Increased nitric oxide production by neutrophils in bronchial asthma

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ABSTRACT: This study was designed to assess the production of nitric oxide (NO) by neutrophils in bronchial asthma.

Thirty asthmatic patients (ten each of mild, moderate and severe asthma) and ten healthy controls were included in the study. Neutrophils from peripheral venous blood were stimulated with latex, and production of nitrite (an NO metabolite) and L-citrulline (a co-product of NO) was studied. It was postulated that peripheral blood neutrophils, being in a primed or activated state in asthma, would reflect the changes occurring in bronchial tree neutrophils.

Nitrite and L-citrulline production by neutrophils was significantly higher in asthmatics (p < 0.001) and increased with disease severity. A strong negative correlation was observed between peak expiratory flow and both nitrite (r = -0.87, p < 0.001) and L-citrulline (r = -0.88, p < 0.001) production.

It is concluded that nitric oxide production by neutrophils is increased in bronchial asthma and can possibly contribute to airway narrowing and disease severity.


Nitric oxide (NO) is implicated in the pathogenesis of bronchial asthma, but its precise role has not been defined [1]. NO acts as a neurotransmitter in the bronchodilator nonadrenergic, noncholinergic nerves [2]. Several experimental studies have shown that NO administration produces bronchodilation [3, 4]. These observations lead to the impression that it is a relative deficiency of endogenous NO that might contribute to airway narrowing in bronchial asthma. Endogenous NO production may, however, act as a double-edged sword. When produced locally by constitutive nitric oxide synthase (NOS) in the bronchodilator neurons, it may be beneficial in relaxing airway smooth muscle in small amounts [2]. However, when produced in high concentrations by inducible nitric oxide synthase (iNOS) present in various inflammatory cells, it leads to hyperaemia, oedema and exudation, and can contribute to airway narrowing in bronchial asthma [5]. Various cells and their mediators are involved in airway inflammation and hyperresponsiveness in asthma. These cells include neutrophils, which play an important role in asthma attacks, especially in the late-phase reaction [6]. Neutrophils have iNOS and are capable of producing NO [7]. This study was designed to look into the endogenous production of NO by neutrophils.

Material and methods

Subjects

The study included 30 known patients of bronchial asthma (10 each with mild, moderate and severe asthma) selected from those attending the Chest Clinic and Medical Emergency Service of the Postgraduate Institute of Medical Education and Research, Chandigarh, India. Patients with history of recurrent attacks of wheezing, breathlessness or chest tightness provoked by various stimuli, in whom physical examination and peak expiratory flow (PEF) measurement supported the diagnosis of bronchial asthma, were included. Ten healthy volunteers were studied for control values. Smokers, patients with other concomitant medical disorders or those with respiratory tract infection in the preceding 6 weeks were excluded. Informed written consent was obtained from each participant. The study protocol had been approved by the Institute’s Medical Ethics Committee.

Study design

Detailed history was obtained and physical examination carried out in each subject. Relevant laboratory investigations and PEF measurements were obtained in all patients to establish the diagnosis and grade the severity of asthma. Severity of asthma was assessed using criteria defined by the International Consensus Report on diagnosis and treatment of asthma [8]. Patients with intermittent brief symptoms (<1 – 2 times a week), PEF >80% predicted, and requiring intermittent inhaled short acting β2-agonists only, were categorized as the mild asthma group. Those with exacerbations >1 – 2 times a week, PEF 60–80% pred, and requiring a daily inhaled anti-inflammatory agent and a long-acting bronchodilator were categorized as the moderate asthma group.
Patients with more frequent exacerbations or continuous symptoms restricting physical activities, PEF < 60% pred, and requiring frequent systemic corticosteroids, were characterized as the severe asthma group. In case of overlap of these features, an individual was assigned to the most severe grade in which any one feature occurred.

Heparinized peripheral venous blood (5 mL) was collected from each subject and the neutrophils were studied for their ability to generate nitrite (an NO metabolite) and l-citrulline (a co-product of NO pathway) upon stimulation with latex.

Estimation of nitrite and l-citrulline

Neutrophils were separated by Ficoll isopaque gradient centrifugation [9] and viability of cells was checked by 0.1% trypan blue dye exclusion [10]. Identification of neutrophils was carried out by Giemsa staining. Around 10^6 neutrophils were stimulated with 20 μL latex and incubated for 2 h.

The accumulation of nitrite in culture supernatant was measured using the method described by GREEN et al. [11]. Absorbance was measured at 546 nm using an Uvicon Spectrophotometer (Model No. 930, Kontron). l-citrulline in the culture supernatant was estimated using the procedure described by BOYDE and RAHMATULLAH [12], by measuring absorbance at 530 nm. For both tests, each standard and culture supernatant sample was analysed in duplicate and an average absorbance value was calculated. The nitrite and l-citrulline concentrations in the culture supernatant were calculated from absorbance values using standard curves. These standard curves were prepared separately using varying concentrations of sodium nitrite in a range of 10 – 700 nM, and of DL-citrulline in a range of 10 – 500 nM.

Statistical analysis

All values were expressed as mean ± SD. One way analysis of variance and unpaired t-tests were used to evaluate differences in PEF, nitrite and l-citrulline production among various groups. Nitrite and l-citrulline levels were also correlated with PEF by calculating Pearson’s correlation coefficient.

Results

Thirty asthmatic individuals (18 males and 12 females) in the age range 15 – 45 yrs and 10 healthy subjects used as controls were studied. The 10 patients with mild asthma had a mean ± SD PEF of 93 ± 14% pred; seven of them were currently using inhaled β₂-agonists on an "as and when required" basis. Ten patients with moderate asthma had a PEF of 67 ± 8% pred; nine of them required regular inhaled β₂-agonists. The 10 patients with severe asthma had a PEF of only 30 ± 13% pred.

Nitrite and l-citrulline production by neutrophils were significantly higher in asthmatics as compared to controls (fig. 1). There was a progressive increase in nitrite production as the severity of asthma increased, with a nitrite level in mild, moderate and severe asthma of 161.4 ± 24.8, 347.6 ± 30.9 and 485.6 ± 54.5 nM·10^6 cells, respectively (p < 0.001). A similar trend was seen in l-citrulline production in mild, moderate and severe asthma (82.4 ± 18.9, 143.8 ± 27.3, 186.7 ± 14.3 nM·10^6 cells, respectively, p < 0.001). PEF among all asthmatics correlated negatively with nitrite and L-citrulline levels (r = -0.87 and -0.88, respectively, p < 0.001) (figs. 2 and 3).

Four patients each in moderate and severe asthma groups were on steroids at the time of study. Patients of severe asthma on steroids had a nitrite level of 443.10 ± 39.1 nM·10^6 cells in culture supernatant, which was significantly lower than those who were not on steroids (513.9 ± 45.1 nM·10^6 cells, p < 0.05).

Fig. 1. – Distribution of nitrite (▲) and L-citrulline (●) levels in asthmatics and controls. Peripheral blood neutrophils of the subjects were stimulated with latex. The nitrite and L-citrulline levels in the culture supernatant are shown (p < 0.001 on comparing each group with each of the other groups).

Fig. 2. – Relation between peak expiratory flow (PEF) and nitrite levels in asthmatics. A significant negative correlation was observed (r = -0.87, p < 0.001).
Similarly, patients of moderate asthma on steroids had a significantly lower nitrite production (317.0 ± 12.7 nM·10^-6 cells) compared to those who were not on steroids (368.0 ± 19.2 nM·10^-6 cells, p < 0.001). However, no significant difference was noted in L-citrulline production.

Discussion

Nitrite and L-citrulline, being the metabolize and co-product of NO respectively, are valid indicators of NO production [13]. In asthma, neutrophils are in a primed or activated state in peripheral blood [14 – 16]. Several investigators have looked for abnormal generation of inflammatory mediators by peripheral blood neutrophils in asthma, and their results were similar to those obtained when bronchoalveolar lavage specimens were processed to assess the local production of those mediators [17, 18]. Peripheral blood neutrophils are easily accessible, easy to isolate and reflect the changes occurring in the inflammatory cells of bronchial tree. On the other hand, performing a bronchoalveolar lavage is not always safe in asthma. This study, therefore, investigated peripheral neutrophils.

The production of nitrite and L-citrulline by neutrophils in this study increased significantly as the severity of asthma increased from mild to severe, and a strong negative correlation was noted with PEF, suggesting an association between NO production and progressive airway narrowing. Other investigators have also observed higher NO levels in exhaled air in asthmatics [19, 20]. NO is a potent vasodilator and may contribute to hyperaemia and oedema leading to airway narrowing in asthmatics. In fact, inhibition of endogenous NO production was shown to decrease plasma exudation and inflammation in guinea-pig airways in one study [21].

Conversely, it can be argued that an increase in NO levels, found with increasing levels of bronchoconstriction, is a host defence response in asthma to counteract bronchoconstriction, in view of the smooth muscle relaxant effect of NO. However, steroids, which inhibit the iNOS [22] and thus decrease NO production, are well known to have a beneficial role in bronchial asthma. This is another strong pointer that favours the hypothesis that increased NO production might actually contribute to airway narrowing in asthmatics. This study found a lower nitrite production by neutrophils among patients treated with steroids in each group. This finding is consistent with the noted effect of steroids on NO production.

In summary, it is concluded that nitric oxide production by neutrophils is increased in bronchial asthma and could partly contribute to airway narrowing.

References


