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Original scientific paper

Malonaldehyde and Erythrocyte Antioxidant Status in Children with Controlled Asthma

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A B S T R A C T

In the pathogenesis of asthma, oxidative stress appears to play an important role and existence of an oxidant/antioxidant imbalance is evident. In this study the key markers of oxidative stress and lipid peroxidation in the pathogenesis of asthma in childhood in comparison to healthy subjects were investigated. Plasma marker of the lipid peroxidation: malondialdehyde (MDA), the erythrocytes antioxidative enzymes: glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), glutathione reductase (GR) and cysteine-containing tripeptide glutathione (GSH) were evaluated by spectrophotometric methods using blood samples collected from 37 healthy children and 44 asthmatic patients. The GSH-Px activity was significantly lower in asthmatic children (3.99±1.0 IU/g Hb) than in healthy controls (4.61±1.3 IU/g Hb; $p<0.034$). Significant difference in activity of the SOD, GR, and concentration of cysteine-containing tripeptide GSH was not confirmed ($p>0.05$). Lower GSH-Px activity in children with controlled asthma showed deficient erythrocyte antioxidant defence and evidence of association between oxidative stress and asthma in childhood. Preserved activity of GR and SOD, together with concentration of GSH and MDA, still seems to be crucial in controlling antioxidant/oxidant balance of the disease.

Key words: *asthma, children, GPx, GR, GSH, MDA, oxidative stress, SOD*

Introduction

Asthma is a chronic inflammatory airway disease which is the most common chronic disease in the childhood in developed countries, with an increasing prevalence in the last few decades[1].

In the pathogenesis of asthma, oxidative stress appears to play an important role. There is a growing evidence of an oxidant/antioxidant imbalance. In asthma, bronchial obstruction is associated with an increased spontaneous and stimulus – induced production of reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide, and hydroxyl radical[2].

ROS are associated with many pathophysiologic changes that are relevant in asthma, such as increased lipid peroxidation, increased airway reactivity and secretions, increased production of chemoatractants, and increased vascular permeability. Also, oxygen radicals that are not neutralized by the antioxidant defence react with polyunsaturated fatty acid residues in phospholipids, resulting in the production of reactive aldehydes. The most abundant of these is malondialdehyde (MDA)[3,4].

The organism, especially lung and blood are endowed with numerous antioxidants, including glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), glutathione (GSH), glutathione reductase (GR), to counter the oxidant – mediated toxicity. On the other hand, changes in antioxidant defences have been reported, including de-

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creased GSH-Px in whole blood, and a deficiency of selenium[5]. GSH-Px also plays an important role in the detoxification of various hydroperoxides[6].

There is consistent evidence that oxidative stress is associated with asthma, but until now in research literature are very little data about oxidative stress and lipid peroxidation in asthma of childhood[7–9].

The house dust mite *Dermatophagoides pteronyssinus* is one of the most significant indoor sensiting agents of allergy[10].

House-mite allergy is a hypersensitivity reaction to proteins excreted by dust-mites. In susceptible individuals these proteins, have been shown to be associated with asthma and other allergic diseases[11].

The aim of this study was to investigate the antioxidant enzymatic activities and concentration of MDA and GSH in controlled asthma in childhood in comparison to healthy subjects, there is reason to believe that there is association between asthma and decreased levels of antioxidant enzymes.

Materials and Methods

Study groups

In this study analyzed antioxidant status and marker of lipid peroxidation, MDA, were performed in samples divided into two groups, patients of asthma and healthy controls. The control group consists of 37 healthy children included 19 girls and 18 boys with a $\overline{X}$±SD age of 10.5±4.7 years. The study population consisted of 44 children with asthma included 16 girls and 28 boys with a $\overline{X}$±SD age of 10.7±3.6 years. Studied groups of children were age-gender related.

The present study included children suffering from allergic asthma who were monosensitized to house dust mite (*Dermatophagoides pteronyssinus*) allergen. Inclusion criteria were: a) age 5–16 years, b) a clinical history of allergic persistent asthma according to the criteria recommended by the PRACTALL[12] and GINA 2007[13], c) positive skin pick test to *Dermatophagoides pteronyssinus,* d) increased serum total IgE[14], e) specific IgE to *Dermatophagoides pteronyssinus* ≥17.1 kIU/L (Pharmacia CAP system, Pharmacia Diagnostics AB, Uppsala, Sweden), f) baseline FEV1/FVC<predicted and FEV1 <70% of predicted (FEV1=forced expiratory volume in one second, FVC=and forced vital capacity.

All patients underwent specific immunotherapy to *Dermatophagoides pteronyssinus* (Croatian national standard, partially purified Der p extract, Imunološki zavod, Zagreb, Croatia)[15]. During the preceding three months patients were on their regular controller therapy (inhaled corticosteroids, ICS or ICS plus long-acting β_2-agonists). At the time of investigation patient group had conrolled asthma (based on daytime/nocturnal symptoms, FEV1≥80%, need for reliever and limitations of daily activities).

Control group included healthy children without clinical signs and symptoms of neither allergic disease nor acute or chronic disease.

Diagnostic work-up was performed according to standardized procedure, and in line with ethical principles and Declaration on Human Rights from Helsinki 1975 and Tokyo amendments 2004[16].

All study procedures were performed in accordance with a protocol previously approved by the Ethics Committee of Children Hospital Srebrnjak, Zagreb. All parents provided written informed consent for the study procedures.

Sample collection

Plasma peripheral blood samples were drown into EDTA-containing tubes after 12h fast, by venipuncture. After centrifugation, the plasma was removed and frozen at –80 °C. Erythrocytes were washed three times with 0.9% NaCl and lysed in ultra pure water. The supernatant obtained from lysed erythrocytes was stored at –80 °C until use.

Malondialdehyde (MDA) was measured in plasma but superoxide dismutase (SOD, EC 1.15.1.1), glutathione peroxidase (GSH-Px, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2) were determined on erythrocyte lysate, and glutathione (GSH) levels were determined on erythrocyte lysate after precipitation of proteins, using Ellman's reagent.

Laboratory assay techniques

The plasma MDA level was determined using the method based on the reaction with thiobarbituric acid (TBA) at 90–100 °C[17]. In the TBA test reaction, MDA or MDA-like substances and TBA react together producing of a pink pigment having an absorption maximum at 532 nm. The sample was mixed with cold 10% (w/v) trichloroacetic acid (TCA) to precipitate proteins. The precipitate was pelleted by centrifugation. The results were expressed as μmol/L of plasma according to a standard graphic, which was prepared with serial dilutions of standard 1,1,3,3-tetramethoxypropane. The TBA and TCA used in this assay procedure were obtained from Sigma Chemical Company.

Hemoglobin (Hb) concentrations were determined by the method of Drabkin.

Measurement of erythrocyte CuZn-SOD activity was performed using Ransod reagents (Randox Laboratories) and is based on the method developed by McCord and Fridovich[18]. Absorbance was monitored in a Beckman DU 7500 spectrophotometer[19].

GSH levels were determined on erythrocyte lysate after precipitation of proteins, using Ellman's reagent. Cayman's GSH assay kit utilizes a carefully optimized enzymatic recycling method, using glutathione reductase for the quantification of GSH. The sulfhydryl group of GSH reacts with DTNB (5.5-diothio-bis-2-nitrobenzoic acid) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH

and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which in turn is directly proportional to the concentration of GSH in the sample[19].

Measurement of erythrocyte glutathione peroxidase (GSH-Px) activity was performed using the Cayman Chemical Glutathione Peroxidase Assay Kit. The activity of GPx was measured indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample[20].

Measurement of erythrocyte glutathione reductase (GR) activity was performed using the Cayman Chemical Glutathione Reductase Assay Kit. The activity of GR was measured by the rate of NADPH oxidation. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm and is directly proportional to the GR activity in the sample[21].

Investigated erythrocyte antioxidant defence: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione (GSH) and glutathione reductase (GR) and their activity is expressed as IU/g Hb.

Results

Results of plasma MDA, as a measure of lipid peroxidation, and erythrocyte antioxydant activity of SOD, GR and GSH-Px, and concentration of GSH and MDA in healthy children and children with asthma were presented in Table 1.

Assessed plasma MDA level showed no statistically significant difference in group of children with asthma compared with control group ($p>0.05$). But, erythrocyte GSH-Px activity was significantly lower in the group of children with asthma than in control group (3.99±0.98 IU/g Hb *vs*. 4.61±1.35 IU/g Hb; $p=0.034$). Determined erythrocyte SOD activity and also activity of GR and concentration of GSH showed no statistically significant difference between studied groups of children ($p>0.05$).

Discussion

The lung is a major target organ for injury by exogenous oxidants such as environmental pollutants (house dust mite). Many environmental pollutants (*Dermatophagoides pteronyssinus*) exert their major effect by causing asthma and oxidative stress in cells and tissues that they contact. Reactive oxygen species (ROS) may damage proteins, lipids, and DNA directly[22,23]. Oxidants can cause airway inflammation and airway hyperresponsiveness, which are major characteristics of asthma. Patients with asthma have increased ROS production[24].

The hydroxyl radical is, by far the most damaging ROS and reacts with biomolecules primarily by hidrogen abstraction and addition reactions. Characteristic products, described as biomarkers of oxidative stress, are formed in these reactions. One of the most sensitive sites of free radical damage is the cell membrane, which is rich in readily oxidized polyunsaturated fatty acids. Peroxidative damage of cell membranes affects the integrity and function of the membrane, compromising cell's ability to maintain ion gradients and membrane phospholipids asymmetry. Lipid peroxides formed in this reaction degrade to form characteristic products, such as malondialdehyde (MDA)[25].

In the study of Jacobson et al. investigated plasma MDA level was higher in asthma subjects (1.30±0.6 μmol/L) than in controls (0.86±0.5 μmol/L), but patients in this study have acute severe asthma[26]. But, in the study of Hanta et al. result was quite opposite. MDA was lower in the asthmatic group than in the healthy subjects[27]. In this study the oxidant-antioxidant balance was investigated in mild asthmatic adult patients. Our results of MDA level in asthmatic children may be explained by controlled status of disease in time of sampling.

Study of Sackesen et al. showed decreased GSH-Px activity in children patients with mild asthma in children[8]. The children in this study were not receiving any controller medication and had not any symptoms of respiratory infection or asthma exacerbation. The same result was achieved in our study, GSH-Px was lower and statistically significant in children with controlled asthma than in controls ($p=0.034$).

Chronic oxidative stress in red blood cells (RBC) results in a significantly increase GSH concentration with

TABLE 1
PLASMA MDA AND ERYTHROCYTE ANTIOXIDANT STATUS IN CHILDREN WITH CONTROLLED ASTHMA AND HEALTHY SUBJECT

	Control group (healthy children, n=37) $\overline{X}$±SD	Patient group (children with asthma, n=44) $\overline{X}$±SD	p-value*
MDA, μmol/L	1.23±1.0	0.97±0.6	0.154
GSH, nmol/g Hb	202.9±18.4	204.6±14.1	0.939
GSH-Px, IU/g Hb	4.61±1.3	3.99±1.0	0.034
SOD, IU/g Hb	26.15±2.9	25.77±3.9	0.825
GR, IU/g Hb	0.44±0.3	0.36±0.2	0.212

* t-test

a concomitantly decreased GSSG level[28]. In our study, erythrocyte GSH level was slightly increased in the children group with asthma vs. control group and this result was not statistically significant ($p>0.05$) (Table 1). Other authors like, Ercan et al. showed that glutathione is significantly decreased in asthma subjects[7].

GSH-Px reduces hydrogen peroxide and lipid peroxide, using GSH as a co-substrate. The GSH is recycled by glutathione-reductase (GR) using NADPH from the pentose phosphate pathway. This enzyme is essential for the GSH redox cycle which maintains adequate levels of reduced cellular GSH. A high GSH/GSSG ratio is essential for protection against oxidative stress. Superoxide is one of the main in the cell and as such, SOD serves a key antioxidant role. SOD is the major superoxide dismutase of pulmonary fluids and interstitial spaces of the lungs[26–28].

Our preliminary results showed that only one (GSH-Px) of all studied parameters was harmed in studied asthmatic children group. But, further investigation is needed to evaluate antioxydant/oxydant balance in asthma and also by disease duration and definitive conclusions of oxidative stress role in pathogenesis of asthma.

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MDA I ERITROCITNI ANTIOKSIDACIJSKI STATUS KOD DJECE S KONTROLIRANOM ASTMOM

SAŽETAK

U patogenezi astme, oksidativni stres i postojanje oksidacijsko/antioksidacijske neravnoteže imaju važnu ulogu. U ovoj studiji analizirana je uloga biljega oksidativnog stresa i lipidne peroksidacije u patogenezi astme dječje dobi u usporedbi sa zdravim ispitanicima iste dobi. Plazmatski biljeg lipidne peroksidacije malondialdehid (MDA), antioksidacijski enzimi iz eritrocita: glutation peroksidaza (GSH-Px), superoksid dismutaza (SOD), glutation reduktaza (GR) i glutation (GSH), određeni su spektrofotometrijskom metodom iz uzoraka krvi sakupljenih od 37 zdrave djece i 44 djece s kontroliranom astmom. Rezultati su pokazali signifikantno nižu aktivnost GSH-Px u skupini djece s kontroliranom astmom (3.99±1.0 IU/gHb) u usporedbi s kontrolnom skupinom (4.61±1.3 IU/gHb; $p<0.034$). Značajne razlike u aktivnosti SOD, GR, GSH i MDA nisu potvrđene ($p>0.05$). Niža aktivnost GSH-Px u skupini djece s kontroliranom astmom ukazuje na smanjeni eritrocitni antioksidacijski status i ukazuje na povezanost između oksidativnog stresa i astme u ispitivanoj skupini. Međutim, sačuvana aktivnost GR, SOD te koncentracija GSH i MDA čini se odlučujuća u kontroli oksidacijsko/antioksidacijske ravnoteže.