Level of Nitric Oxide in Bronchoalveolar Lavage Fluid of Asthmatic Mice Model

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ABSTRACT

Background: Asthma is a chronic inflammatory disease with multifactorial and complicated mechanisms. Elevated level of exhaled Nitric Oxide (NO) in asthma and other inflammatory lung diseases has led to many studies examining NO as a potential marker of airway inflammation. **Objective:** This study was designed to determine the level of NO in Bronchoalveolar Lavage (BAL) fluid during early and late stages of asthmatic attack in mouse model. **Methods:** In this study male BALB/c mice were used. The level of NO was determined in BAL fluid of asthmatic mice five minutes, six and sixteen hours after challenge with methacholine, as irritant and smoke and 5% ovalbumin as allergens, using colorimetric assay. **Results:** The level of NO increased upon exposure to all three irritants used in this study (52.3 μM for smoke and 49.5 μMfor methacholine) as compared to 22.8 μM for the baseline. Our results showed that NO levels were increased during early phase of asthmatic condition and reached to its maximum level after six hours and decreased at the late stage of asthma (16hrs) possibly by activating a feedback regulatory loop. In addition, high level of NO led to the hypertrophy of smooth muscle that can account for the pathological changes associated with asthma. **Conclusion:** Thus, NO is an inflammatory marker in asthma and its measurement, as a non-invasive method during asthmatic attack is suggested. A careful development of specific inhibitors for iNOS enzyme during asthmatic attack is also necessary.

Keywords: Asthma, BAL fluid, Inflammation, Methacholine, NO, Ovalbumin

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INTRODUCTION

Asthma is a clinical syndrome defined physiologically by episodic reversible airway narrowing and hyperresponsiveness of the airway to a variety of stimuli (allergens, irritants and infections). It is also defined pathologically by the presence of certain recognizable microscopic features including infiltration of the airway and lamina propria with chiefly eosinophils, and presence of mast cells, neutrophils, and T lymphocytes (1). In addition, hypertrophy and hyperplasia of airway smooth muscles, mucous secretory apparatus, and overall thickening of the airway wall are also important pathophysiological components of asthma (1).

Research evidence has shown that the concentration of nitric oxide (NO) in exhaled gas is elevated in asthmatic patients (2). Nitric oxide is a gaseous signaling molecule and activator of soluble guanylyl cyclase in the cardiovascular system. It is also ubiquitously present in the respiratory system where it functions as a regulatory molecule in health and disease (3). Numerous studies over the past years have provided direct evidence for a modulatory role of NO and its synthesising enzymes in asthma (4,5). However, it is still controversial whether this is beneficial or deleterious (4,6).

Nitric oxide is generated by the three different NOS isoforms, which are all expressed in pulmonary cells. In the respiratory tract and in normal stages NO is generated by two constitutive NOS (cNOS) isoforms, including neuronal NOS (nNOS or NOS-1), and endothelial NOS (eNOS or NOS-3), and controls the relaxation of smooth muscles and modifies the function of central and peripheral nervous system (7). The third NOS isoform is inducible (iNOS or NOS-2) which is responsible for the massive and prolonged generation of NO (6,7).

In the respiratory tract NO is produced by a wide variety of cell types including epithelial cells, airway nerves, inflammatory cells (macrophages, neutrophils, mast cells) and vascular endothelial cells (4). After its formation, NO decomposes into other nitrogen oxides, namely nitrite ($NO2^-$) and nitrate ($NO3^-$) (6,7). NO also reacts with superoxide anion to produce peroxynitrite ($ONOO^-$), which is a potent cytotoxic molecule (8). Peroxynitrite causes AHR and epithelial damage, enhances inflammatory cell recruitment, and inhibits pulmonary surfactants (4).

There is increasing evidence that endogenous nitric oxide (NO) plays an important role in physiological regulation of the airway and is implicated in the pathophysiology of airway disease (9,10). NO mediated protective physiological responses include antimicrobial activity (11), increased mucin secretion and increased ciliary motility (10). This NO is generated by cNOS. However, prolonged exposure to large concentrations of NO which are generally produced by iNOS enzyme may lead to tissue injury by the formation of NO derived metabolites.

Exhaled NO is significantly elevated in acute asthma, or steroid-resistant severe asthma (12). Atopic asthmatic patients have much higher levels of exhaled NO than subjects with nonatopic asthma (13,14). In airway inflammation, NO is not only a marker but may have anti-inflammatory and pro-inflammatory effects (12). The relationship between NO and FEV1 depends on the severity of asthma (15). It has been shown that NO can be significantly reduced or even normalized whereas other markers of airway inflammation, e.g. number of eosinophils in sputum, would still be high (12). In addition, iNOS is up-regulated in asthma and there is evidence for increased expression of iNOS protein and mRNA in asthmatic airway, particularly in macrophages and epithelial cells (16). High concentrations of NO may have adverse effect on immune system and hence, the onset of inflammatory

responses. NO inhibits Th_1 lymphocytes in mice and thus favors the development of a Th_2 response with eosinophilia (17).

Currently, all the direct methods for assessing airway inflammation, bronchial biopsy specimens, and analysis of BAL fluid, are invasive tests. The noninvasive tests are needed for assessing inflammatory markers, and candidates include induced sputum analysis and measurement of exhaled mediators (18). The measurement of NO is noninvasive and a sensitive marker for early airway inflammation and can be performed repeatedly, even in patients with severe asthma (19).

When asthmatic mice were exposed to allergens, the early-phase of AHR began in 5 minutes, reached to its peak within 15 minutes, and normalized in 60 minutes, whereas the late-phase developed after 3.5 hours (20). Cytokines have been considered to have an important role in immune response, inflammation and probably a role in asthma (21). Thus, this study was designed to measure the levels of NO in BAL fluid during early-phase and late-phase of asthmatic mouse model using methacholine, smoke and 5% ovalbumine challenge as irritants and allergens.

PATIENTS AND METHODS

Materials. Aluminium Hydroxide and Acetyl- -Methacholine Bromide (MCH) was purchased from Sigma Co. (US). Egg Ovalbumin was obtained BDH Co. and nitric oxide kit for the measurement of nitric oxide was the product of Roche Co. High tar Winston cigarette was purchased from market. Distilled water was used for preparation of all solutions.

Methods.

I: Generation of Asthmatic Mouse: Sensitization Protocol. Male BALB/c mice (6 weeks of age) were immunized by a combination of 4μg ova and 2mg/ml aluminum hydroxide gel (22) (alum, as adjuvant) by IP injection on day 0, and again on day 11. Control mice received pyrogen-free saline injection instead of the ova/alum. Solution of 3% ova diluted in PBS was administered by a compressor/ nebulizer for 10 minutes. The aerosol was generated in a Plexi glass chamber connected to a nebulizer with a maximum aerosol output of 5ml/min and an average particle size of 4μm. Control mice inhaled PBS under the same conditions. Ninty-six hours after the fifth ova challenge (on day 26), a final ova inhalation challenge was given in the same way, and the control group was challenged with PBS (23).

II: Collection of BAL Fluid. Forty eight hours after the last series of ova challenges (e.g.; on day 28) BAL fluid was collected. Animals were anesthetized by IP injection of 10% ketamin (44mg/kg). Then BAL fluid was collected by cannulating the upper part of the trachea through tracheal catheter and lavaging three times with 0.5 ml pyrogen- free PBS (75%-85% of the 0.5 ml input volume was retrieved). The cell suspension was centrifuged at 300 x g for 10 minutes at 4°c. The supernatants were collected and stored at -70°c until analyzed.

III: Cell Analysis of BAL Fluid. The cells were resuspended in 1 ml of PBS, and the total cell numbers were counted with a cell counter (Sys. Mex.). The samples were centrifuged at 1000 rpm and smears from sediments were obtained using May-Grumwaled Giemsa staining. Differential cell count was determined with at least 100 leukocytes in each sample.

IV: Histological Studies. Lung were removed from the chest cavity and fixed in 10% formalin (10 ml), and embedded in paraffin; 3μm sections from left lung were obtained and stained with Wright-Geimsa stain to identify eosinophils in prevascular and peripheral areas of lung tissue.

V: Exposure to Irritants. The preliminary study showed that exposure to 3% ova did induce asthma in mice. Animals were assigned into 10 groups, each group included 8 mice. One group was assigned as control and other groups were exposed to three irritants.

VI: Cigarette Smoke Challenge. Three groups of asthmatic mice were exposed to cigarette smoke. Each animal received 20 puffs for ten minutes by poar. BAL fluid was collected from the first group after 5 minutes, from the second group after 6 hours and third group after 16 hour upon exposure to smoke.

VII: Methacholine Challenge. The asthmatic mice were exposed to nebulized Mch (25 mg/ml) for 3 minutes each. BAL fluid was collected with Mch challenge as before.

VIII: 5% OVA Challenge. The asthmatic mice were exposed to nebulized 5% ova for 5 min. Each BAL fluid was collected with ova challenge as before.

IX: Irritants. Smoke was selected as an areoirritant, and 5% ova was selected as a specific allergen. The Mch was selected because of the stimulation of smooth muscles via muscarinergic M3 receptors and for comparison with other irritants.

X: NO Measurements. Nitric oxide were measured by spectrophotometric assay (Griess reaction) using NO kit.

XI: Data Analysis. Data were analyzed by ANOVA and student's t-tests and $P \leq 0.05$ was used to assess significant differences between treated and control groups.

RESULTS

Cell Analysis of BAL Fluid. Total WBC count in the asthmatic group was higher ($1700/mm^3$) as compared with control group ($250/mm^3$). On day 28 the percentages of eosinophils, lymphocytes and neutrophils were 38.6%, 25% and 36%, respectively. As compared with the control group there were only 2-3 eosinophils in each 10 microscopic fields (Fig. 1).

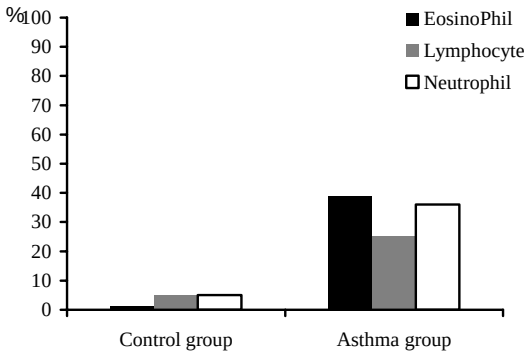

Figure 1. The percentage of WBC in BAL fluid of asthmatic and control group

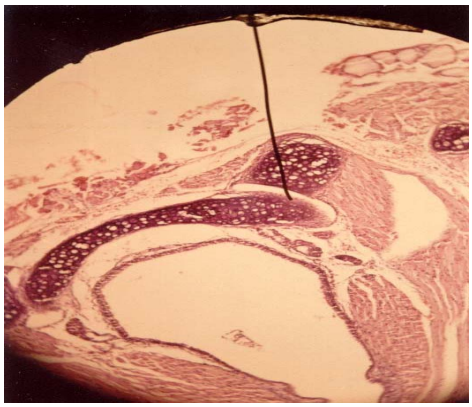

Figure 2. Hypertrophy in smooth muscle cell layer in asthmatic group

Edema and Inflammation. On day 28, a marked bronchial inflammation (+++) in the Ova-treated mice was detected as compared with control group (+) in microscopic study. In addition, the infiltration of inflammatory cells (most of them eosinophils) into the peribronchial interstitial area and into the mucosal and submucosal areas of the bronchus was observed. Also, on day 28 a marked hypertrophy in smooth muscle cell layer in the Ova-treated mice was detected (Fig. 2)

NO Measurements. The mean baseline level of NO in control group was 22.8µm. The mean level of NO after 5 minutes of exposure to Mch smoke and 5% Ova were 49 µm, 25 µm and 34 µm, respectively. Our results shows significant increase of NO level in methacholine

treated mice ($p \leq 0.0001$) after 5 minutes of exposure as compared to control group (Fig. 3). In addition, NO level in BAL fluid of mice treated with 5% Ova was significantly increased as compared to control group ($p \leq 0.004$). The NO level of BAL fluid in smoke treated mice

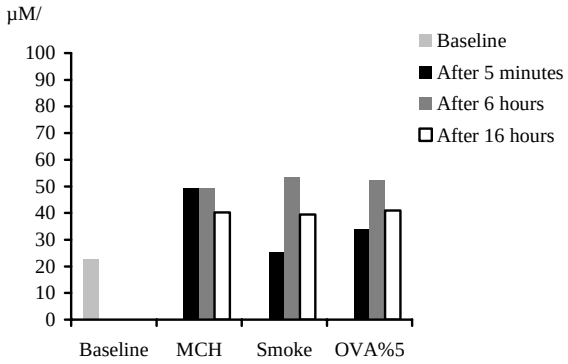

Figure 3. The level of NO in BAL fluid of treated mice and control group at different interval times

was slightly increased but not significantly. The mean levels of NO in BAL fluid after 6 hours of exposure to Mch, smoke and 5% Ova were 49.5 µm, 53.4 µm and 52.4 µm, respectively. After 16 hours of exposure to the above irritants the mean levels of NO in BAL fluid were 40.3 µm, 39.5 µm and 40.9 µm, respectively (Fig. 3). The comparison of the level of NO in treated groups showed the significant increase of NO in both smoke treated mice (53.4 µm) and 5% Ova treated mice as compared to control group after 6 hours of exposure (Fig. 4). A decrease in NO level after 16 hours of exposure to the above irritants was observed as com-

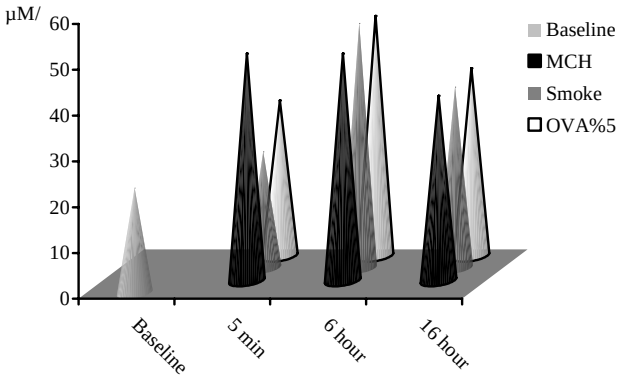

Figure 4. Comparison of NO level between treated group and baseline

pared to 6 hours of exposure. However, there was a significant increase in NO level after 16 hours of exposure compared to control group. The peak levels of NO upon exposure to Mch, smoke and 5% Ova treated mice were during the 6 hours of contact (Fig. 4).

DISCUSSION

It has been reported that nitric oxide (NO) may play an important role in regulating airway function and in the pathophysiology of inflammatory airway diseases (1). Barnes PJ et al. (24) and Kharitonov SA et al. (12) have studied exhaled nitric oxide in asthmatic patients. They found that asthmatic patients exhaled significantly higher level of NO. Thus, measurement of NO or other inflammatory markers which may induce the production of NO could be used as a diagnostic tool for the screening of asthmatic patients. Recently the measurement of exhaled NO has been proposed as a non-invasive, simple test and a sensitive marker for airway inflammation in asthma (3). The present study was designed to investigate the level of NO in BAL fluid during early and late stages of asthmatic attack in mouse model upon exposure to Mch, smoke and 5% ova.

The results of this study showed significant increase of NO after 5 minutes, 6 hours and 16 hours intervals. The level of NO increased during early phase of asthma and reached to its maximum level after 6 hours and decreased at the late stage (16 hours) of asthmatic attack as compared to the baseline. These increased levels of NO in BAL fluid of asthmatic mice are the consequences of exposure of immune cells, especially inflammatory cells, to the Mch, smoke and 5% ova. These stimulatory agents have induced type II NOS (iNOS) enzyme in inflammatory cells and thus the release of large amounts of NO. These data confirms the other reports concerning the expression of type II NOS which would lead to increased level of NO in biopsy specimens from asthmatic patients (25). Other reports indicate the induction of iNOS by INF-γ, TNF-α, TNF-β, IL-β and endotoxin, as well as by a variety of other cytokines (4,5), allergens or even environmental air pollutants and subsequent release of large quantities of NO several hours after exposure (15).

NO may amplify allergic inflammation by selective inhibition of Th_1 lymphocytes which allows the activation of Th_2 cells. Th_2 cells are normally suppressed by INF-γ which is secreted by Th_1 (11). Th_2 cells secrete IL-4 and IL-5 which lead to mast cell sensitization by IgE formation and eosinophil recruitment within airways and ultimately production of high level of NO in asthma (26). The results obtained in this study support the above reports concerning the high level of NO which may correlate with asthma.

Recently, in-vitro and in-vivo studies have shown increased AHR (Airway Hyper Responsiveness) induced by allergen 6 hours after exposure (27). In asthmatic mice after exposure to allergen (early phase) AHR begins in 5 min., reaches its peak within 15 minutes and normalizes after 60 minutes whereas, the late-phase develops after 3.5 hours, maximizes in 6 hours and returns to the baseline by 8.5 hours (20,28). Other findings have shown that peritoneal macrophages in mouse produce large amounts of NO after 6 hours when stimulated with LPS (7). Fig. 4 shows the maximum level of NO after 6 hours of exposure as compared to baseline. The generation of these high levels of NO upon exposure to stimulants used in this study could also be explained by the secretion of cytokines such as INF-γ, IL-2 and IL-3 by the cells which ultimately induce the iNOS enzyme and hence, production of high level of NO. This high level of NO may function as a cytotoxic agent and evoke inflammation (12,29).

Cytokine-mediated NO release at the early-phase of asthma onset is a characteristic of positive feedback in this condition. On the other hand, NO has an inhibitory effect on Th_1 cells thus, constituting a negative feedback mechanism. Cytokines down regulated by NO include macrophage inflammatory protein 1-a (MIP-1a) and GM-CSF, both of which are among the chemoattractants for inflammatory cells such as eosinophils, basophils and T-lymphocytes implicated in asthma inflammation (14).

However, our findings suggest that application of Mch, smoke (non-specific allergen) and 5% ova (specific allergen) induce the type II NOS enzyme (iNOS) and subsequent increased production

of NO in an asthmatic mouse model leading to the hypertrophy of smooth muscle and inflammation which can account for the pathological changes that are associated with asthma. It is noteworthy that high level of NO may be cytotoxic to other NO producing cells resulting in shedding of those cells. Our conclusion remark is that the control of NO production during asthmatic attack would be beneficial to asthmatic patients thus NOS inhibitors could represent a novel therapeutic approach for the inflammation of asthmatic condition. Although NO is a bronchodilator and seems to be a great candidate as a marker for determining the severity of asthma, a careful development of specific inhibitors for type II NOS (iNOS) and measurement of NO for diagnosis of asthmatic patients will open a new horizon in the area of inflammatory diseases

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