

BIOMARKERS OF OXIDANT STRESS IN BRONCHIAL ASTHMA

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Summary: Oxidant stress is believed to contribute to both the initiation and development of bronchial asthma (BA). As such, the pathophysiology of BA is characterised by the large generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), predominantly by inflammatory cells of asthmatic airways. These species play an important role in remodelling airways and in orchestrating the type of inflammatory response. Oxidants influence the specific balance of Th1/Th2 cytokines, and together with Th2-cytokines and Th2 induced cells can cause many of the features typical of asthma. They induce bronchoconstriction, mucus secretion, effects on airway vasculature, and increase airway responsiveness to several agonists. This review discusses the necessity of oxidant stress evaluation in BA using reliable biomarkers, which provide an appropriate tool for studying oxidant stress. It is desirable to determine prooxidants and antioxidant systemic and local parameters in lung specific media such as bronchoalveolar lavage (BAL), sputum, exhaled air and breath condensate. These airway biomarkers may be representative indicators which could show the processes occurring on the airway surface, the initial site of oxidation. Thus, the examination of biomarkers is essential for establishing the potential target for antioxidant supplementation that would be able to normalise the oxidant/antioxidant imbalance.

Key words: bronchial asthma, biomarkers, oxidant stress, antioxidants

Introduction

Many lung diseases, including bronchial asthma (BA), are associated with chronic inflammation and oxidant stress. Currently, it is believed that oxidant stress and the response of antioxidant enzymes play a key role in the initiation and development of asthma (1, 2). Oxidant stress is responsible for the main characteristics of asthma: chronic inflammation, variable airflow obstruction and increased airway responsiveness to a variety of stimuli. These events are being increasingly recognized as important in remodelling airways and playing a critical role in orchestrating the type of the inflammatory response (3).

Lung possesses some specificity vs. other organs. First, lung is directly exposed to ambient air, irritants and pollutants, and partial pressure of oxygen is much higher in the alveolar space than in

other vital organs. Also, the large surface area of lung (about 70 m²), presence of lung inflammation (usually associated with BA), and oxygen therapy may generate additional free radicals; all of them are additional factors for increased generation of free radicals in this organ (4).

Oxidants and antioxidants in asthma

Many experimental models showed that oxidants associated with a specific pattern of Th1/Th2 cytokines could produce numerous features typical of asthma: they can induce bronchoconstriction, increased permeability and responsiveness of the airways (5, 6). The inflammatory cells stimulated by cytokines and recruited to the asthmatic airways have an exceptional capacity for producing oxidants. BA is characterised by activated eosinophils, neutrophils, monocytes and macrophages, as well as resident cells such as bronchial epithelial cells, and these specific cells can also generate oxidants (7, 8). First of all, the univalent reduction of oxygen to superoxide anion (O₂⁻) is an important step in the formation of oxidants. Sources of superoxide anion include primarily the membrane-associated NADPH oxidase-dependent complex, the cytosolic xanthine oxidase system, and the mitochondrial respiratory chain. Superoxide

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anion is then converted to hydrogen peroxide (H_2O_2), either spontaneously or under the influence of superoxide dismutase (SOD) (4). Although O_2^- and H_2O_2 themselves are moderate oxidants, both species are critical for the formation of potent cytotoxic radicals in biological systems through their interaction and reaction with other molecules.

Activation of inflammatory cells is the main source of oxidants production in asthma. Once recruited in the airspaces, inflammatory cells may be activated and may generate reactive oxidants in response to various stimuli. In addition, experimental exposure to oxidants induces different degrees of bronchial epithelial cells injury, including cell death. Oxidants also induce increased apoptosis of bronchial epithelial cells in asthmatic subjects (9, 10), stimulate the release of tachykinins and neurokinins into the airways, and decrease β_2 adrenergic receptors, cholinesterase and neutral endopeptidase activities. *In vitro* exposure of asthmatic structural and inflammatory lung cells to oxidants induces the release of proinflammatory mediators including cytokines, chemokines (and their receptors), growth factors, arachidonic acid metabolites, and adhesion molecules (and their ligands) involved in inflammatory cell recruitment (1, 11). All of these processes either lead to cell injury (necrosis) or programmed cell death (apoptosis). On the other hand, reducing agents exert a relaxing effect on the airway smooth muscle, and can inhibit bronchial smooth muscle contraction, preventing airway hyperresponsiveness as well as cell injury in several experimental models.

Lung tissue is protected against these oxidants by a variety of antioxidant mechanisms. Enzymatic antioxidants play a significant role against oxidant stress, particularly SOD. There are three SODs: cytosolic CuZnSOD, mitochondrial MnSOD and extracellular SOD, that convert superoxide into H_2O_2 (12, 13). Catalase and glutathione peroxidase (GSH-Px) are the most important H_2O_2 scavenging enzymes. GSH-Px is closely associated with the maintaining of reduced glutathione (GSH) by glutathione reductase, γ -glutamyl cysteine synthetase and glutathione synthase. Beside »classical« antioxidant enzymes, lungs display antioxidant activities from the thioredoxin-thioredoxin reductase system, thioredoxin peroxidase and glutaredoxin, with the highest activity of these enzymes in the airway (especially in macrophages).

Glutathione represents the major thiol antioxidant with a characteristically high concentration in epithelial lining fluid (about 150 times higher than in blood) (4, 14). Except GSH, the non-enzymatic lung system involves surfactant protein G, albumin, and vitamins C and E (inhibit IgE response). Simultaneously, the properties of Fe and Cu are strongly linked to proteins (via transferrin, ferritin, ceruloplasmin, laktoferin) and this decreases the possibility of ROS generation. Both nitric oxide in lung tissues and thiol compounds form S-nitroso thiol, which is resistant to oxidants, and this feature contributes to the antioxi-

dant stability of lung. This stability is supported by a higher concentration of cyclin-dependent kinase inhibitor (p21CIP1/WAF1), protein that protects against oxidant stress (15).

A number of studies indicate that BA may be a consequence of antioxidant deficiency. For example, an evident fall in plasma antioxidant capacity occurs during the initiation and exacerbation of this chronic disease. Many investigators emphasize lower CuZn-SOD activity in asthmatics than in normal subjects. Also, the peroxyxynitrite inhibitory activity, a known antioxidant system, is reduced in plasma and sputum of patients with stable asthma, and its values are positively related to airway responsiveness and negatively related to the degree of sputum eosinophilia (16).

Nitric oxide level is generally low in the respiratory system if the NO is coupled with reducing agents, mainly with glutathione, to form S-nitrosothiols (SNOs) which are relatively resistant to oxidants. A recent study showed decreased levels of the bronchodilator S-nitrosoglutathione (GSNO) in patients with near fatal asthma. It is believed that GSNO could be broken down by oxidants in the asthmatic patients and its catabolism could have an inhibitory effect on the airway smooth muscle relaxation (17).

Biomarkers of oxidant stress in asthma

Traditionally, oxidant stress has been monitored by measuring increased production of reactive oxygen species or end products of oxidation in circulating cells and plasma. Recently, several techniques have been developed to detect oxidant stress using breath samples, induced sputum, BAL and breath condensate, which present more directly samples of local oxidant production in the lungs. Current measurement of asthma biomarkers includes different media, such as blood (plasma, erythrocytes, platelets, and whole blood), bronchoalveolar lavage, induced sputum, exhaled air and breath condensate.

Each of them provides useful data, which have systemic or local significance. However, there are many problems with the standardisation of parameter determination in exhaled air and condensate. Also, the not-uniform methods of sputum collection present an additional problem. Traditionally, the most frequent and the most important biomarkers in the blood include isoprostane (8-izo-PGF 2α), malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), GSH (total, reduced, oxidised), GPx, SOD and catalase activities, oligoelements (Se, Zn) and vitamins (C, E, A).

The investigation of all these different parameters often gives dissimilar results or contradictory information in the various and in the same media. In *in vitro* studies, many problems occur because the studies may include patients with various clinical findings or medical treatment. Some results of oxidant stress biomarkers investigated with BA patients in separate studies are given in *Table 1* (18–25):

Table I Markers of oxidant stress in BA

Biomarker	Medium	vs. control
Isoprostane (8-izo-PGF _{2α})	plasma	↑↑↑
	condensate	↑↑↑
MDA	plasma	↑↔
	condensate	↑↑↑
Ethane	condensate	↑↑↑
Pentane	condensate	↑↑↑
GSH (total)	BAL	↑↑↑
	sputum	↔
GSH (oxidised)	BAL cells	↔
	BAL	↑↑
	sputum	↑
GSH (reduced)	erythrocytes	↔
	BAL	↑
	erythrocytes	↑↔
GPx (activity)	whole blood	↓
	erythrocytes	↔↔↓
	whole blood	↔↔↓
	platelets	↔
	BAL	↔
SOD (activity)	BAL cells	↔
	BAL	↓↓
	BAL	↔
	BAL cells	↓
	platelets	↓
Catalase (activity)	erythrocytes	↔↔↓
	plasma	↔
	erythrocytes	↔↔↓
	BAL cells	↔
Se	BAL	↔
	sputum	↔
	whole blood	↓
	erythrocytes	↓
	plasma	↔↔↓
Zn	platelets	↔
	plasma	↓
Vitamins C, E, A	serum	↓
	BAL	↓
TEAC	plasma	↓↓

TEAC: trolox equivalent antioxidant capacity
 ↓ decrease value
 ↑ increase value
 ↔ unchanged value

A general characteristic of these results is the uniform increase in markers of lipid peroxidation. Elevated 8-iso-PGF_{2α} concentrations have been observed in various media, because its levels are detectable in all human biological fluids. Isoprostanes are structurally stable. They are produced *in vivo* and are present in relatively high concentrations. As a marker of oxidant stress, 8-iso-PGF_{2α} has been shown to be 20 times more sensitive than the measurement of TBARS. In experimental models of oxidant stress, the levels of isoprostanes (free and esterified) are dramatically increased (19, 26).

Isoprostanes possess good properties for quantifying: they are stable for up to six months (at -70 °C) and they may be determined by quick, simple and relatively low-cost enzyme immunoassay (EIA) or by the method of gas chromatography-mass spectrometry. A recent study promotes isoprostanes as an excellent tool for monitoring effects of antioxidants agents. Several studies have demonstrated that isoprostane levels can provide beneficial information about doses and possible combination of antioxidants in asthma therapy (27).

Results from *Table I* show the increased concentration of other lipid peroxidation biomarkers in different media: MDA, TBARS, ethane and pentane. One of the most frequently used parameters is an indirect determination of MDA concentration, using the TBARS assay. MDA is an end product of the oxidation and decomposition of polyunsaturated fatty acids, and a part of TBARS, and it may form the MDA-TBARS adduct. It is not an ideal parameter because it is possible for it: to interfere with haemoglobin or biliverdin, or with iron present in the reagents which are used for analysis; its metabolism is rapid; MDA represents <1% of lipid peroxides. There is a recommendation that MDA should be investigated with some other markers of lipid peroxidation (22). New methods provide direct measurement of MDA by high-performance liquid chromatography (HPLC) to separate MDA from other interfering chromogens and to improve the specificity of the test.

Many investigators prefer parameters from breath because their determination includes non-invasive methods and involves determination of pentane and ethane as the end products of fatty acid peroxidation (28). Nevertheless, these methods are problematical as follows: hydrocarbon gas production depends on the presence of metal ions to decompose lipid peroxides; contamination of the atmosphere is produced by these gases; the metabolism of pentane in the liver by cytochrome P450 contributes to inaccurate results.

Corradi et al (29) compared acrolein, *n*-hexanal (C6), *n*-heptanal (C7), *n*-nonanal (C9), 4-hydroxynonanal (HNE) and 4-hydroxyhexenal (HHE) in both induced sputum and exhaled breath condensate, and concluded that aldehydes can be detected in both fluids, but their relative concentrations were different and not correlated with each other.

Recent studies investigated some of non-classical parameters: oxidant resistance of LDL (estimate protection of polyunsaturated fatty acids by antioxidants *in vivo*), antibodies against oxidised LDL, total antioxidant status, the total radical trapping antioxidant potential (TRAP), and trolox equivalent antioxidant capacity (TEAC). Last two assays were designed to describe the total capacity to withstand free-radical stress. The TEAC assay compares the antioxidant capacity of plasma with the antioxidant potential of trolox. These assays indicate the strength of the antioxidant screen, however, they are only indirect markers of the degree of oxidant stress (31).

SOD is one of the most »popular« investigated antioxidant enzymes in the evaluation of asthma. The lower activities of SODs were noted in different media and these findings are in correlation with the incidence of asthma. Primarily, the decrease in asthmatic airways is reserved for CuZnSOD while MnSOD is lower or frequently unchanged in asthmatic patients (31). MnSOD is elevated in alveolar epithelial cells type II and macrophages, and in proliferating epithelial cells type II. It is the most abundant SOD within this compartment.

It is known that extracellular glutathione peroxidase is increased in the bronchial epithelial cells of asthmatic patients. A link between asthma and selenium deficiency has also been hypothesised. Many studies showed lower levels of selenium in asthmatic patients, but controlled trials did not show any significant benefit of short-term selenium supplementation in patients with intrinsic asthma (32).

Numerous investigators give glutathione a central place in the lung antioxidant defence. The results related to glutathione mainly showed lower levels in all biological fluids, while some investigators noted transitory increase in GSH but only in the initial phase of the disease. Other investigators noted that GSH level did not differ significantly in the sputum of BA patients vs. healthy controls (14, 25).

Vitamin C has also been studied because its potential lies in the inverse relationship with increased risk for asthma, but confirmations from controlled studies are not enough to recommend the use of vitamin C in the antioxidant treatment of asthma (33). On the other hand, vitamin E plays an important role by inhibiting IgE response to allergic stimuli. Both vitamin E and vitamin C are inversely related to the incidence of asthma. In addition, the supplementation with vitamin C and E reduces ozone-related decrement in lung function in asthmatic subjects, particularly in those with genetically determined increased susceptibility to oxidant stress (34).

An important point in the investigation of asthma biomarkers is the determination of Th1/Th2 cytokines in blood, induced sputum and in BAL. These data demonstrate that there are increased numbers of CD4+ IL-4 producing T cells in induced sputum from patients with BA, in accordance with the shift towards the Th2 response known to exist in BAL and peripheral blood. The positive correlation between the CD4+ IL-4 producing T-cells in BAL and in induced sputum suggests that asthmatic patients could be followed up by this noninvasive method.

Today, nitric oxide presents the most studied biomarker in asthma. Measurement of fractional exhaled NO (FE_{NO}) has some advantages: it is a noninvasive, instantly repeatable and safe method. Exhaled NO has been used to monitor the effect of antiinflammatory treatment in asthma (35) and asthma exacerbation. It behaves as a »rapid response« marker, which is extremely sensitive to steroid treatment. It may be significantly reduced even 2 to 3 days after inhaling cortico-

steroids, reaching its maximal effect after 2 to 4 weeks of treatment (36). Potential problems of NO measuring in expired air include possible contamination with high concentrations of NO in the nasal and paranasal sinus, as well as the fact that the concentration of NO is lower with increased flow rate. Nowadays, there are recommendations for standardized procedures for FE_{NO} measuring and many problems are minimized.

Systemic versus airway markers of oxidant stress

Systemic biomarkers present the classical parameters in evaluation of oxidant stress. However, an important question is how correctly they represent the conditions at the airway surface, the site of oxidant attack. Some reports suggest that a more accurate picture may be obtained with parameters from the lung-lining fluid. Since the deficiencies of antioxidants and vitamins do not reflect in plasma, for some parameters large deficiencies in BAL, accompanied with increase in oxidized GSH, were noted. For many parameters, the relationship between plasma and lung-lining fluid antioxidant pool is unknown. It is likely that an accurate estimation of the antioxidant defence status requires sampling directly from the site of oxidant damage – in asthma, it is the airway.

Biomarkers in induced sputum may be useful for studying the lower respiratory tract, and provide a noninvasive alternative against BAL samples. Induced sputum is collected following saline inhalation. Thus the noninvasive nature of the sample collection is advantageous, compared with the collection of BAL fluid which must be performed under anaesthesia. Isoprostanes measured in induced sputum showed higher values than in plasma.

Collection of breath condensate in asthma is also noninvasive, however, the method is limited. Technical modifications may overcome some of the practical problems, including the elimination of ambient contamination by exhaled breath, and breath condensate samples are not suitable for a comprehensive analysis of both oxidant stress and antioxidant defences.

Conclusion

Oxidant stress and disturbed antioxidant status in asthmatics are well established. A complete biochemical evaluation of the antioxidant defence is needed to identify the nature and extent of any deficiency. Much of the reported data has been obtained from various blood components, but cannot be representative of the events at the airway surface, the initial site of oxidation. Thus, the examination of airway biomarkers is critical to determine the potential targets of antioxidant supplementation for restoring the oxidant/antioxidant imbalance. Measurement of isoprostanes in the breath condensate or induced sputum should provide useful information concerning the degree of oxidant stress and success of antioxidant therapy in asthma.

BIOMARKERI OKSIDANTNOG STRESA U BRONHIJALNOJ ASTMI

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Kratak sadržaj: Veruje se da oksidantni stres učestvuje u inicijaciji i razvoju bronhijalne astme (BA). Uostalom, patofiziologija BA odlikuje se ogromnom produkcijom reaktivnih kiseoničkih vrsta (ROS) i reaktivnih azotnih vrsta (RNS), uglavnom od strane inflamatornih ćelija vazdušnih puteva astmatičara. ROS i RNS igraju važnu ulogu u remodeliranju vazdušnih puteva, kao i u orkestriranju vrste inflamatornog odgovora. Oksidanti utiču na specifičnu ravnotežu Th1/Th2 citokina i zajedno sa Th2 citokinima i Th2 indukovanim ćelijama mogu uzrokovati mnoge specifičnosti tipične za astmu. Oni indukuju bronhokonstrikciju, sekreciju mukusa, deluju na vaskulaturu vazdušnih puteva i povećavaju hiperreaktivnost prema pojedinim agonistima. Ovaj članak ispituje neophodnost evaluacije oksidantnog stresa u BA korišćenjem pouzdanih biomarkera koji omogućuju podesno praćenje oksidantnog stresa. Poželjno je određivanje prooksidanata i antioksidanata, i to sistemskih i lokalnih, u specifičnim medijumima pluća kao što su bronhoalveolarni lavat (BAL), sputum, izdahnuti vazduh i kondenzat izdahnutog vazduha. Ovi biomarkeri vazdušnih puteva mogu biti reprezentativni indikatori dešavanja na površini vazdušnih puteva, na inicijalnom mestu delovanja oksidanata. Ispitivanje biomarkera važno je i za utvrđivanje mogućih ciljeva delovanja antioksidantnih suplemenata koji mogu biti u stanju da normalizuju oksidantnu/antioksidantnu ravnotežu.

Ključne reči: bronhijalna astma, biomarkeri, oksidantni stres, antioksidanti

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