Serum Prolidase Activity and Oxidative Status in Patients With Bronchial Asthma

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Asthma is a disease where there is an accumulation of collagen in the reticular basal membrane of the airway leading to chronic inflammation. The enzyme prolidase plays an important role in the breakdown of collagen and the breakdown of intracellular protein especially in the final stage when peptides and dipeptides contain a high level of proline. To evaluate the relationship between prolidase activity and oxidative status in asthma patients. Comparison was made between 42 patients diagnosed with bronchial asthma and 32 healthy children of similar age and gender. Serum prolidase activity was measured spectrophotometrically. Oxidative status was determined using total antioxidant capacity (TAC) and total oxidant status (TOS) measurement. The prolidase activity of the asthma patient group was statistically significant compared with the control group

($P \leq 0.001$). TAC and TOS levels in the asthma patient group were higher than the control group ($P \leq 0.001$, $P \leq 0.002$, respectively). No correlation was found between the prolidase and oxidative levels of the two groups. A positive correlation was determined between the prolidase activity and TAC in the asthma patient group ($P \leq 0.001$, $r = 0.501$). The prolidase enzyme activity, which plays a role in the collagen turnover, was low in the asthma patients; therefore, their collagen metabolism had undergone a change and this indicates that there may be an effect on the accumulation of collagen in the reticular basal membrane. Moreover, the high level of TOS indicates that these patients were exposed to severe oxidative stress with an increased TAC response. J. Clin. Lab. Anal. 23:132–138, 2009. © 2009 Wiley-Liss, Inc.

Key words: asthma; prolidase activity; airway remodeling; oxidative status

INTRODUCTION

The importance of tissue remodeling as an early and consistent component of childhood asthma has been emphasized in a recent biopsy study that describes collagen deposition in the lamina reticularis and underlying fibroblast proliferation, rather than eosinophil infiltration, as diagnostic features of this disease (1). Although remodeling has been considered to be caused by long-standing inflammation, airway biopsy studies in young children have shown tissue restructuring up to 4 years before the onset of symptoms (2), indicating that this process begins early in the development of asthma and might occur in parallel with inflammation or even be required for the establishment of persistent inflammation.

Thickening of the lamina reticularis in bronchial biopsy specimens from young children is present several years before asthma becomes clinically manifest (2). Accumulation of collagen III, I, and IV and fibronectin

at the basal membrane level occurs in patients with asthma (3). In the submucosa of asthmatic patients, electron microscopy studies show that collagen is not completely normal. In some patients hyperplasia of collagen fibers can be observed (4). Although it is well recognized that airway inflammation is a prominent feature of asthma, the relationship between individual components of airway inflammation and the progression of inflammation to remodeling of the airways in asthma is not well understood.

Remodeling of the asthmatic airway is associated with accumulation and activation of subepithelial fibroblasts

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(5,6). However, the origin of the increased number of these cells is unknown. One hypothesis is that mesenchymal stem cells are precursors for tissue resident fibroblasts and certainly this has been shown to occur in pulmonary fibrosis (7). Fibrosis may occur in some lung diseases; therefore, prolidase activity may be linked to some lung diseases (8).

Prolidase is a manganese-requiring homodimeric iminodipeptidase that releases carboxy-terminal proline or hydroxyproline from oligopeptides and was first identified in 1937 (9). It has been shown that an increase in prolidase enzyme activity is correlated with increased rates of collagen turnover (10). Prolidase, an iminodipeptide cleaving cytosolic enzyme found in human and animal tissue, plays an important role in the collagen catabolism process by recycling dipeptide-bound proline for collagen synthesis, and is considered as a rate limiting factor to collagen turnover, which takes place at the final step of collagen degradation (10). Studies have shown that prolidase activity is altered in fibrotic diseases and chronic wounds (11). Prolidase deficiency is an autosomal recessive disorder that is associated with chronic cutaneous ulcers, mental retardation, unusual facial appearance, skeletal deformities, joint dislocations, hematological anomalies, splenomegaly, and chronic infections. The most typical finding is chronic, recurrent leg ulcers appearing in early childhood. Its deficiency would hinder recycling of proline for collagen biosynthesis and leads to impairment in connective tissue of the skin, capillaries, and lymphatic vessels (12).

Prolidase is a ubiquitous enzyme and its activity has been documented in erythrocytes, leukocytes, plasma, dermal fibroblasts, the kidney, brain, heart, thymus, and uterus (13). The enzyme plays an important role in the recycling of proline for collagen synthesis and cell growth (10). Prolidase enzyme activity has been investigated in various disorders (14). In subjects with liver disease, serum prolidase activity has been shown to increase, especially in the early stage of fibrosis, and it has been suggested that plasma prolidase activity might be useful in evaluating fibrotic processes in chronic liver disease in humans (14).

To the best of our knowledge, there are no data concerning the serum prolidase activity in young children with bronchial asthma. Asthma is a chronic inflammatory pulmonary disease related to increased oxidative stress (15). The association between chronic inflammation and oxidative stress is well documented. Elevated levels of reactive oxygen species (ROS) such as hydroxyl radicals, superoxides, and peroxides in inflammatory conditions have been reported previously (16).

In a recent study of 11 moderately severe pediatric asthmatic patients, aged 8–16 years, evaluated in the emergency department, receiving either nebulized budesonide

or albuterol, improvement was based on a pulmonary index score peak flow and spirometry. Fraction of exhaled nitric oxide (FENO) as a measure of oxidant stress was measured in three patients. In all nonresponsive patients admitted, specific IgE (sIgE) levels were determined. Increased FENO levels in two had a pulmonary index of 8 and polysensitization developed in 89% to inhalants and various foods (17).

The cells infiltrating the bronchial mucosa in patients with asthma produce a variety of mediators including ROS (18). Increased production of ROS leading to an imbalance between the oxidative forces and the antioxidant defense systems favoring an oxidative injury has been implicated in the pathogenesis of asthma (19,20). However, when the production of damaging ROS exceeds the capacity of the body's antioxidant defenses to detoxify them, a condition known as oxidative stress occurs (21). Numerous studies have implicated nitric oxide to be involved in lung tissue damage and injury from O₂ free radicals can occur in bronchopulmonary dysplasia, which can lead to asthma (22). The formation of peroxynitrate can act as an antioxidant. Nitric oxide levels in neonates with bronchopulmonary dysplasia and respiratory distress syndrome have also been correlated with disease severity (23).

ROS may exert a number of toxic effects that have been demonstrated in many different biological systems. Oxidative stress leads to changes such as modification of receptor activity and signaling and release of endogenous mediators of inflammation. An imbalance in oxidant–antioxidant activity is involved in much free radical-mediated pathology, e.g., ischemia–reperfusion and asthma (24).

The lungs are directly exposed to higher oxygen concentrations than most other tissues. Increased oxidative stress is a significant part of the pathogenesis of obstructive lung diseases such as asthma and chronic obstructive pulmonary disease, parenchymal lung diseases (e.g., idiopathic pulmonary fibrosis and lung granulomatous diseases), and lung malignancies. Lung tissue is protected against these oxidants by a variety of antioxidants (25).

Thus, in this study, we aimed to determine serum prolidase activity and oxidative stress markers including total oxidant status (TOS) and total antioxidant capacity (TAC) and to establish if there is any association between serum prolidase activity and these oxidative stress parameters.

METHODS

Study Groups

Parents of all patients signed informed consent forms and the Ethics Committee of the Hospital approved the

study. A total of 42 subjects between 6 and 15 years of age who had been attending the Pediatric Allergy Unit of the Medical Faculty of Harran University for at least 1 year were included in this study. The clinical severity of the asthma was determined using the criteria (appropriate clinical and respiratory function tests) defined in the Global Initiative for Asthma guidelines (26). Family history of atopy was considered as positive if atopy was present in parents and/or siblings (bronchial asthma, allergic rhinitis, and atopic dermatitis). In all patients, allergen sensitivity was performed with sIgE and skin prick test (SPT) to aeroallergens. Patients with clinical signs of asthma who had a positive sIgE in addition to sensitivity against at least one aeroallergen on the SPT were included in the atopic asthma groups. Immunocompromised patients, patients with a history of chronic inflammation/rheumatological disorder, and patients with autoimmune diseases were excluded. Asthma patients were not receiving any controller medication and had not had any symptoms of lower or upper respiratory tract infection or asthma exacerbation within the previous 4 weeks.

The control group consisted of 32 age-matched healthy children (6–16 years). Healthy children were chosen from those referred to a pediatric outpatient clinic in Harran University Hospital, where all children periodically undergo check-ups for their growth and development. Control patients were evaluated with regard to chronic and/or severe infections, rheumatological and autoimmune disorders, and familial and personal history of atopy, and also by laboratory tests. Children were included in the control group if they had no personal and familial history of atopy and no signs of atopic disorder, and if they were negative for sIgE and SPT.

As it is known to have an effect on oxidative status, the patients came from nonsmoking households, and the control group were also selected from nonsmoking households.

All the patients in the study were weighed and measured and then a calculation was made according to the Body Mass Index Standard Deviation Score (BMI SDS) (27).

Study Measurements

sIgE levels

Serum allergen sIgE measurements were performed using CAP FEIA method (Pharmacia, Uppsala, Sweden) and evaluated with regard to the standard deviations (SD) listed in the user's manual according to age-sIgE levels reported by the World Health Organization. The sensitivity of the kits used for measurements was standardized to 0.35–100 kU/L for serum sIgE, which is used to detect the sensitization in the serum against

inhaled allergens (house dust mite, yeasts, animal dander, grass pollen, trees, and wild grass); the result was considered as positive if the measured value was greater than 0.35 kU/L.

Skin prick test

Prick test for aeroallergen sensitivity was done using lancets (Stallerpoint, Paris, France) providing a standard puncture of 1 mm. Commercial allergen solutions manufactured by Allergopharma (Joachim Ganzer KG, Reinbeck, Germany) were used for the skin test. A total of 44 different allergens consisting of house dust mite, grass, wild grass, tree pollens, fungi, animal dander, and insects were tested. Tests were evaluated 20 min after allergen application using European Academy of Allergy and Clinical Immunology criteria (28). The diameter of the induration on the volar aspect of the forearm was scored between 1 and 4 if it was at least half of the diameter of the induration caused by a positive control (1 mg/L histamine). A score equal to or greater than 3 was considered as significant.

Blood Samples

Blood samples were obtained following overnight fasting. Blood samples were collected into empty tubes and immediately stored on ice at 4°C. The serum was then separated from the cells by centrifugation at 3,000 rpm for 10 min. Serum samples for measurement of TOS and TAC levels and prolidase activity were stored at –80°C until they were used.

Measurement of the TAC

TAC of serum was determined using a novel automated measurement method, developed by Erel (29). In this method, hydroxyl radical, which is the most potent biological radical, is produced. In the assay, ferrous ion solution, which is present in Reagent 1, is mixed with hydrogen peroxide, which is present in Reagent 2. The sequentially produced radicals such as brown-colored dianisidiny radical cation, produced by the hydroxyl radical, are also potent radicals. Using this method, the antioxidative effect of the sample against the potent-free radical reactions, which is initiated by the produced hydroxyl radical, is measured. The assay has excellent precision values of lower than 3%. The results are expressed as mmol Trolox Eq./L.

Measurement of TOS

TOS of serum was determined using a novel automated measurement method, developed by Erel (30). Oxidants present in the sample oxidize the ferrous ion-*o*-dianisidine complex to ferric ion. The oxidation

reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter ($\mu\text{mol H}_2\text{O}_2 \text{ Eq./L}$).

Measurement of Total Peroxide Concentration of Plasma (LOOHs)

Serum LOOHs levels were measured with the ferrous ion oxidation–xylenol orange (FOX-2) assay. The principle of the assay depends on the oxidation of ferrous ion to ferric ion via various oxidants and the produced ferric ion is measured with xylenol orange. LOOHs are reduced by triphenyl phosphine (TPP), which is a specific reductant for lipids. The difference between with and without TPP pretreatment gives LOOHs levels (31).

Determination of Prolidase Activity

Serum was diluted 40-fold with 2.5 mmol/L Mn^{2+} , 40 mmol/L trizma HCl buffer (pH 8.0) and preincubated at 37°C for 2 hr. The reaction mixture containing 30 mmol/L gly-pro, 40 mmol/L trizma HCl buffer (pH 8.0), and 100 μL of preincubation serum in 1 mL was incubated at 37°C for 30 min. Adding 0.5 mL of 20% trichloroacetic acid solution then stopped the incubation

reaction. The supernatant was used for measurement of proline by the method proposed by Myara et al. (32,33), which is a modification of Chinard's method (34). Intraassay coefficient of variation (CV) of the assay was 3.8%.

Statistical Analyses

Data were analyzed using the SPSS® for Windows computing program (Version 11.5; SPSS, Chicago, IL). The values are expressed as mean $\pm$ SD for the asthma patients and the healthy population, separately. The differences between the different groups of controls and patients were analyzed by Student's *t*-test or Mann–Whitney *U*-test. Correlation analyses were performed using Pearson's correlation test and Spearman's rank correlation test. Statistical significance was defined for *P* values of less than 0.05.

RESULTS

The demographic and clinical data of the study population are given in Table 1. There were no statistically significant differences between the two groups with regard to age, gender, and BMI SDS ($P > 0.05$) (Table 1). The prolidase level of the asthma patient group was significantly low compared with the control group ($1,150 \pm 415$ and $1,408 \pm 162 \text{ U/L}$, $P \leq 0.001$) (Table 2). The levels in the asthma patient group were TAC ($5.5 \pm 2.5 \mu\text{mol Trolox Eq./L}$), TOS ($9.0 \pm 3.5 \mu\text{mol H}_2\text{O}_2 \text{ Eq./L}$), and LOOHs ($9.9 \pm 3.4 \mu\text{mol H}_2\text{O}_2 \text{ Eq./L}$). The levels in the control

TABLE 1. Demographic Data of Bronchial Asthma Patients and Healthy Subjects

	Patients (<i>n</i> = 42)	Controls (<i>n</i> = 32)	OR (95% CI)	<i>P</i> *
Age (yr)	9.0 $\pm$ 2.8	9.4 $\pm$ 3.8		0.60
Gender (M/F)	20/22	10/22	0.50 (0.17–1.45)	0.15
BMI SDS	0.77 $\pm$ 0.25	0.61 $\pm$ 0.20		0.14
Atopy in family (%)	17 (40.4)	–		

The values represent the mean $\pm$ SD. BMI SDS, Body Mass Index Standard Deviation Score.

*Significance was defined as $P < 0.05$.

TABLE 2. Prolidase Activity and Oxidative/Antioxidative Parameters in Asthma Patients and Control Groups

	Asthma patients (<i>n</i> = 42)	Control groups (<i>n</i> = 32)	<i>P</i> *
Prolidase (U/L)	1,150 $\pm$ 415	1,408 $\pm$ 162	≤ 0.001
TOS ($\mu\text{mol H}_2\text{O}_2 \text{ Eq./L}$)	13.4 $\pm$ 7.0	9.0 $\pm$ 3.5	$\leq 0.002^{**}$
LOOHs ($\mu\text{mol H}_2\text{O}_2 \text{ Eq./L}$)	9.9 $\pm$ 3.4	4.4 $\pm$ 1.5	≤ 0.001
TAC ($\mu\text{mol Trolox Eq./L}$)	5.5 $\pm$ 2.5	1.0 $\pm$ 0.6	$\leq 0.001^{**}$

TOS, total oxidative status; TAC, total antioxidant capacity; LOOHs, total peroxide concentration of plasma. The values represent the mean $\pm$ SD.

*Significance was defined as $P < 0.05$.

**Difference between two groups was compared using Mann–Whitney *U*-test.

group were TAC ($1.0 \pm 0.6 \mu\text{mol Trolox Eq./L}$), TOS ($13.4 \pm 7.0 \mu\text{mol H}_2\text{O}_2 \text{ Eq./L}$), and LOOHs ($4.4 \pm 1.5 \mu\text{mol H}_2\text{O}_2/\text{L}$). There was no correlation between the TOS and LOOH levels and the prolidase activity between the patient and control groups (Table 2). TAC and TOS levels in the asthma patient group were higher than the control group ($P \leq 0.001$, $P \leq 0.002$, respectively). No correlation was found between the prolidase and oxidative levels of the two groups. SPT of the asthma patient group were 45% grass pollen, 30% polysensitization, 15% mites, and 10% alternaria. No difference was determined in the prolidase activity and oxidative level between the mite-positive group and patients positive for other allergens ($P > 0.05$). In the asthma patient group a significant correlation was determined between prolidase ($1,150 \pm 415 \text{ U/L}$) and TAC ($5.5 \pm 2.5 \mu\text{mol Trolox Eq./L}$) ($P \leq 0.001$, $r = 0.501$). There was no significant correlation between sIgE, prolidase, TAC, TOS, and LOOH ($P > 0.05$). No difference was determined when the results were evaluated separately according to gender.

DISCUSSION

Bronchial asthma has become an increasing concern for public health, especially in industrialized countries. The prevalence of asthma has increased significantly, particularly among children, and it is now the most frequent chronic medical condition in the pediatric age group (35).

Despite intensive research in recent years, the pathogenic mechanisms of asthma are still poorly understood. It is well known that pathologic changes include both airway inflammation and remodeling, which may lead to thickening of the airway wall, therefore playing an important role in the pathophysiology of the disease (36). Prolidase enzyme activity in various tissues has been demonstrated (37), but to the best of our knowledge there is no report concerning prolidase activity in pediatric bronchial asthmatic patients. Prolidase enzyme activity plays an important role in its degradation and its activity might be correlated with the rate of collagen degradation (38).

In our study the serum prolidase level of the patients with bronchial asthma was found to be statistically significantly low ($P = 0.001$) in comparison with the control group. In a previous study prolidase activity was shown as an indicator of collagen metabolism and was found to be increased in patients with asthma who were using theophylline (39).

Our study results support the study where prolidase activity increased following theophylline treatment. In another study, serum prolidase activity in patients with moderate persistent bronchial asthma increased compared with the controls and a decrease was seen after treatment (40). Our study results do not conform with

this study. In an early study theophylline was demonstrated to inhibit the production of nitric oxide by peripheral blood in mononuclear cells from patients with asthma (41).

To the best of our knowledge there are only two published studies concerning adult asthma patients (39,40) and these both emphasize the connection between the treatment and prolidase. Our study evaluated prolidase activity independently of the treatment in a group of asthmatic children. In contrast to the other two studies, steroids or other asthma treatment medication had not been in regular use up to the time of taking the blood samples. Our study group is of a different age group to other known studies. In our study, when the prolidase activity was evaluated it was found to be statistically significantly low in the group diagnosed with asthma in comparison with the healthy control group.

The balance between oxidant-antioxidant system is impaired in patients with asthma (22). Many observations suggest that levels of oxidative stress are increased in children and in adults with asthma, not only in the lungs but also in the circulation (22). Excessive production of oxidants occurs spontaneously or after stimulation in blood leukocytes of stable asthmatic patients compared with normal subjects (42). Several published studies have put forward the idea that when the oxidant system increases, there is a decrease in the antioxidant system (43). In our study group it was determined that TOS and TAC increased together. In a study similar to ours, Nadeem et al. (44) recorded that when there was an increase in the oxidant system there was also an increase in the antioxidant system.

Owing to the socio-economic conditions in the region, our cases were diagnosed with asthma at a late stage and were not having regular check-ups. In this situation they are vulnerable to long-term oxidative stress and so the development of the antioxidant system could be in response to that.

In a study measuring sIgE levels in 66 moderate to severe adult asthmatics evaluated in the emergency room, the frequency of class III or higher sIgE serum levels was as follows: cat epithelium, 56.1%; *D. farinae*, 50.0%; *Alternaria tenuis*, 10.7%; cockroach, 9.7%; *Aspergillus fumigatus*, 6%. Thus, patients with acute severe asthma have a relatively high frequency of polysensitization (45). In our study the levels were 45% grass pollen, 15% mite, 30% polysensitization, and 10% alternaria. The majority of patients were sensitive to grass.

It is known that there is reduced antioxidant activity in dust mite-positive asthmatic patients compared with those who are dust mite negative (46). In our study there was no difference between the dust mite-positive patients and the other group. This could be owing to the small number of the study group.

In our study, it is thought that in the asthmatic patients, because of the increased level of oxidative stress with no correlation to the prolidase activity, prolidase was not affected by the oxidative status or that the oxidative stress together with the strengthened antioxidant capacity prevented any effect.

In the asthmatic patient group, a positive correlation was determined between prolidase ($1,150 \pm 415$ U/L) and TAC (5.5 ± 2.5 μ mol Trolox Eq./L) ($P \leq 0.001$, $r = 0.501$); as TAC increased so did prolidase activity. There was no correlation between sIgE, prolidase, TAC, TOS, and LOOH in the patient group.

In conclusion this study was carried out with the aim of evaluating prolidase activity as the most important enzyme playing a role in accumulated collagen turnover in remodeling. The role of prolidase in remodeling will be better indicated by studies evaluating prolidase activity in asthma remodeling together with parameters.

This study is important as it is a preliminary work, which shows that prolidase activity in bronchial asthma and pathogenesis is significant as a collagen turnover indicator, and can be used in both to evaluate pathogenesis and prognosis.

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