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DIMETHYL SULFOXIDE IN A 10% CONCENTRATION HAS NO EFFECT ON OXIDATION STRESS INDUCED BY OVALBUMIN-SENSITIZATION IN A GUINEA-PIG MODEL OF ALLERGIC ASTHMA

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In allergic asthma, activated cells produce various substances including reactive oxygen species (ROS). As heterogenic pathophysiology of asthma results to different response to the therapy, testing novel interventions continues. Because of water-insolubility of some potentially beneficial drugs, dimethyl sulfoxide (DMSO) is often used as a solvent. Based on its antioxidant properties, this study evaluated effects of DMSO on mobilization of leukocytes into the lungs, and oxidation processes induced by ovalbumin (OVA)-sensitization in a guinea-pig model of allergic asthma. Guinea-pigs were divided into OVA-sensitized and naive animals. One group of OVA-sensitized animals and one group of naive animals were pretreated with 10% DMSO, the other two groups were given saline. After sacrificing animals, blood samples were taken and total antioxidant status (TAS) in the plasma was determined. Left lungs were saline-lavaged and differential leukocyte count in bronchoalveolar lavage fluid (BAL) was made. Right lung tissue was homogenized, TAS and products of lipid and protein oxidation were determined in the lung homogenate and in isolated mitochondria. OVA-sensitization increased total number of cells and percentages of eosinophils and neutrophils in BAL fluid; increased lipid and protein oxidation in the lung homogenate and mitochondria, and decreased TAS in the lungs and plasma compared with naive animals. However, no differences were observed in DMSO-instilled animals compared to controls. In conclusion, OVA-sensitization increased mobilization of leukocytes into the lungs and elevated production of ROS, accompanied by decrease in TAS. 10% DMSO had no effect on lipid and protein oxidation in a guinea-pig model of allergic asthma.

Key words: *allergy, asthma, eosinophil, dimethyl sulfoxide, ovalbumin-sensitization, oxidation stress, reactive oxygen species*

INTRODUCTION

Bronchial asthma is a respiratory disease with a complex pathophysiology (1, 2). It is characterized by airway hyperresponsiveness, enhanced irritability of sensory nerves in the airways and increased mucus secretion, as well as by acute and chronic inflammation, edema and airway remodeling (1). In airway inflammation, various types of cells, mainly eosinophils, neutrophils, T-lymphocytes and mast cells contribute to these processes. Activation of the cells during inflammation is associated with increased generation of reactive oxygen species (ROS) and reduced antioxidant status, as it has been recently described (3, 4). ROS, such as superoxide anion, hydrogen peroxide, hydroxyl radicals *etc.*, may directly damage proteins, lipids and DNA, forming products that can be used as biomarkers of oxidative stress. In addition, oxidative stress through the activation of mitogen-activated protein kinase (MAPK) signaling as well as by increase of nuclear factor erythroid-2 related factors (NRF)-1 and -2, and nuclear factor- κ B (NF- κ B) promotes inflammation (5) and modulates airway responses (6). Even, higher ROS may act as a contributor to the induction of allergic asthma (5, 7).

Heterogenous participation of the individual pathomechanisms results in the heterogeneity of clinical expressions of bronchial asthma and finally relates to the different response to administered therapies (1). Because of rather high proportion of patients not responding to the therapy, developing and testing novel therapeutic interventions continues. Considering that mobilization and activation of various cells in allergic inflammation is associated with overproduction of ROS, administration of anti-inflammatory and antioxidative treatment may be of benefit. Nevertheless, some of potentially beneficial drugs, such as several phosphodiesterase inhibitors, are not soluble in water and their efficacy is evaluated when dissolved in other solvent, *e.g.* in dimethyl sulfoxide (DMSO). DMSO is a colorless liquid, which is both water soluble and able to dissolve lipophilic compounds thanks to containing a hydrophilic sulfoxide group and two hydrophobic methyl groups (8). It has low toxicity and well penetrates through the membranes without damaging them and could carry other compounds into a biological system (9). These properties have destined it for a wide use as a solvent particularly in the development and testing efficacy of new drugs (8). However, because DMSO may act also as an

antioxidant (8, 10), an extent of potential antioxidant action of the solvent should be carefully studied to distinguish between actions of the therapeutic agent dissolved in DMSO and DMSO itself.

Thus, the goal of this study was to evaluate whether the 10% concentration of DMSO used as a solvent may influence, firstly, the mobilization of eosinophils and neutrophils into the lungs; and secondly, the severity of oxidation processes induced by OVA-sensitization in a guinea-pig model of allergic asthma. An extent of oxidation of lipids and proteins was determined in the lung homogenate as well as in isolated lung mitochondria, where the oxidation is most prominent. To assess a systemic impact of oxidation generated in the lungs, total antioxidant status (TAS) was estimated in the blood plasma, as well.

MATERIAL AND METHODS

General protocol of the study

The study protocol was approved by a local Ethics Committee at Jessenius Faculty of Medicine, Comenius University in Martin. In total, twenty four healthy male guinea pigs (250-350 g) were used in the study, divided into four groups. They were kept in an animal house and had food and water *ad libitum*. In twelve animals, model of allergic asthma was induced with ovalbumin allergen (Albumin from chicken egg white, crystallized and lyophilized, salt free >98%; Sigma-Aldrich, Germany). Other twelve animals served as non-sensitized (naive) controls. Sensitization of animals with ovalbumin was performed during 14 days (11, 12). Ovalbumin in 1% concentration (10 mg of ovalbumin in 1 ml) was administered on the 1st day of sensitization intraperitoneally (0.5 ml) and subcutaneously (0.5 ml), on the 3rd day intraperitoneally (1 ml), and on the 14th day only by the inhalation (3 min). Ovalbumin challenge by the inhalation was performed using an Aerosol Jet Nebulizer (HSE, Germany), with 100% particles below 10 µm, 60% particles below 2.5 µm. On 15th-21st day, in one group of OVA-sensitized animals (n=6) and one group of naive animals (n=6), dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany) was given in a 10% concentration at a dose of 3 ml/kg b.w. intraperitoneally in a daily interval. The second ovalbumin challenge by the inhalation was performed on the 21st day, 30 minutes after the last dose of DMSO. Normal saline was given to control animals at the same dose and regimen as in OVA-sensitized ones. Animals were killed one hour after the administration of the last dose of DMSO or saline.

After sacrificing the animals, samples of blood were taken and centrifuged at 3000 rpm for 20 min. Blood plasma was stored at -70°C till an analysis of total antioxidant status (TAS) was made. Left lungs were saline-lavaged (see below). Right lung tissue was homogenized, TAS and markers of lipid and protein oxidation were determined. Additionally, several oxidation markers were evaluated also in the lung mitochondria (see below).

Bronchoalveolar lavage

After placing a cannula, left lungs were lavaged with saline (2x10 ml/kg b.w.). Total number of cells in bronchoalveolar lavage (BAL) was evaluated microscopically using a Bürker's chamber and expressed per 1 µl of BAL fluid. BAL fluid was then centrifuged at 1500 rpm for 10 min. In the sediment of BAL fluid, differential leukocyte count was evaluated microscopically after panchromatic staining by Pappenheim, as described previously (13).

Biochemical assay

1. Preparation of the lung homogenate

Lung tissue was washed, minced and homogenized in 50 mmol of phosphate buffer (pH=7.4) and 1 mmol butylated hydroxytoluene (BHT) in a ratio 1:5 using a homogenizer (Potter, B. Braun Melsungen A.G., Germany) at a temperature 0-4°C. Protein assay was performed by a method of Lowry *et al.* (14). For dilution of the homogenate, 1% sodium dodecyl sulfate was used. Concentration was calculated using a bovine serum albumine as a standard.

2. Fluorescence measurements

Fluorescence measurements were performed in a solution containing 50 µg of membrane protein per ml, 10 mmol/l HEPES, 100 mmol/l KCl, pH 7.0 at 25°C using spectrofluorimeter (RF-540, Shimadzu, Japan). Fluorescence emission spectra (380-440 nm, slit width 5 nm) of dityrosine, a product of tyrosine oxidation, were measured at excitation wavelength 325 nm (slit width 5 nm). Fluorescence intensity was expressed in arbitrary units (A.U.) (15). Emission spectra (from 425 to 480 nm, slit width 5 nm) of lysine conjugates with lipid peroxidation (LPO) end-products were recovered at excitation of 365 nm (5 nm slit width). Excitation spectra (from 325 to 380 nm, 5 nm slit width) were measured at 440 nm (5 nm slit width). Fluorescence intensity was expressed in arbitrary units (A.U.) (16).

3. Measurement of lipid peroxidation

Thiobarbituric acid-reactive substances (TBARS) in the lung homogenate were determined from the absorbance at 532 nm and expressed in nmol/mg protein (17).

4. Preparation of mitochondrial fraction

Mitochondria were prepared from a tissue homogenate by a differential centrifugation. Lung homogenate was centrifuged at 400g for 5 min and supernatant was collected. The supernatant was then centrifuged at 12,000g for 10 min. Resulting pellet was resuspended in a homogenisation buffer (25 mM 4-morpholinepropanesulfonic acid, 250 mM sucrose, 4 mM MgCl₂, 0.05 mM EGTA, pH 7.4) and centrifuged at 12,000g for 10 min. Final pellet was resuspended in a homogenisation buffer (see above) and stored on ice. Protein concentration was determined by methods of Lowry *et al.* (14).

5. Measurements of oxidation in the lung mitochondria

Fluorescence of 8-anilino-1-naphthalenesulfonic acid (ANS) was measured following 10 min. incubation of ANS as a probe with mitochondria membrane proteins. The excitation and emission wavelengths were 365 and 480 nm, respectively (5 nm slit widths) (18). Concentrations of dityrosines and lysine-LPO products in mitochondria were measured by the fluorescence methods described above (15, 16).

6. Total antioxidant status

Quantification of total antioxidant status (TAS) in the lung homogenate and in the plasma was carried out using ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) radical formation kinetics (Randox TAS kit, Randox Laboratories Ltd., UK) and were expressed in mmol/l.

Statistics

Statistical analysis was made by Systat for Windows. Because of relatively small number of animals in the groups and non-parametric distribution of some data, Kruskal-Wallis test for between-group comparisons was used. Association

between several parameters was evaluated by Pearson's correlations and expressed as Pearson's correlation coefficient (r) and Bonferroni probability (p). A $p < 0.05$ was considered statistically significant.

RESULTS

Cells in the lavage fluid

OVA-sensitization increased a total number of cells (both $p < 0.01$; *Fig. 1A*), as well as percentages of eosinophils (both $p < 0.01$; *Fig. 1B*) and neutrophils (both $p < 0.05$; *Fig. 1C*) in the lavage fluid in both OVA-control and OVA-DMSO groups in comparison with naive groups. No significant differences between the controls (naive-control vs. DMSO-control) and DMSO-instilled animals (OVA-control vs. OVA-DMSO) were observed (all $p > 0.05$).

Oxidation markers in the lung homogenate

OVA-sensitization increased concentrations of thiobarbituric acid-reactive substances (in OVA-control vs. naive-control $p < 0.01$, in OVA-DMSO vs. naive-DMSO $p < 0.05$; *Fig. 2A*) and lysine-lipoperoxidation end-products (in OVA-control vs. naive-control $p < 0.01$, in OVA-DMSO vs. naive-DMSO $p < 0.01$; *Fig. 2B*) in the lung homogenate compared to naive controls. However, only non-significant increase in dityrosine formation was observed in OVA-sensitized groups vs. naive animals (both $p > 0.05$, data not shown). In these markers, no significant differences between the controls (naive-control vs. DMSO-control) and DMSO-instilled animals (OVA-control vs. OVA-DMSO) were observed (all $p > 0.05$).

Oxidation markers in the lung mitochondria

Sensitization with ovalbumin significantly increased a formation of dityrosines (in OVA-control vs. naive-control $p < 0.05$,

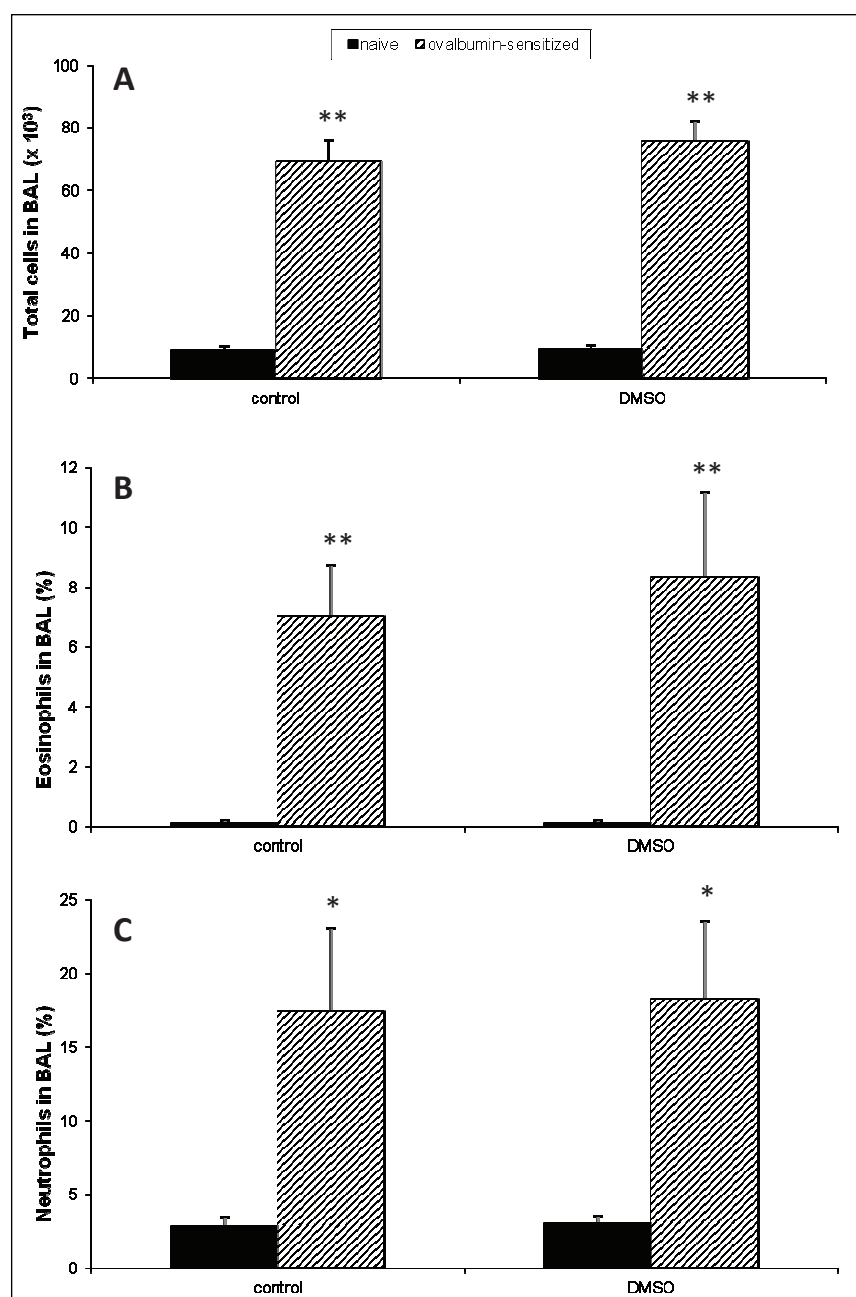


Fig. 1. Total number of cells (A) and percentages of eosinophils (B) and neutrophils (C) in the BAL fluid. For differences between OVA-control vs. naive-control and OVA-DMSO vs. naive-DMSO: $p < 0.01$, $p < 0.05$. DMSO-instilled animals vs. controls: $p > 0.05$.

in OVA-DMSO vs. naive-DMSO $p < 0.05$; Fig. 3A), conjugates of lysine-lipoperoxidation products (in OVA-control vs. naive-control $p < 0.01$, in OVA-DMSO vs. naive-DMSO $p < 0.01$; Fig. 3B) and fluorescence of 8-anilino-1-naphthalenesulfonic acid (in OVA-control vs. naive-control $p < 0.01$, in OVA-DMSO vs. naive-DMSO $p < 0.01$; Fig. 3C), a parameter showing change in a tertiary structure of proteins. In the mentioned parameters, no significant differences between the controls (naive-control vs. DMSO-control) and DMSO-instilled animals (OVA-control vs. OVA-DMSO) were observed (all $p > 0.05$).

Total antioxidant status in the lung homogenate and blood plasma

Total antioxidant status (TAS) slightly decreased in the lung of ovalbumin-sensitized animals (in OVA-control vs. naive-control $p > 0.05$, in OVA-DMSO vs. naive-DMSO $p < 0.05$; Fig. 4A). However, more obvious trend of decreased total antioxidant status was observed in the blood plasma of ovalbumin-sensitized animals (in OVA-control vs. naive-control $p < 0.05$, in OVA-DMSO vs. naive-DMSO $p < 0.05$). Value of plasma TAS was borderly, but non-significantly higher in OVA-DMSO group than in OVA-control group ($p = 0.056$; Fig. 4B). No other obvious differences between the controls (naive-control vs. DMSO-control) and DMSO-instilled animals (OVA-control vs. OVA-DMSO) were observed (all $p > 0.05$).

Association between the evaluated parameters (Pearson's correlations)

1. Eosinophils and neutrophils in the lavage fluid vs. oxidation markers

Pearson's evaluation of association between the variables showed positive correlations between thiobarbituric acid-reactive substances in lung homogenate as a marker of lipid oxidation and counts of eosinophils (in control groups $p = 0.030$, $r = 0.682$, and in DMSO groups $p = 0.004$, $r = 0.814$), and neutrophils in the lavage fluid (in control groups $p = 0.004$, $r = 0.813$; in DMSO groups $p = 0.021$, $r = 0.712$). Similarly, 8-anilino-1-naphthalenesulfonic acid as a marker of oxidation changes of proteins positively or nearly-significantly correlated with both eosinophils (in control groups $p = 0.004$, $r = 0.849$, and in DMSO groups $p = 0.055$, $r = 0.657$) and neutrophils (in control groups $p = 0.034$, $r = 0.705$; in DMSO groups $p = 0.059$, $r = 0.648$) in the lavage fluid.

2. Total antioxidant status in the blood plasma vs. oxidation markers

On the other hand, negative correlations were observed between TAS in the plasma and several oxidation markers: thiobarbituric-acid reactive substances (in control groups $p = 0.009$, $r = -0.687$; in DMSO groups $p = 0.007$, $r = -0.784$) and lysine-lipoperoxidation products (in control groups $p = 0.001$,

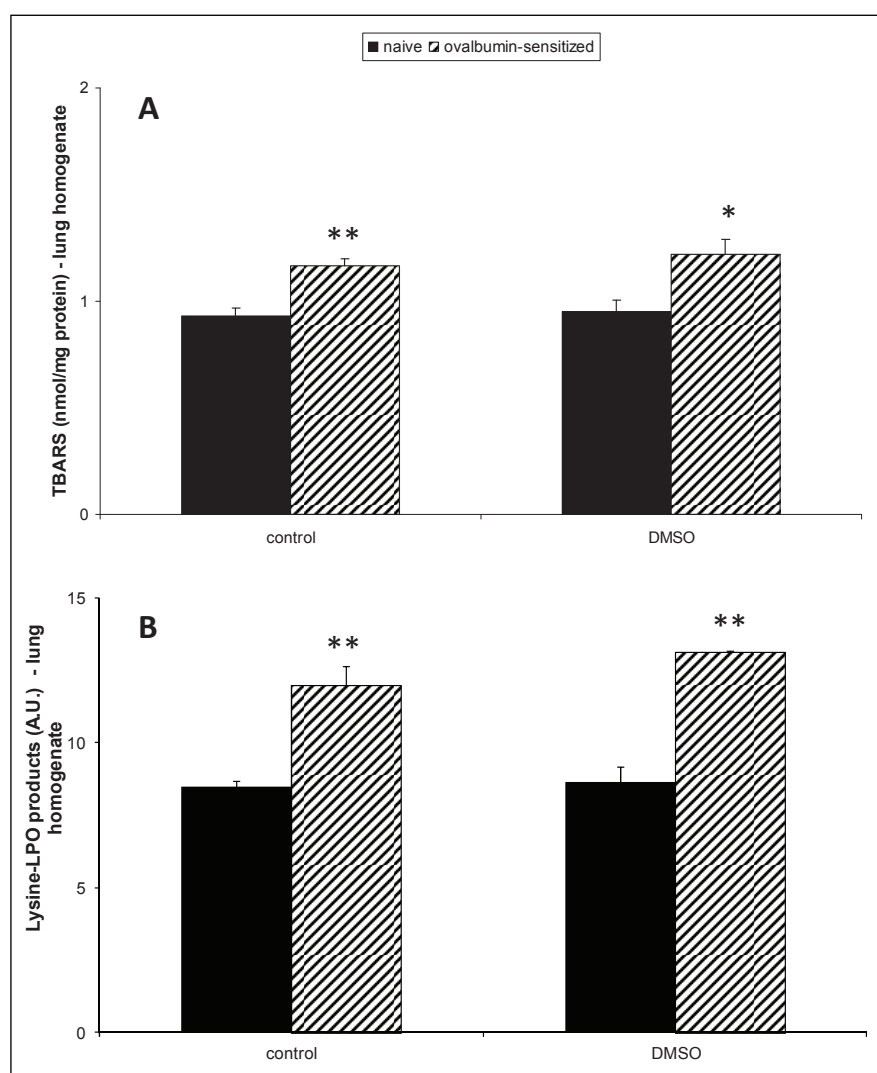


Fig. 2. Formation of TBARS (A) and lysine-LPO end-products (B) in the lung homogenate. For differences between OVA-control vs. naive-control and OVA-DMSO vs. naive-DMSO: $p < 0.01$, $p < 0.05$. DMSO-instilled animals vs. controls: $p > 0.05$.

$r=-0.852$; in DMSO groups $p=0.006$, $r=-0.793$) in the lung homogenate. In the mitochondria, negative or nearly-significant correlations between TAS in blood plasma and lysine-liperoxidation products (in control groups $p=0.002$, $r=-0.851$; in DMSO groups $p=0.057$, $r=-0.653$) and 8-anilino-1-naphthalenesulfonic acid (in control groups $p=0.008$, $r=-0.776$; in DMSO groups $p=0.018$, $r=-0.758$) were observed.

DISCUSSION

Experimental sensitization with ovalbumin is used to evoke an airway hyperresponsiveness, however, it induces inflammation and oxidation in the lung tissue, as well. In this study, OVA-sensitization increased total number of cells and percentages of eosinophils and neutrophils in the BAL fluid

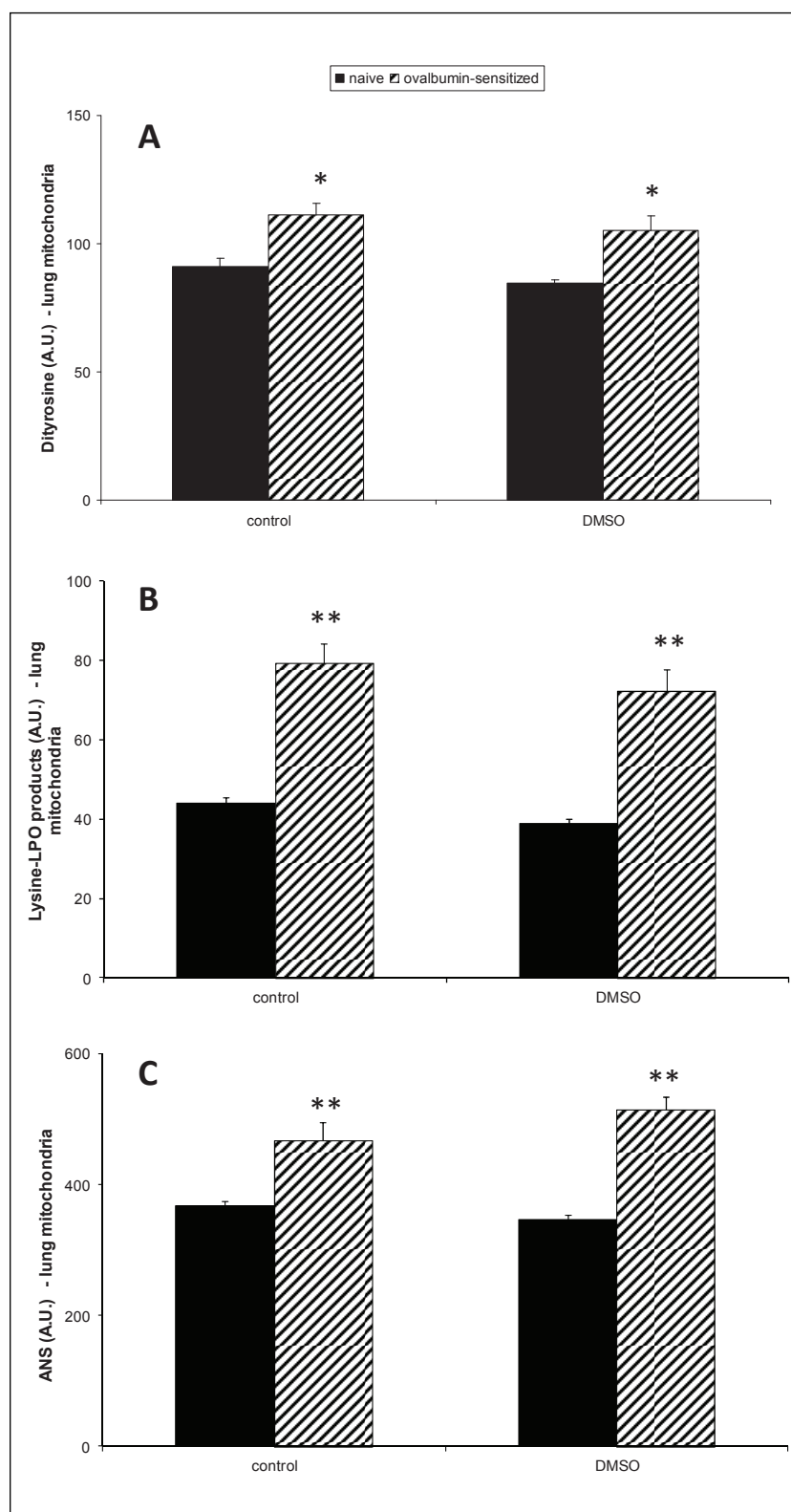


Fig. 3. Fluorescence of lysine-LPO end-products (A), dityrosine (B), and ANS (C) in the isolated lung mitochondria. For differences between OVA-control vs. naive-control and OVA-DMSO vs. naive-DMSO: $p<0.01$, $p<0.05$. DMSO-instilled animals vs. controls: $p>0.05$.

compared to naive animals. Increase in BAL cells correlated well with increased lipid peroxidation in the lung tissue, as proven by higher thiobarbituric acid-reactive substances in the lung homogenate, accompanied by an accumulation of conjugates of lipoperoxidation end-products with lysine. Additional analysis of mitochondrial fraction of the lung tissue has shown higher formation of dityrosines, lysine-lipoperoxidation products and change in 8-anilino-1-naphthalenesulfonic acid fluorescence in comparison with naive animals. Increase in lipid and protein oxidation was associated with a decrease in total antioxidant status in the lung homogenate and the plasma. However, in the mentioned markers, no differences between the controls and DMSO-instilled animals were observed.

Ovalbumin-sensitization in our study significantly increased eosinophils and slightly elevated neutrophils in the BAL fluid in both controls and DMSO-instilled animals. In allergic asthma, increased eosinophils may be found not only in the peripheral blood, but also in the BAL fluid, sputum and airway wall (1). Eosinophils are a rich source of granule basic proteins, but they generate prostacyclin, cysteinyl leukotrienes, reactive oxygen species, growth factors and a wide range of cytokines and chemokines, as well. Despite eosinophils produce lower amounts of cytokines and free radicals than the other leukocytes, they play an essential role in the immune response, airway hyperreactivity, airway remodelling and mucus production in

allergic asthma (19). On the other hand, neutrophils are potent producers of reactive oxygen species, however, their role as the secondary effector cells in "classical" allergic asthma of Th2-type, particularly mild-to-moderate asthma, is questionable (1). Nevertheless, neutrophilic inflammation was noted in patients with severe asthma and during acute exacerbations, as well as in patients treated with glucocorticoids (1, 20). Finding increased neutrophils in an absence of eosinophils suggests that the disease becomes more aggressive and chronic, representing non-eosinophilic asthma of Th1-type (1). More prominent increase in the eosinophils in our study is, definitively, in accordance with characteristics of the used model of allergic asthma.

After ovalbumin-sensitization, eosinophils and neutrophils rapidly migrate into the lungs, where they activate and produce a variety of biologically active substances including reactive oxygen species. In the higher concentrations, reactive oxygen species may damage lipids, proteins and nucleic acids. Several by-products of oxidation may be used as biochemical markers of oxidative processes in tissues. To evaluate a lipoperoxidation, generation of an end-product - malonyldialdehyde - was determined. Free malonyldialdehyde may react with free sulfhydryl (-SH) groups of cysteine or amino (-NH₂) groups of lysine and generate complexes reacting with thiobarbituric acid, creating thiobarbituric acid-reactive substances (TBARS). In this study, higher concentrations of TBARS have proven an overproduction of free radicals after ovalbumin-sensibilization

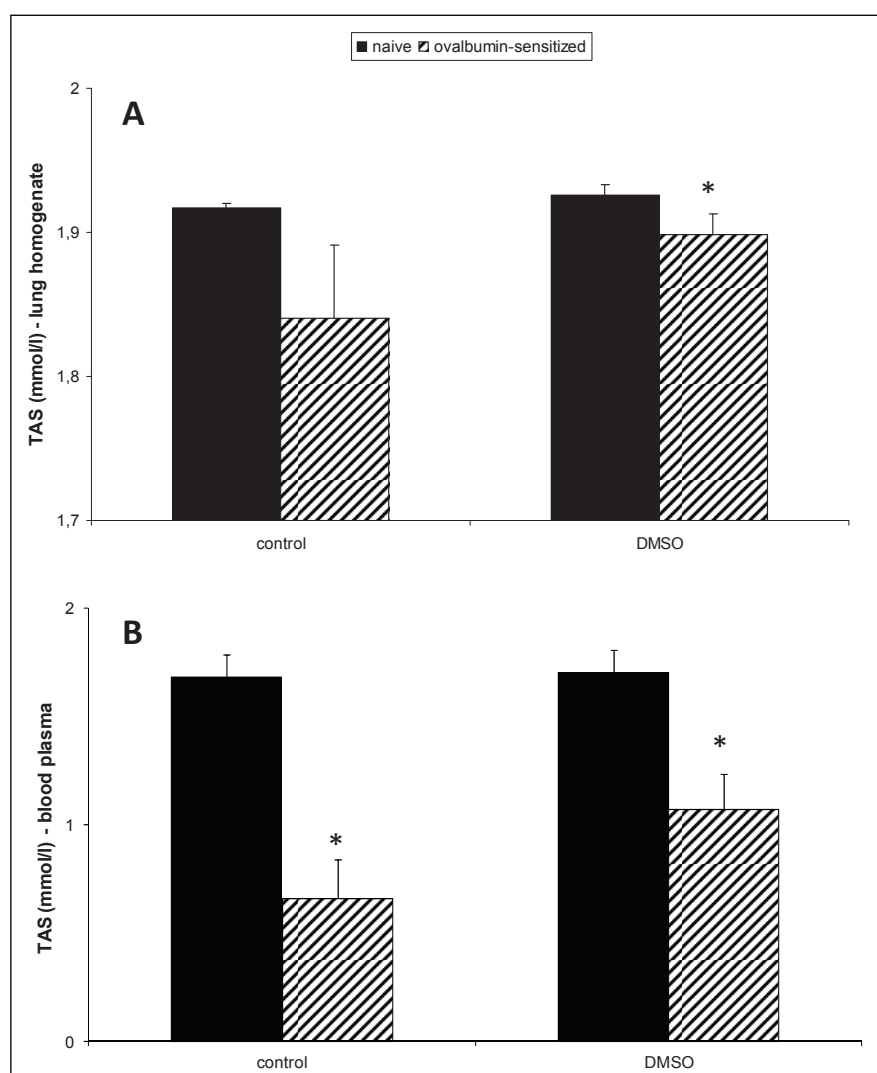


Fig. 4. TAS in the lung homogenate (A) and blood plasma (B). For differences between OVA-control vs. naive-control and OVA-DMSO vs. naive-DMSO: $p < 0.01$, $p < 0.05$. DMSO-instilled animals vs. controls: $p > 0.05$.

in guinea-pigs. Similarly, intensive oxidation stress characterized by increased TBARS and decreased amount of SH-groups was found in asthmatic children compared with healthy controls (21).

Protein oxidation by reactive oxygen species may lead to a modification of amino acid side chains and to a formation of new groups or to a formation of covalent protein-protein cross bonds. Aromatic amino acids (e.g. tyrosine) are easily modified by oxidation and products of these changes may be detected, such as dityrosine in our study. In addition, oxidation-induced conformational changes of a tertiary structure of proteins were determined using 8-anilino-1-naphthalenesulfonic acid (ANS), a fluorescent "hydrophobic probe" (18). Thus, increased concentrations of the mentioned markers in our study indicate significant lipid and protein oxidation in the lungs after OVA-sensitization in a guinea-pig model of allergic asthma.

Total antioxidant status (TAS) represents a cumulative action of all antioxidant systems in the plasma (22) and may thereby help to identify conditions affecting oxidative-antioxidative status *in vivo*. Contrary to a healthy individual with a balance between the production of free radicals and antioxidant capacity, oxidant-antioxidant imbalance is an important sign of asthma (23, 24). In our study, OVA-sensitization elevated generation of reactive oxygen species, resulting in higher by-products used as markers of oxidation stress, which negatively correlated with total antioxidant status in the plasma. Similarly, increased TBARS, decreased plasma total antioxidant capacity and total protein sulfhydryls were found in patients with asthma, particularly in acute exacerbations (23).

However, a decrease in antioxidants may be detected also at a local level. In our study, total antioxidant status was reduced also in the lungs of OVA-sensitized animals, but more pronounced decrease was observed in the plasma. In a recent study, malonyldialdehyde as an indicator of oxidation stress increased and glutathione as an indicator of antioxidant defense decreased in exhaled breath condensate of asthmatic children compared with healthy controls (24). In a mouse model of asthma, increased reactive oxygen species in the bronchial epithelium, reduced ratio of reduced-to-oxidized glutathione, elevated inflammatory cells in BAL fluid and airway hyperresponsiveness were found (4). Similarly, in a guinea-pig model of early asthmatic reaction, decreased lung levels of glutathione associated with increased airway reactivity were found (25). On the other hand, sputum glutathione levels of stable asthmatic patients did not differ significantly from healthy controls (26). Thus, determination of antioxidants in the blood may not necessarily reflect concentrations in the target tissues, where the oxidation stress is most intensive. In addition, accumulation of antioxidants in the selective tissues may not be apparent from plasma measurements. Therefore, it is better to combine assays for total antioxidant capacity in plasma with indices of oxidative damage to lipids, proteins and DNA providing complex information on the oxidant/antioxidant imbalance (27).

As several substances potentially beneficial in influencing inflammation and airway hyperresponsiveness are water-insoluble, dimethyl sulfoxide (DMSO) is often used as a solvent and vehicle for these drugs. However, DMSO itself may show some antioxidant (8, 28) and anti-inflammatory effects (9, 29). In our study, no clear differences in BAL cells and oxidation products between the saline-instilled and DMSO-instilled groups were observed suggesting that the administered dose and concentration of DMSO is too low to elicit any obvious antioxidative effects. This is in accordance with a finding, that *in vitro* incubation with DMSO may decrease TBARS, protein oxidation and production of hydroxyl radicals in the brain tissue, but the antioxidative effect is dependent on the concentration of DMSO (8).

Taken together, our study shows that ovalbumin-sensitization in guinea-pigs is associated with a powerful oxidative stress and reduction of an antioxidant status locally in the lungs, but also in the systemic circulation. DMSO, widely used as a solvent of drugs tested in asthma, had in a concentration and dosage used in our study no significant antioxidative effects when compared to saline-instilled animals.

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