Antioxidant Enzymatic Activities in Human Blood Cells after an Allergic Reaction to Pollen or House Dust Mite

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José M. Mateés 1, José M. Segura 1, Cristina Pérez-Gómez 1, Rafael Rosado 1, Lucia Olalla 1, Miguel Blanca 2, Francisca M. Sánchez-Jiménez 1

ABSTRACT: Several diseases have been related to oxidative stress. Recently, antioxidant functions have also been linked to anti-inflammatory properties. Cell defenses against reactive oxygen species include antioxidant enzymes. We studied the enzymatic antioxidant capacity in human blood of both red blood and mononuclear cells from patients suffering from an allergic reaction to pollen or house dust mite. We determined superoxide dismutases (SODs), glutathione peroxidase (GSHPx) and catalase (CAT) activities in each cell type. We also determined the extent of thiobarbituric acid reactive substances (TBARS), in order to study the correlation between the cellular enzymatic activities, the redox status and the disease. In mononuclear cells from allergic patients, SODs and CAT activities were enhanced compared to controls. Conversely, a decrease in GSHPx activity was found. In erythrocytes, higher values for GSHPx and SODs and similar CAT activities were found in allergic patients and controls. Interestingly, CuZnSOD and MnSOD activities were enhanced in the same proportion for both, erythrocytes and mononuclear cells. TBARS were also enhanced in both types of cells. The respective enzymatic imbalances in mononuclear cells and erythrocytes, namely, GSHPx/SOD and CAT/SOD, and their consequences are discussed. To our knowledge, this is the first global study of antioxidant enzymes, including TBARS level determinations, in allergy. © 1999 Academic Press

Keywords: allergy, catalase, CuZn superoxide dismutase, glutathione peroxidase, house dust mite, human blood cells, lipid peroxidation, Mn superoxide dismutase, pollen, reactive oxygen species.

INTRODUCTION

At the molecular level, reactive oxygen species (ROS) are constantly generated in all aerobic organisms in response to both external and internal stimuli. Low concentrations of radicals may be beneficial or even indispensable in processes such as intracellular messaging and defense against micro-organisms, contributing to phagocytic bactericidal activity. In contrast, high doses and/or inadequate removal of active oxygen results in oxidative stress, which may cause severe metabolic malfunctions.

Reactive oxygen intermediates, including hydroxyl radicals (OH), superoxide anions (O$_2^{-}$), hydrogen peroxide (H$_2$O$_2$) and nitric oxide (NO), lead to lipid peroxidation and specific oxidation of some enzymes and protein oxidation and degradation (1,2). ROS generation through normal cellular metabolism and by means of exogenous insult is a constant problem for which cells have developed multiple protective mechanisms to survive (3).

In fact, free radical production and disturbance in redox status can modulate the expression of a variety of inflammatory molecules (4, 5) affecting certain cellular processes leading to inflammatory processes, both exacerbating inflammation and effecting tissue damage (6). Recently, antioxidant functions have been definitively linked to anti-inflammatory and/or immunosuppressive properties (7–11). The wide array of enzymatic and non-enzymatic antioxidant defenses, includes superoxide dismutase (SOD), glutathione peroxidase (GSHPx), catalase (CAT), ascorbic acid (vitamin C), α-tocopherol (vitamin E), reduced glutathione (GSH), β-carotene, and vitamin A (12–14).

The function of antioxidant enzymes is to protect cells from toxic oxygen. In this project, we studied the enzymatic antioxidant capacity in human erythrocytes and mononuclear cells. We measured total superoxide dismutase (SOD), as well as CuZnSOD and MnSOD, catalase (CAT) and glutathione peroxidase (GSHPx) activities. We also determined the extent of thiobarbituric acid reactive sub-

1 Department of Molecular Biology and Biochemistry, Sciences, University of Málaga, Málaga, Spain.
2 Allergy Unit of Hospital Carlos Haya, Málaga, Spain.
Reprint requests to: José M. Mateés, Department of Molecular Biology and Biochemistry. Sciences Faculty, University of Málaga, Campus de Teatinos, s/n 29071 Málaga, Spain; Tel: +34–95–213–7135; Fax: +34–95–213–2000; email: jmates@uma.es.
staces (TBARS) as the amount of malonaldehyde (MDA) in mononuclear cells and erythrocytes, in order to verify the possible correlation between the cellular enzymatic antioxidant capacity and the degree of membrane lipid peroxidation. Thus, the aim of this study was to evaluate the status and the interrelationships of the different antioxidant enzymatic activities, TBARS levels, and inflammation, due to allergic reactions in blood samples from patients allergic to pollen or house dust mite.

MATERIAL AND METHODS

Patients

The studies were performed on more than thirty allergic subjects suffering from rhinitis and/or asthma and with skin tests positive to at least one of the inhalant allergen mentioned below. At the moment they were included in the study all of them were symptomatic and were not taken any drug for the relief of these symptoms. A standard battery of allergens, including house dust mites, grass and tree pollen, cat and dog epithelia and molds, was used in this skin test for the diagnostic evaluation of the subjects. Patients ranged in age from 19 to 51 (mean 34.2 ± 6.3 years). Ten healthy and age-matched donors served as controls (mean age 31.1 ± 3.8 years).

Chemicals and Apparati

All solutions used for enzymatic assays were prepared using deionized water with a resistance greater than 15 megohms/cm to eliminate trace metal contamination. Reagents were of analytical grade. Xanthine oxidase, glutathione reductase, catalase, xanthine, H2O2, tert-butyl hydroperoxide, diethylene triamine pentaacetic acid (DETAPAC) and nitro blue tetrazolium (NBT) were purchased from Fluka. Thiobarbituric acid (TBA), the reduced form of nitro blue tetrazolium (NBT), the reduced form of nitro blue tetrazolium (NBT), the reduced form of nitro blue tetrazolium (NBT) were purchased from Fluka.

Blood Samples and Lysates

Venous peripheral blood samples (9 ml) were collected in EDTA-coated tubes, and were centrifuged at 800 x g for 30 min through a density gradient for the separation of mononuclear cells from whole blood (Lymphopred).

Mononuclear cells were washed by centrifugation at 250 x g at 4 °C, with 0.9 % NaCl and with 0.05 M potassium phosphate buffer, pH 7.0, and finally resuspended in 500 µl of the above phosphate buffer. We sonicated at 4 output (60 % duty cycle) for 2 min in 20-sec bursts, while on ice. Extract was centrifuged at 50,000 x g for 20 min at 4 °C. Supernatant, diluted or not in the appropriate buffer, was used as the enzymatic extract.

Erythrocytes were washed three or four times with isotonic saline. Centrifugation for each wash was at 1400 x g at 4 °C. After the final wash, the red blood cells were lysed by hypotonic shock (15), and different dilutions were used as hemolysate.

Measurement of Catalase Activity

Immediately after obtaining the mononuclear cell extract or the red blood cell hemolysate, quantitative determination of CAT was performed. Measurement of H2O2 concentration was recorded by using UV absorbance at 240 nm as previously described (16). 300 µl was used for each determination and activity was expressed as µmol H2O2 min-1 mg-1 protein (U/mg protein) or as µmol H2O2 min-1 g-1 hemoglobin (U/g Hb), respectively.

Monitoring of Glutathione Peroxidase

GSHPx was quantified in 50 λ of each sample, with continuous photometric monitoring of oxidized glutathione (GSSG) at 37 °C. The conversion of NADPH to NADP was evaluated using UV absorbance at 340 nm (17). GSHPx activity was calculated after subtraction of the blank value, as µmol of NADPH oxidized min-1 g-1 protein (U/mg protein) or µmol of NADPH oxidized min-1 g-1 Hb (U/g Hb).

Determination of Superoxide Dismutases

SOD was determined in mononuclear cell extracts and in erythrocytes hemolysates by using the inhibition by SOD of the reaction of O2- with NBT following the technique published by Oberley and Spitz (18) with slight modifications. The rate of NBT reduction in the absence of Lysate was used as the reference rate. When increasing amounts of protein (containing SOD activity) were added to the system, the rate of NBT reduction was progressively inhibited. One unit of activity was considered as the amount of protein that gives half-maximal inhibition. The assay mixture also contained CAT to remove H2O2 and DETAPAC to chelate metal ions capable of redox cycling and interfering with the assay system. NaCN (5 mM final concentration) was added 1 hour before the measurement in order to assay MnSOD.
activity. The MnSOD activity was subtracted from the total SOD activity to calculate the CuZnSOD activity. Both values were expressed as U mg⁻¹ protein or as U g⁻¹ Hb.

Quantification of Lipid Peroxidation

We determined TBARS as a measurement of the oxidative stress assayed for malondialdehyde. Mononuclear cell extract or erythrocyte lysate in the volume of 0.1 ml was mixed with 2 volumes of cold 10 % (w/v) TCA to precipitate proteins. The precipitate was pelleted and the supernatant was reacted with 0,67 % (w/v) TBA. After cooling, the absorbance at 532 nm was recorded. A standard curve was prepared using malonaldehyde bisdimethylacetal as the source of MDA. To ensure that no lipid oxidation occurs during the assay, BHT [0.01 % (v/v) of a 2% stock solution in ethanol] and EDTA (1 mM final concentration) was added to the sample prior to TCA precipitation (19). Results are expressed as nmol TBARS/mg protein and nmol TBARS/g Hb, respectively.

Protein concentrations were determined by the method of Bradford (20); 0.5 mg/ml bovine serum albumin was used as the standard protein. Hb concentrations were determined in a modification of the method of Drabkin (15).

Statistical Methods

Statistical comparisons between groups were done with one-way analysis of variance (ANOVA). All data are reported as Mean ± SD. A $p$ value of less than 0.01 was considered to be significant.

RESULTS

We investigated endogenous levels of antioxidant enzymes in mononuclear and red blood cells of patients suffering from an allergic reaction. No significant differences were found between patients with allergies to pollen or house dust mite nor between asthmatics and patients suffering from rhinitis neither significant correlation with disease activity and/or severity (results not shown). We used disease-free donors as controls. Patients suffering from allergy were diagnosed with the disease and had undergone no treatment prior to the time that blood samples were drawn. The objective of this study was to see whether allergic patients possess characteristics similar to other patients suffering from other pathologies related with oxidative stress (21–23).

The activities of CAT, GSHPx and SOD in mononuclear cells of allergic patients and healthy donors are given in Figure 1A, 1B and 1C, respectively. The activities of SOD and CAT enzymes in allergic patients were found to be significantly enhanced compared to the levels in controls. Conversely, decreases in the mean value compared to the
levels in controls. The figure clearly illustrates the differences found in our study between patient and control plots.

CuZnSOD and MnSOD activities were determined in mononuclear cells and erythrocytes of both allergic patients and healthy donors. It is interesting to note that although a remarkable enhancement was detected in both activities in mononuclear cells, they were increased in the same ratio (Figure 2). Curiously, similar results were obtained in erythrocytes (Table 1). So, the proportion for erythrocyte activities was nearly identical in cytosolic and mitochondrial SOD activities, although MnSOD was 10% of total SOD activity, instead of 6% found for mononuclear cells.

On the other hand, Table 1 shows similar CAT activities in controls and allergic patients, and higher values for GSHPx and SOD activities in erythrocytes from patients suffering from the disease. GSHPx and both SODs activities increased significantly ($p<0.005$ and $p<0.001$, respectively). Conversely, no significant variation was obtained for CAT activity ($p>0.01$).

Expected significantly higher levels of TBARS were found in both, mononuclear cells and erythrocytes from allergic patients. In our patients, a remarkable enhancement was obtained, especially, in red blood cells (Figure 3).

**DISCUSSION**

In mononuclear cells, decreased GSHPx activity agrees with previously reported finding where authors argue that reduced activity of this enzyme in blood may reflect mechanisms associated with the pathogenesis and severity of asthmatic disorders (24).

The imbalance of SOD/GSHPx ratio causes an increase of peroxide availability and, thus, of Fenton’s and Haber-Weiss reactions with the consequence of higher ROS production (25). So, the enhancement of CAT and SOD activities could act as a defense mechanism against radicals.

To our knowledge, this is the first report that finds how MnSOD (inducible) and CuZnSOD (constitutive) activities are induced in the same ratio to protect mitochondria and cytosol from oxidative stress. It is also noteworthy that the enhancement of MnSOD activity was slightly higher in erythrocytes than in mononuclear cells.

In erythrocytes, higher GSHPx activities are in accordance with those of previous findings. Thus, in animal cells, and specially in human erythrocytes, the principal antioxidant enzyme for the detoxification of H$_2$O$_2$ has been considered for a long time to be GSHPx, as CAT has much lower affinity for H$_2$O$_2$ than GSHPx (26).

Interestingly, although SOD, a specific inhibitor of oxygen radicals, was increased in both mononuclear cells and erythrocytes, CAT and GSHPx act in a different manner. Therefore, erythrocytes, in spite of increased GSHPx activity, can not compensate the overproduction of H$_2$O$_2$ by SOD. So, the imbalance of SOD/GSHPx ratio in mononuclear cells converts to a SOD/CAT imbalance for red blood cells.

**Table 1.** Activities of catalase, glutathione peroxidase and superoxide dismutases in erythrocytes from healthy donors and patients suffering from allergic rhinitis and bronchial asthma as a reaction to pollen or house dust mite exposure.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Controls</th>
<th>Patients</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT (U/g Hb)</td>
<td>5.8 ± 3.8 (n=10)</td>
<td>6.3 ± 3.9 (n=37)</td>
<td>NS (&gt;0.01)</td>
</tr>
<tr>
<td>GSHPx (U/g Hb)</td>
<td>16.4 ± 4.8 (n=5)</td>
<td>31.7 ± 8.9 (n=8)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>CuZnSOD (U/g Hb)</td>
<td>3.83 ± 1.67 (n=5)</td>
<td>7.94 ± 1.98 (n=8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MnSOD (U/g Hb)</td>
<td>0.40 ± 0.64 (n=5)</td>
<td>0.82 ± 0.84 (n=8)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

As controls we used healthy age-matched donors. Results are expressed as µmol H$_2$O$_2$/min g Hb (CAT), µmol NADPH/min g Hb (GSHPx), and units/g Hb (SOD).
that could equally explain the oxidative stress. In fact, erythrocytes are very susceptible to oxidative damage due to the high degree of polyunsaturated fatty acids in their membranes, and the high concentration of intracellular oxygen and Hb whose redox chemistry is known to produce oxyradicals (27). The general conclusion from recent studies is that lipid peroxidation potential is mainly a reflection of the degree of unsaturation of fatty acids present in the membranes (28).

Increase of TBARS levels, as a marker of endogenous lipid peroxidation, shows the important significance of the oxidative damage caused by the imbalance in antioxidant functions. Higher levels found in erythrocytes could be consequence of the before described characteristics of their membranes (27).

Concerning the differences observed in antioxidant enzymes of mononuclear cells versus erythrocytes, the main ones were found in GSHPx and CAT activities. As stated above GSHPx activity is, in human erythrocytes, the principal antioxidant enzyme for the detoxification, because of which it could equally be the most affected activity. However, a deeper study must be done before to state definitive conclusions. Additionally, decreased GSHPx activity, compared to the levels in controls, only was found in mononuclear cells. This fact could be interesting because mononuclear cells are more closely related to immune system than erythrocytes, for which reason mononuclear cells may play a more important role in the pathophysiology of allergic diseases.

An unbalanced production of ROS (small deviations from the physiological activity ratios of GSHPx/SOD and CAT/SOD) plays a role in the pathogenesis of a number of clinical disorders such as neurodegenerative diseases, ischemia/reperfusion injury, atherosclerosis, cancer, and many others pathologic processes (8, 27, 29–37). Therefore, we can also conclude that similar global stress occurs in patients suffering from allergy to a pollen or house dust mite exposure. In fact, it is also established that, in asthma, inflammatory cells release oxygen radicals that cause tissue damage (38).

In conclusion, when antioxidant, free radical scavenging systems are overwhelmed, inflammatory, hypersensitivity, and “autoimmune” conditions may result. The observed differences in the in vivo defense systems against free radicals in allergic patients are a proof of the main role of antioxidant enzymes in blood cells detoxification, showing the coordinated enzymatic mechanisms and the interrelationships between all these enzymatic activities in allergy.

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REFERENCES


35. Wang P, Chen H, Qin H, et al. Overexpression of


37. Offen D, Beart PM, Cheung NS, et al. Transgenic mice expressing human Bcl-2 in their neurons are resistant to 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity. *Proc Natl Acad Sci USA* 95:5789–5794, 1998.