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Superoxide release and cellular glutathione peroxidase activity in leukocytes from children with persistent asthma

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Abstract

Asthma is an inflammatory condition characterized by the involvement of several mediators, including reactive oxygen species. The aim of the present study was to investigate the superoxide release and cellular glutathione peroxidase (cGPx) activity in peripheral blood granulocytes and monocytes from children and adolescents with atopic asthma. Forty-four patients were selected and classified as having intermittent or persistent asthma (mild, moderate or severe). The spontaneous or phorbol myristate acetate (PMA, 30 nM)-induced superoxide release by granulocytes and monocytes was determined at 0, 5, 15, and 25 min. cGPx activity was assayed spectrophotometrically. The spontaneous superoxide release by granulocytes from patients with mild (N = 15), moderate (N = 12) or severe (N = 6) asthma was higher at 25 min compared to healthy individuals (N = 28, $P < 0.05$, Duncan test). The PMA-induced superoxide release by granulocytes from patients with moderate (N = 12) or severe (N = 6) asthma was higher at 15 and 25 min compared to healthy individuals (N = 28, $P < 0.05$ in both times of incubation, Duncan test). The spontaneous or PMA-induced superoxide release by monocytes from asthmatic patients was similar to healthy individuals ($P > 0.05$ in all times of incubation, Duncan test). cGPx activity of granulocytes and monocytes from patients with persistent asthma (N = 20) was also similar to healthy individuals (N = 10, $P > 0.05$, Kruskal-Wallis test). We conclude that, under specific circumstances, granulocytes from children with persistent asthma present a higher respiratory burst activity compared to healthy individuals. These findings indicate a risk of oxidative stress, phagocyte auto-oxidation, and the subsequent release of intracellular toxic oxidants and enzymes, leading to additional inflammation and lung damage in asthmatic children.

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Key words
- Children
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- Respiratory burst
- Granulocytes
- Monocytes
- Inflammation

Introduction

The major characteristics of asthma are reversible airflow obstruction, bronchial hyperresponsiveness, and airway inflammation (1). This is a complex inflammatory disease that involves leukocytes, airway epithelial and smooth muscle cells, and several inflammatory mediators with multiple effects. The airway remains edematous and infiltrated with inflammatory cells, which are predominantly eosinophils and lymphocytes. Mast cells play a key role in asthma symptoms, whereas eosinophils, macrophages and T-helper 2 cells are involved in the chronic inflammation that underlies airway hyperresponsiveness (2). Resident or infiltrating inflammatory phagocytes in the airways release reactive oxygen species and other mediators with pleiotropic effects (1).

The role of phagocytes in the pathophysiology of asthma is not completely understood. They release enzymes, including extracellular matrix-degrading proteases, elastase, cytokines, and reactive oxygen species, which have been implicated in lung injury (3). The reported effects of reactive oxygen species in asthma include a decrease of beta-adrenergic function in lungs, airway smooth muscle contraction, increased vascular permeability, bronchial hyperresponsiveness, increased mucus secretion, impaired ciliary activity, generation of chemotactic factors, lipid peroxidation, and secondary production of mediators with a bronchoconstrictor effect (4,5). Airway inflammation is also associated with increased activity of the inducible nitric oxide synthase found in respiratory epithelium and activated macrophages (6). Superoxide and nitric oxide may rapidly combine to form peroxynitrite, a potent oxidant, leading to the depletion of antioxidants. For instance, the antioxidant glutathione (GSH), which is 100-fold more concentrated in the airway epithelial lining fluid compared with plasma, may be converted to its oxidized form (GSSG) (7). Thus, subsequent lung injury and more inflammation may take place.

Glutathione peroxidases (GPx) play an important role in the detoxification of various hydroperoxides. Four types of GPx have been identified: cellular GPx (cGPx), gastrointestinal GPx, extracellular GPx, and phospholipid hydroperoxide GPx (8,9). cGPx (EC 1.11.1.9), also termed GPX1, is ubiquitously distributed. It reduces hydrogen peroxide as well as a wide range of organic peroxides derived from unsaturated fatty acids, nucleic acids, and other important biomolecules (8). At peroxide concentrations encountered under physiological conditions, it is more active than catalase (which has a higher K_m for hydrogen peroxide) and is also active against organic peroxides. Thus, cGPx represents a major cellular defense against toxic oxidant species (9).

The ability of cGPx to reduce peroxides and hydroperoxides plays a particularly important role in the anti-oxidant defense system of phagocytes, which are subject to autooxidation by their own respiratory burst products (10,11), leading to damage of the surrounding tissues when a great amount of oxygen radicals are released (12). Thus, oxidative stress is a relevant risk factor for lung damage in chronic inflammatory diseases such as asthma (6,13).

The aim of the present study was to investigate superoxide release and cGPx activity of granulocytes and monocytes from children and adolescents with atopic asthma, classified according to the Global Initiative for Asthma (GINA) criteria for evaluation of disease severity.

Subjects and Methods

Subjects and study design

The study included atopic asthmatic patients with the following characteristics: 22 males and 22 females, 20 Caucasians and 24 Blacks. The age ranged from 6 to 16 years

(mean = 10.8 ± 2.85 years, median 11 years). The height ranged from 115 to 173 cm, and weight from 21 to 59 kg. Our clinical protocol included patients with a combination of clinical history of recurrent and reversible symptoms of airway obstruction, high serum IgE levels, positive skin tests to recognized allergens, and a family history of allergy (14). We were not allowed to collect blood samples from healthy children. Thus, we selected 28 healthy individuals for the comparative group, with an age range of 22-43 years.

The experimental protocol included assays of superoxide production and cGPx activity in peripheral blood granulocytes and monocytes. Written informed consent was obtained from all patient's parents and healthy individuals prior to the study. The Medical School Ethics Committee approved the experimental protocol in accordance to the Ministry of Health of Brazil (resolution 196/96) and the Helsinki Convention.

Severity of disease was classified according to GINA criteria into mild intermittent, mild persistent, moderate persistent, and severe persistent (15). Patients with moderate persistent asthma used inhaled $beta_2$-adrenergic and/or inhaled steroids (equivalent to 400-800 µg/day budesonide). Patients with severe persistent asthma used inhaled $beta_2$-adrenergic and inhaled, but not systemic steroids (equivalent to 800-1600 µg/day budesonide).

None of the patients had received non-steroidal anti-inflammatory drugs, theophylline, leukotriene modifiers, systemic steroids, systemic $beta_2$-adrenergic agents, or blood transfusion for at least one month prior to the study. Patients with infections, airway foreign bodies, or known systemic or pulmonary chronic diseases such as immunodeficiency, diabetes, ciliary dyskinesia syndromes, cystic fibrosis, or $alpha_1$-antitrypsin deficiency were excluded. Smokers were excluded from both patient and control groups.

Isolation of granulocytes and monocytes

Granulocytes and monocytes were isolated by centrifugation of blood samples over a discontinuous density gradient (Histopaque 1.077 and 1.119 g/ml; Sigma, St. Louis, MO, USA) followed by adherence of monocytes to polystyrene plates (16). Contaminating erythrocytes were removed by hypotonic lysis. Both cell populations were washed three times in Hank's balanced salt solution (HBSS) without phenol red and the final leukocyte count was adjusted to 2 x 10^7 cells/ml. Trypan blue exclusion showed greater than 90% cell viability.

Superoxide anion production

The spontaneous or phorbol myristate acetate (PMA, 30 nM)-induced superoxide anion production by leukocytes was assayed by a spectrophotometric method based on the superoxide dismutase inhibitable reduction of cytochrome c, according to McCord and Fridovich (17), as modified (18). The absorbance at 550 nm of the supernatants was monitored at 0, 5, 15, and 25 min. The amount of superoxide was calculated using an extinction coefficient of 21,100 M/cm. The results are reported as nmol of superoxide released per 10^6 cells per sampling time. This assay was performed in granulocytes and monocytes from 44 patients and 28 healthy individuals.

Cellular glutathione peroxidase activity

cGPx activity was assayed in peripheral blood granulocytes and monocytes according to the method of Beutler (19), as modified (20). Leukocytes (10^6) were incubated for 5 to 10 min in cuvettes containing 0.05% Triton X-100, 0.2 nmol GSH, 1 U/ml GSH reductase and HBSS, pH 7.4. The reaction was initiated by the addition of t-butyl-hydroperoxide. The change in absorbance at 340 nm was monitored at 0 and 1 min.

Results are reported as nmol of oxidized NADPH min^{-1} 10^6 $cells^{-1}$. This assay was performed in granulocytes and monocytes from 20 patients and 10 healthy individuals according to cell number availability.

Statistical analysis

Comparisons between the patient groups and healthy individuals were made using the Duncan or Kruskal-Wallis (21) test as indicated, with the level of significance set at $P < 0.05$.

Results

The spontaneous superoxide production by granulocytes from severe, moderate or mild persistent asthmatic patients was higher at 25 min of incubation compared to healthy individuals (Figure 1A, $P < 0.05$, Duncan test).

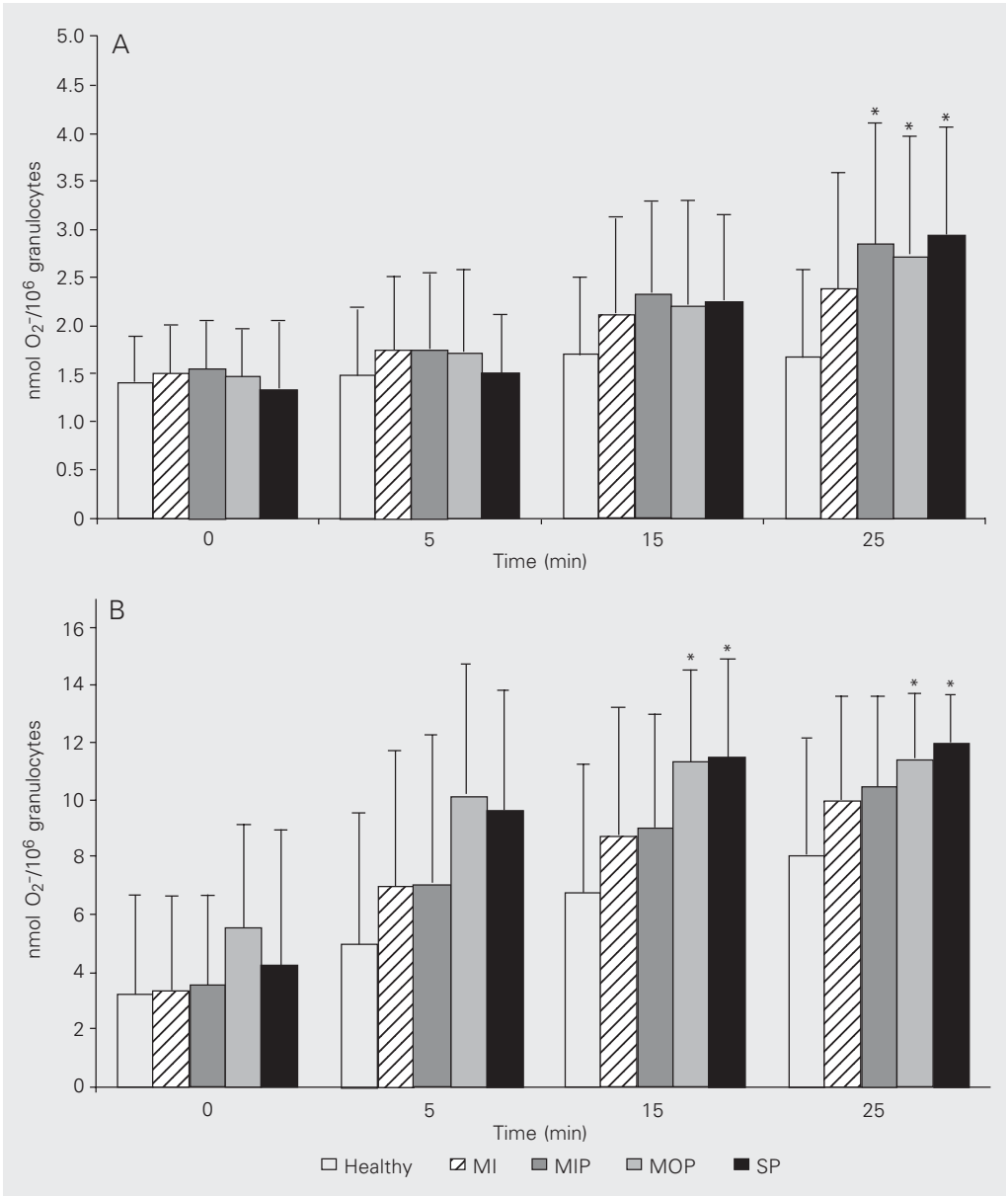

Figure 1. Superoxide release by granulocytes and monocytes from patients with asthma, grouped according to GINA diagnostic criteria. *Panel A*, Spontaneous superoxide (O_2^-) production was measured in granulocytes from patients with asthma grouped according to GINA criteria and from healthy individuals at the indicated time points. The spontaneous superoxide production by granulocytes from SP, MOP or MIP asthmatic patients was higher at 25 min of incubation, compared to healthy individuals (*$P < 0.05$, Duncan test). *Panel B*, Phorbol myristate acetate (PMA)-stimulated O_2^- production was measured in granulocytes from patients with asthma grouped according to GINA criteria and from healthy individuals at the indicated time points. The PMA-stimulated superoxide production by granulocytes from SP and MOP asthmatic patients was higher compared to healthy individuals at 15 and 25 min of incubation (*$P < 0.05$, Duncan test). SP = severe persistent (N = 6), MOP = moderate persistent (N = 12), MIP = mild persistent (N = 15), and MI = mild intermittent asthma (N = 11). Healthy individuals (N = 28).

The PMA-stimulated superoxide production by granulocytes from severe and moderate persistent asthmatic patients was higher compared to healthy individuals at 15 and 25 min of incubation (Figure 1B, $P < 0.05$, Duncan test).

However, neither spontaneous nor PMA-stimulated superoxide production by monocytes from asthmatic patients, grouped according to GINA criteria, differed statistically from healthy individuals ($P > 0.05$, Duncan test).

The cGPx activity of granulocytes from mild persistent (N = 12), moderate persistent and severe persistent asthmatic children (N = 8), and healthy individuals (N = 10) were (mean ± SD, nmol of oxidized NADPH min^{-1} 10^6 $cells^{-1}$): 171 ± 75, 181 ± 33, and 197 ± 68, respectively. The cGPx activity of monocytes from mild persistent (N = 12), moderate persistent and severe persistent asthmatic children (N = 8), and healthy individuals (N = 10) were (mean ± standard deviation, nmol of oxidized NADPH min^{-1} 10^6 $cells^{-1}$): 142 ± 52, 151 ± 75, and 136 ± 36, respectively.

Thus, the cGPx activity of granulocytes or monocytes from children and adolescents with persistent asthma did not differ from that of healthy individuals ($P > 0.05$ in all situations, Kruskal-Wallis test).

Discussion

Our results show that the spontaneous or stimulated superoxide production by granulocytes from children and adolescents with persistent asthma is elevated compared to healthy individuals.

Ethical restrictions were much greater than ordinary because the study concerned samples from children. We were not allowed to obtain bronchoalveolar lavage cells from our patients with asthma, an apparently more sensitive approach to the questions we asked. Thus, we chose to investigate the superoxide release and cGPx activity of blood leukocytes. Indeed, one must consider that both bronchoalveolar lavage cells and blood leukocytes are not intralesional lung tissue cells.

In addition, we were also not allowed to collect blood samples from healthy children. Thus, we selected 28 healthy individuals for the comparative group, which could not be age matched. Previous studies have shown that the respiratory burst activity of leukocytes from children and adolescents does not differ significantly from that of adults (22,23). This similarity allowed us to compare the NADPH oxidase activity of leukocytes from children with asthma to measurements in healthy adult individuals.

Considering the limitation of volume when collecting blood samples from children, we were unable to separate neutrophils (predominant cells), eosinophils and basophils from the granulocyte pool. Thus, we chose to perform those tests in granulocytes. It is generally accepted that eosinophils and neutrophils produce equivalent amounts of superoxide, although this is still a controversial issue. Because of differences in the assembly of the NADPH oxidase components, eosinophils show a trend to release more extracellular superoxide compared to neutrophils (24).

Other studies have demonstrated that leukocytes from asthmatic patients show higher superoxide production compared to healthy individuals, particularly during asthma attacks (6,24-31). In the present, more detailed, study, pediatric patients with chronic asthma were for the first time carefully classified according to GINA criteria. In addition, we have assessed both respiratory burst and cGPx activity in granulocytes and monocytes. Only patients with persistent asthma showed a significant increase in superoxide release by granulocytes. These findings demonstrate at least a partial relationship between asthma severity and respiratory burst activity in peripheral blood granulocytes from pediatric patients.

Several studies have demonstrated that GPx activity in asthmatic patients varies ac-

cording to cell lineage or body fluid. GPx activity may be lower in platelets, normal or lower in red blood cells, lower or increased in plasma, and normal or lower in whole blood (6,13,26,32,33). These conflicting data may be the result of assaying distinct isoforms of GPx in different blood components from heterogeneous asthmatic patients. To date, we have shown that cGPx activity of leukocytes from pediatric asthmatic patients, carefully classified according to GINA criteria, was similar to healthy controls and did not follow the up-regulation of NADPH oxidase activity in the same cells.

We conclude that under specific circumstances granulocytes from children and adolescents with persistent asthma produce more reactive oxygen species compared to healthy individuals. These reactive species have the potential to overwhelm the antioxidant system, with resultant oxidative stress and damage of surrounding pulmonary tissue. This process may be a relevant and still uncontrolled risk factor in childhood asthma pathophysiology. In children with asthma, key markers of inflammation are present early in life, highlighting the importance of early intervention and the appropriate use of drugs that modulate the NADPH oxidase activity (34), preventing the irreversible lung remodeling that contributes to this chronic disease.

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