS2 by L-NAME reduced airway hyperreactivity and eosinophil infiltration in allergic lung inflammation in mice suggesting that NO has a pro-inflammatory effect. Similar studies in guinea pigs have shown that NOS inhibitors potentiated acute bronchoconstriction induced by allergen inhalation [32,33], whereas other studies *in vitro* and *in vivo* showed that the hyperreactivity induced by allergens is not affected by pretreatment with NOS inhibitors [33,34].

These discrepant results could be attributed to the different models employed but also to the phase of the allergic inflammation analyzed. Although NO may exhibit an immediate anti-inflammatory function in some situations of oxidative stress, persistently elevated levels of NO synthesis increase tissue injury over time [35,36].

Several pieces of evidence indicate that the biological activities of NO may vary depending on the NO concentration (low or high doses exert pro- or anti-inflammatory effects, respectively), the cell types, enzymes, and transcription factors involved [17,37].

Ours results show that following antigen challenge there is a transient expression of NOS2 in lung tissue and airways cells, coincident with NOS2 expression by BALF cells, and that neutrophils infiltrated in the airways produce NO. The biological significance of these findings remains to be determined. One possibility is that this early generation of NO by infiltrating neutrophils is somehow responsible for the migration of eosinophils into the airways, which follows neutrophils. We have previously observed that L-NAME, given before antigen challenge, reduced significantly (93%) the eosinophils infiltration measured at 24 h. It is also possible to speculate that the NO generated early after antigen challenge would counteract the bronchoconstriction caused by the burst of mediators released by allergen stimulation. Moreover, this peak of NO following antigen challenge could activate Th2 lymphocytes to release cytokines that would be important in the late phase of asthma. Thus, it is important to increase our understanding on the role of NO in asthma before proposing the use of NOS inhibitors in this disease.

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