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Evaluation of oxidative–antioxidative status and the L-arginine–nitric oxide pathway in asthmatic patients

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Summary *Background:* Reactive nitrogen, oxygen species and oxidative stress are related to many pulmonary diseases. Nitric oxide (NO) may be involved in either the protection against or the induction of oxidative stress within various tissues. It is derived from the amino acid L-arginine by the action of NO synthase (NOS). L-arginine can also be metabolized by arginase with the production of ornithine and urea. Because of the competition between NOS and arginase for the same substrate, their activities are regulated reciprocally. Therefore, the rate of NO generation associated with oxidative stress is dependent on the relative activities of both NOS and arginase. The objective of this study is to investigate the L-arginine–NO pathway, evaluate oxidative–antioxidative status in the patients with asthma and demonstrate their reciprocal regulation.

Methods: 30 voluntary asthmatic patients and 30 healthy control subjects with similar age range and sex were included in the study. A total of 10 ml venous blood was drawn, plasma and packed erythrocytes were prepared for the biochemical analyses. Plasma arginase activities and NO levels, and erythrocyte malondialdehyde and reduced glutathione levels were detected.

Results: Plasma malondialdehyde levels were significantly higher and glutathione levels were lower in patients with asthma than those of the control subjects ($P<0.001$ and $P<0.01$, respectively). Arginase activities were significantly lower and NO levels were higher in the patients than those of the controls ($P<0.001$ for both). The negative correlation between arginase and NO levels in the patients was significant ($r=-0.47$; $P<0.01$). There was also a positive correlation between malondialdehyde and NO levels in the patients ($r=0.51$; $P<0.01$).

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Conclusions: The results suggest that the L-arginine–NO pathway is involved in the pathophysiology of asthma; the arginase activities decrease which causes an increase in the L-arginine levels thereby up-regulation of NO production may contribute to the increase of oxidative stress in asthma.

Introduction

It has been proposed that reactive oxygen and nitrogen species, both formed in infected and inflamed tissues, play roles in oxidative stress which cause damage on biomolecules such as lipids, proteins and nucleic acids, each by a different mechanism. Nitric oxide (NO), a potentially toxic gas with free radical properties, is generated from L-arginine by constitutive or inducible NO synthase (NOS). It is important to note that NO is a modulator of cellular function and can act either as a cellular messenger or, when produced in excess quantities, as pro-oxidant, inducing oxidative stress.[1–3] Under normal conditions, NO interacts with superoxide anion to protect lungs against superoxide, but when NO and superoxide are both produced in very high quantities they react rapidly to form the very powerful oxidant peroxynitrite, which may contribute to lung injury.[2–5]

The relation between exhaled NO and asthma status was recently defined in several studies of asthma.[1–8] The NO was found to be elevated in asthmatic patients with evidence of atopy, whereas non-atopic asthmatic patients, atopic non-asthmatic patients, and non-atopic non-asthmatic patients did not have raised levels of exhaled NO. These data imply a relation between asthma severity, NO production, and oxidative stress.[7] However, as cited by Baraldi and Zanconato, patients with asthma are clinically diverse, have variable levels of exhaled NO, and it is difficult to distinguish asthmatic patients using exhaled NO levels.[8] Moreover, the source of NO in airways is not only endogenous but also exogenous as a consequence of air pollution and smoking.[6,9] Therefore, a further step is required to evaluate NO as an indicator for the disease.

Since NO is synthesized from L-arginine by NOS which competes with arginase for the same substrate, we should theoretically expect a reciprocal relation between them. Changes in L-arginine metabolism during inflammation are not limited to effects of NOS but can also involve arginases whose two types are well known; arginase I is termed as liver-type arginase and located in the cytosol, and arginase II is termed as non-hepatic type and located in the mitochondrial matrix, which can modulate NO synthesis and produce ornithine for the generation of polyamines and proline. The latter are important molecules involved in tissue damage and repair during inflammation. The links with the L-arginine–NO pathway and collagen generation make L-arginine metabolism a rising star in the asthmatic process. Arginase I and II were recently shown to contribute to the development of mouse lung fibrosis as cited by Vercelli.[10] Thus, the L-arginine–NO pathway should be elaborated to better understanding the correlation of NO, asthma and oxidative stress, which is still unresolved, and we will try to put a light on it in this work.

Material and methods

The study was conducted on 30 patients with asthma who applied to the outpatients department of the Chest Diseases Clinic, Harran University-Research Hospital and evaluated according to the International GINA Asthma Consensus Guidelines.[11] and on 30 healthy volunteers. The ages and sexes and the rate of smoking of the patients and controls were similar. None of them had allergic rhinitis, eczema or hay fever. All the patients had mild asthma without having an acute exacerbation during the last 3 months. They had no other infectious condition and/or diagnosed disease. All patients were regularly taking inhaled corticosteroids at low doses and short-acting β_2 agonist when they needed. None of them used teophylline, antihistaminik, oral corticosteroid and leukotriene receptor antagonist. All of the patients and controls were put on a standard diet and given up the treatment for 3 days before laboratory analyses. After 3 days with a standard diet, all subjects were taken to a fasting duration between 16.00 p.m. and 08.00 a.m. for about 16 h. All patients and control were examined physically and analysed for forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1), FEV_1/FVC, peak expiratory flow (PEF), early reversibility test, skin prick test and biochemical analyses. None of the patients had allergy profile. After their physical and laboratory examination, all patients were taken to the treatment according to the Asthma Consensus guidelines.[11]

Fasting blood samples were drawn into EDTA-containing tubes and centrifuged at 1500*g* for 10 min, to separate the plasma. Packed erythrocytes were washed with normal saline three times. Preparation of washed erythrocyte and biochemical analyses were performed immediately after blood collection.

NO reacts with molecular oxygen and accumulates in the plasma as nitrite (NO_2^-) and nitrate (NO_3^-) ions. Thus, the stable oxidation end products of NO, NO_2^- and NO_3^- can be readily measured in biological fluids and used in vitro and in vivo as indicators of NO production. Plasma nitrite levels were measured with Griess reaction.[12] Briefly, samples were initially deproteinized with Somogyi reagent.[13] Total nitrite (nitrite+nitrate) was measured after the conversion of nitrate to nitrite by copporized cadmium granules by a spectrophotometer at 545 nm (Ultraspect Plus, Pharmacia LKB Biochrom Ltd, UK). Results were expressed as micromoles per litre plasma.

Plasma arginase activity was measured according to the method of Geyer and Dabich[14] with some modification for plasma. Briefly, plasma was diluted 10 times with a solution of 5 mmol/l Mn^{2+} and incubated for 8 min at 55 °C (preincubation). Then 0.2 ml preincubated plasma, 0.4 ml 25 mmol/l L-arginine, and 0.4 ml 40 mmol/l carbonate buffer (pH: 9.7) were incubated for 60 min at 37 °C. After incubation, the reaction was stopped and the sample was deproteinized by adding 0.5 ml of 1 N $HClO_4$. Urea level was measured spectrophotometrically through the method of thiosemikarbazid-diasetilmonoksim-urea in the supernatants. The specific enzyme activity was expressed as U/g protein.

The erythrocyte MDA was estimated by the method of Jain et al.[15] based on thiobarbituric acid (TBA) reactivity. MDA reacts with TBA to form a coloured complex that has a maximum absorbance at 532 nm. The MDA concentration in erythrocytes was expressed as U/g Hb.

The erythrocyte reduced glutathione (GSH) content was determined by the method of Beutler et al.[16] Virtually all of the non-protein sulphydryl groups of red blood cells are in the form of GSH. 5,5′-Dithiobis(2-nitrobenzoic acid) is a disulphide chromogen that is readily reduced by sulphydryl compounds to an intensely yellow compound. The absorbance of the reduced chromogen is measured at 412 nm and is directly proportional to the GSH concentration.

Data were analysed using SPSS® 10.0 for Windows program. Parametric statistical methods were used to analyse the data. The student's *t*-tests were used for pair-wise comparisons. Bivariate comparisons were done using Pearson's rank correlation coefficients (*r*) and values were corrected for ties. Two-tailed significance values were used. A *P* value of 0.05 or less was considered to be significant.

Results

The social and demographic data (age, sex, etc.) of the patients and their controls showed homogeneity, and there were no significant differences between the groups ($P > 0.05$). The patients demonstrated 79.44 ± 18.43 FVC and 73.85 ± 6.89 FEV_1. The general characteristics of the patients and controls are shown in Table 1.

The erythrocyte MDA concentrations were increased in asthmatic patients compared with the controls ($P < 0.001$). GSH levels were decreased in erythrocytes of the asthmatic patients compared with the controls ($P < 0.01$).

Plasma arginase activities of patients with asthma were significantly lower ($P < 0.001$) whereas

Table 1 Characteristics of the patients with asthma and the healthy controls.

Parameters	Asthmatic patients ($n = 30$)	Healthy controls ($n = 30$)
Sex, M/F	13/17	12/18
Smokers, yes/no	0/30	0/30
Age (years)	35.8 ± 11.72	34.9 ± 9.79
FEV_1 (%)	73.85 ± 6.89	106.1 ± 19.26
FVC (%)	79.44 ± 18.43	101.5 ± 12.12
FEV_1/FVC	59.22 ± 16.41	106.8 ± 8.46
PEF (l/s)	56.70 ± 17.72	85 ± 15.78
Short-acting beta-2 agonists use	0/30	0/30
Inhaled corticosteroids use	30/30	0/30

The values represent the mean $\pm$ SD.

Table 2 Plasma arginase activities, nitric oxide levels and erythrocyte MDA and GSH levels of the asthmatic patients and the healthy controls.

Parameters	Control groups ($n = 30$)	Asthma ($n = 30$)	P^* value
Arginase (U/g protein)	10.12 ± 5.4	2.75 ± 3.2	<0.001
Total nitrite (μmol/L)	12.45 ± 2.0	16.46 ± 2.1	<0.001
MDA (nmol/g Hb)	1.36 ± 0.33	1.80 ± 0.37	<0.001
GSH (μmol/g Hb)	20.65 ± 4.86	15.92 ± 6.63	<0.01

The values represent mean ± SD.
*Significance was defined as $P < 0.05$.

Table 3 Correlation of arginase–NO and MDA–NO in patients with asthma.

Parameters	r	P^* value
Arginase–NO	−0.47	<0.01
MDA–NO	0.51	<0.01

*Significance was defined as $P < 0.05$.

plasma total nitrite, a product of L-arginine–NO pathway, levels were significantly higher ($P < 0.001$) than those of controls. The results were summarized in Table 2. A significant negative correlation ($r = -0.47$; $P < 0.01$) was detected between plasma arginase activities and plasma NO levels indicating their reciprocal regulation in the L-arginine–NO pathway and positive correlation ($r = 0.51$; $P < 0.01$) was detected between plasma MDA and NO levels (Table 3).

Discussion

It is well known that many cell types utilize L-arginine to generate NO, which plays important roles in many diverse processes including vasodilatation, bronchodilatation, immune responses, neurotransmission, and adhesion of platelets and leukocytes.[17,18] The discovery of the novel L-arginine-dependent NO pathway has stimulated renewed interest in the biochemistry and physiology of L-arginine metabolism in which NOS, whose product NO, and arginase, whose products urea and ornithine, compete for the same subtract. Due to the relationship between NO and L-arginine metabolism, NO level alone would not be an indicator for the disorders. In particular, changes in the activities of arginase, which is thought to be a marker for asthma,[10] and the products of NO namely NO_2^- and NO_3^- might play major roles in determining metabolic fates of the L-arginine–NO pathway in health and diseases, and the current study was designed with the aim to investigate the possible changes of this pathway in asthma.

Asthma is a consequence of an underlying chronic inflammatory disorder of the airways involving many cells, in particular mast cells, eosinophils, neutrophils, and T lymphocytes, and the airway epithelium, with the participation of a number of mediators.[4–7] The mediators released from activated mast cells and eosinophils such as ROS generate oxidative stress, known as oxidants, that contributes to tissue injury and asthma symptomatology. Other airway oxidants in asthma include NO, a free radical released by activated leukocytes and the airway epithelium as a consequence of chronic inflammation, peroxynitrite and hypochlorous acid.[2,3,8,9] Important exogenous oxidants include nitrogen dioxide and ozone as a consequence of air pollution. Cigarette smoke is a particularly rich source of NO and nitrogen dioxide.[6,8] Gutierrez et al.[6] reported that when cultured pulmonary epithelial cells and lung tissue in vivo are subjected to inflammatory mediators or acute oxidative stress, NO can play a protective role by inhibiting O_2^--dependent toxicity. In their in vitro rodent model of pulmonary oxidant injury, they demonstrated that NO decreases epithelial permeability caused by ROS and thus helps defend against tissue injury subsequent to oxidative stress.[6] In the present study, we found that NO levels were higher in asthmatic patients than in healthy controls. However, it is still controversial whether this increase is beneficial or damaging.[7–9,19] As a bronchodilator, inhalation of high concentrations of NO may be beneficial in asthmatic patients by counteracting the bronchoconstriction produced by inflammatory mediators such as leukotriene D4.[7,8,20] On the other hand, there are several controversies in the literature about the damaging effects of increased NO levels in asthma indicating that the NO may amplify asthmatic inflammation by altering the balance between T helper type 1 and T helper type 2 cells and increase bronchial blood flow thereby

plasma exudation in the airways.[19,21] In this study, we detected an imbalance between oxidative and antioxidative system by measuring LPO that was significantly high in the asthmatic patients, and GSH that reduces free radical and was very low in the patients compared to the controls. We found a significant positive correlation between plasma MDA and NO levels which provided an evidence for the contribution of NO to the increased oxidative stress in asthma.[21] The susceptibility of the body to oxidative injury depends largely on its ability to up-regulate protective free radical and ROS scavenging systems such as GSH. However, in asthma, the body cannot increase the antioxidative capacity and/or it increases but not enough to cope with the oxidative stress caused by asthmatic inflammation.

Owing to the competition between arginase and NOS, it is supposed that these two enzymes of the L-arginine–NO pathway may control each other's levels.[18,22] Therefore, we thought that the high NO levels in this work may result from a decrease in the arginase activity because of their reciprocal regulation in the pathway, and we measured plasma arginase activities of the asthmatic patients and controls. We found low arginase activity in our study that supports this relationship between arginase activity and NO levels. The possible regulation of NO production by arginase in various tissues is of great interest. Several diseases associated with the impaired production or overproduction of NO, are also shown to be related to alterations of the L-arginine metabolism.[22–25] In the light of these findings, it would be useful clinically to prevent the overproduction of NO and hence oxidative stress by using NOS inhibitors and/or depressing L-arginine recycling or providing forced induction of arginase if the L-arginine–NO pathway is involved in the pathogenesis of disease whereby NO levels increased and the activities of arginase decreased.[26,27]

As conclusion, it is possible to underline that the increased NO levels and decreased arginase activities indicate the involvement of the L-arginine–NO pathway in asthma. Overproduction of NO contributes to oxidative stress present in asthma that causes worse progression of the disease giving oxidative damage. These data may provide a new approach to the treatment of the disease, because the metabolic enzymes of the L-arginine–NO pathway appear to be pertinent therapeutic targets to control NO production in various diseases associated with disordered NO production like asthma. It may also be useful to add some antioxidants to the treatment such as antioxidant vitamins like vitamins A, C, and E.

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