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EVALUATION OF BLOOD ENZYME AND NON-ENZYME ANTIOXIDANT STATUS, IN GUINEA PIGS WITH BRONCHIAL ASTHMA AND TREATED WITH A NO DONOR

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Abstract. Attempts have made to characterize the antioxidant potential of the early phase of bronchial asthma. An experimental model trying to mimic allergic bronchial asthma was immunologically induced with ovalbumin (OA) in guinea pigs which were then treated with a NO donor (ammonium salt of N- nitrosophenyl-N-hydroxilamine) at a concentration of 0,1 mg/Kg (1/400 of DL50) for 2 weeks. The animals were killed afterwards, for assessing the presence of cellular oxidative stress and free radicals (FR). The changes in the activity of superoxide-dismutase (SOD), glutathione-peroxidase (GPx), reduced glutathione (GSH) and malon-dialdehide (MDA), were followed in the heparinized blood of guinea pigs series (5-6 animals in series): L1 = nebulized with OA; L2 = hypersensitized with OA; L3 = hypersensitized with OA and treated intraperitoneal for 2 weeks with NO donor; L4 = controls, healthy guinea pigs.

The final data have revealed: The increased activity of SOD at series L3 compared to series L1 (+120.79 %) and to series L2 (+69.95 %) becoming closer to the activity of the control series; the increased activity of GPx at series L3, compared to series L1 (+2.48 %), to series L2 (+24.77 %) and to series L4 (+8.64 %); the decreased level of GSH at series L3, compared to series L2 (-22.18 %) and to series L1 (-15.83 %), but with an increased level compared to series L4 (+ 36.85 %); the decreased concentration of MDA at series L3 compared to series L1 (-6.52 %), to series L2 (-17,64 %) and to series L4 (-9.40 %), all these proving a lower level of the lipid peroxides and damaging free radicals. The positive effect of the NO- donor, with the above specific concentration, is due to the stimulation of the synthesis of the oxidative stress biomarkers which have an important antioxidant rol.

Key words: nitric oxide (NO), superoxide-dismutase (SOD), glutathione-peroxidase (GPx), malon-dialdehide (MDA), S-nitroso-glutathione (S-NO-GSH), bronchial asthma, oxidative stress

Rezumat. S-a încercat o caracterizare a potențialului antioxidant al fazei de început al astmului bronşic, indus imunologic cu ovalbumină (OA), pe cobai, în paralel cu administrarea intraperitoneală timp de două săptămâni, a unui donor de NO (sarea de amoniu a N-nitrozofenil-N-hidroxilaminei), în concentrație de 0,1 mg/ Kg corp cobai (l/400 din DL50 care este de 110 mg). Cobaii au fost ținuți în condiții obişnuite de crescătorie, primind o alimentație adecvată. Experimentele s-au efectuat pe cobai de ambele sexe, în vîrstă de 2-3 luni, cu o greutate corporală de 400-500g, furnizați de crescătoria "S.C.GARIBO PREST" Tg. Mureş.

După sacrificare, s-a urmărit în sîngele total heparinizat, modificarea activităţii antioxidanţilor enzimatici superoxid-dismutaza (SOD), glutation-peroxidaza (GPx) şi a antioxidantului neenzimatic glutationul (GSH), în paralel cu nivelul dialdehidei malonice (MDA), ca o apreciere a prezenţei stres-ului oxidativ celular declanşat de lipoperoxidarea membranară datorată prezenţei radicalilor liberi (RL), prejudicianţi, induşi de astmul bronşic alergic, în faza de debut, la nivel sanguin.

Datele obţinute pe cele 4 loturi de cobai (5 - 6 animale/lot: L1 = cobai nebulizaţi (aerosolizaţi) cu OA; L2 = cobai hipersensibilizaţi cu OA; L3 = cobai hipersensibilizaţi cu OA şi injectaţi intraperitoneal cu donor de NO timp de două săptămîni; L4 = lot martor, cobai normali), au relevat creşterea activităţii SOD la L3 faţă de L1 (cu +120,79 %) şi L2 (cu +69,95 %), apropiindu-se de activitatea lotului martor; creşterea activităţii GPx la L3, faţă de L1 (cu +2,48 %), faţă de L2 (cu +24,77 %) şi faţă de L4 (cu +8,64 %); scăderea nivelului GSH la L3, faţă de L2 (cu –22,18 %) şi faţă de L1 (cu –15,83 %), dar cu o creştere faţă de L4 (cu +36,85 %); scăderea concentraţiei MDA la L3 faţă de L1 (cu –6,52 %), L2 (cu –17,64 %) şi faţă de L4 (cu –9,40 %), relevînd o diminuare a peroxizilor lipidici şi RL prejudicianţi, la acest lot.

Efectul benefic al donorului de NO, în concentraţia utilizată, se datorează stimulării sintezei biomarkerilor de stres oxidativ, cu rol antioxidant important.

Cuvinte cheie: oxid nitric (NO), superoxid-dismutaza (SOD), glutation-peroxidaza (GPx), dialdehida malonică (MDA), S-nitrozo-glutation (S-NO-GSH)

INTRODUCTION

NO, discovered in1987 by H.PALMER and S.MONCADA, is now considered an intra- and intercellular messenger with double role, beneficial in low concentration (physiologic dose = 80 ppm, DL_{50} = 110 mg), and harmful in high doses (over 300 μg), depending on the location of synthesis, produced quantity, and target cells in the environment (1). Nitric oxide has several roles at pulmonary level, yet not completely understood, because it is synthesized at cellular levels, such as: vascular endothelium, epithelium of the airways, proinflammatory cells, nerve fibres (2), and also by the activation of some different synthesis enzymes (cNOS and iNOS) (3).In literature, NO is considered an aggressor molecule in chronic inflammatory processes, but the therapeutic benefit may be obtained by iNOS inhibition (4-6). As the neurotransmitter of the broncho-dilator nerves in the airways, when produced in small amount by cNOS, it relaxes the smooth muscle fibres and modulates the movement of the cilia in the respiratory epithelium. As a pulmonary vasodilator, NO opposes to vasoconstriction in pulmonary hypoxia. In this case, it has a paracrine effect, relaxing the smooth muscle cells in the vascular wall via GMP_c/ protein kinase (1,2,7). In the pathogenesis of bronchial asthma there is an increase in local endogenous production of NO after the stimulation of iNOS isoforme, exerting its damaging action by proinflammatory and cytotoxic effects (3,8). NO produced in high concentration and for long time in asthma patients may cause a significant increase in the intracellular concentration of GMP_c, able to activate various biochemical AMP_c/ protein-kinase-dependent processes, causing a harmful cellular oxidative stress (10). NO activates soluble

guanylate cyclase, by bonding to its heme group.
Thus it initiates a series of changes in the tridimensional structure of the enzyme, as well as its activation and enhancement of GMP_c synthesis (8). In the last years some agonists were identified (9). They are released by the alveolar macrophages, causing an increase in the frequency of ciliary movement, which exerts its effect by a NO-dependent mechanism, NO release, via activation of cNOS and iNOS from epithelial cells. Also identified were a series of compounds of the nitrosovasodilators class (such as nitroglycerin, nitropruside), which inhibit the contractility of vascular smooth muscles, causing relaxation or reduction of the vascular tone. These agents, named "NO donors" act by releasing NO, with major beneficial consequences at cellular and blood level.

MATERIALS AND METHODS

An experimental model attempting to mimic allergic bronchial asthma in guinea pigs was designed. Asthma was immunologically induced with ovalbumin (OA) and a NO donor at a concentration of 0.1 mg / Kg body weight (1/400 of DL_{50}) was intraperitonealy administrated for 2 weeks (8,12). The experiments were made on guinea pigs of both sexes, provided by "S.C.GARIBO PREST", Tg.Mureş. They are 2-3 months old and 400 – 500 g weight. The guinea pigs were kept in good environmental conditions, receiving adequate food (carrots, barley, cabbage and bread with milk). The animals were divided into 4 groups: L1 = guinea pigs nebulized with OA before lavage and sacrifice (nebulized controls); L2 = guinea pigs hypersensitized with intraperitoneal OA and 2 weeks later, before lavage and sacrifice, nebulized with OA; L3 = guinea pigs hypersensitized with OA, and receiving daily, for 2 weeks, a NO donor; L4 = normal, healthy guinea pigs, control group. After the animals were sacrificed, we determined in the heparinized total blood, the changes in the activity of some antioxidants, considered to be oxidative stress markers: superoxide-dismutase (SOD), according MINAMI-YOSHIKAWA's method (11), glutathione-peroxidase (GPx) according FUKUZAWA's method (12), glutathione (GSH), according to J.SEDLAK's spectrophotometric method (13) and malon-dialdehide (MDA), according to PLACER's method (14). The hemoglobin (Hb) was determined using DRABKIN reagent. The obtained biochemical data were then statistically processed for calculating the standard deviation (X_M +/- SD) and statistical significance, P (15).

RESULTS AND DISCUSSION

Superoxide dismutase (SOD,E.C.1.15.1.1.), enzyme with a major antioxidative role, quickly decomposes or dismutes the superoxide anion radical ($\overline{O}_2^{\bullet}$), a toxic radical species of O_2 (16) to H_2O_2, however being inhibited by large concentrations of H_2O_2. SOD reacts not only by decomposing $\overline{O}_2^{\bullet}$ excess, it may also inhibit singlet oxygen ($^1O_2$)

and indirectly, the peroxidation of AGPN from membrane phospholipids (16).

In the heparinized total blood of guinea pigs with bronchial asthma immunologically induced with OA, we found a decreased SOD concentration in groups L1 (-64.34 %) and L2 (-53.67 %) as compared to the control group L4, healthy animals, similar, but more significant than at pulmonary level (17) (table 1). This is probably due to the type of cells with different oxidative potential, as well as to SOD susceptibility to oxidative stress, the inactivation being sometimes irreversible if H_2O_2 concentration is higher than 10 μM (18).

SOD activity in group L3 was less decreased (-21.25 %) compared to group L4, but increased as compared to groups L2 (+63.95 %) and L1 (+120.79 %). Thus, it appears that NO donor has a beneficial action on the enzyme, probably due to a lower concentration of H_2O_2 or other harmful radical species occurring in blood under the above mentioned circumstances.

Table 1. Variation of SOD and GPx activity in total blood of guinea pigs with immunologically-induced bronchial asthma, treated with NO donor

Groups of guinea pigs; No. guinea pigs/group	SOD activity ($Ux10^{-2}$/min./g Hb)	Stimulation (+) or inhibition (-) %, compared to L_x (100 %)	GPx activity (μmols/g Hb)	Stimulation (+) or inhibition (-) %, compared to L_x (100 %)
L1. n= 5	4.28 +/-0.70	- 64.34 (L4)	4.03 +/- 1.05	+ 6.05 (L4)
L2. n= 5	5.56 +/-0.92 P< 0,001	- 53.67 (L4) + 29.90 (L1)	3.31 +/- 0.97	- 12.9 (L4) - 17.87 (L1)
L3. n = 4	9.45 +/- 0.55 P < 0.0015	- 21.25 (L4) +69.95 (L2) +120.79 (L1)	4.13 +/- 2.36	+ 8.64 (L4) +24.77 (L2) +2.48 (L1)
L4. n = 6	12.00 +/- 2.3	100 %	3.80 +/-0.29	100 %

Antioxidative enzyme **glutathione peroxidase (GPx, E.C. 1.11.1.9.)** has a major role in the regulation of intracellular H_2O_2 concentration (16,18) and protection of target cells against the presence of damaging lipid peroxides (LP). Some authors (19) believe that GPx has a much more important role than catalase (CAT) in the protection of target cells against extracellular H_20_2 flow, probably due to its greater affinity for this compound.

The activity of the enzyme in whole blood (table 1) follows the same pattern in both L1 and L2 groups, corresponding to the results obtained in the pulmonary homogenate (17). Thus, in group L1 we found an increase (+6.05%) as compared to group L4, while in group L2 there was a decrease (-17.87%) compared to groups L1 and to L4 (-12.9%).

By injecting the NO donor, the enzyme activity in group L3 significantly increased as compared to

group L1 (+2.48%), L2 (+24.77%) and L4 (+8.64%), unlike the values obtained at pulmonary level (12). The stimulating tendency in group L3 may be a consequence of the extracellular release of proinflammatory cytokines, leading both to a stimulation of the synthesis (induction) of this enzyme at blood level, and to a lower level of lipid peroxides. This phenomenon is beneficial because the presence of LP in high concentration acts at blood level as an oxidative stress amplifier.

The major functions of **non-enzymatic GSH** antioxidant and its involvement in numerous disorders and types of cellular oxidative stress accounts for its inclusion in the category "oxidative stress biomarkers" traced at blood level (20,21), in the above mentioned circumstances.

Table 2 Variation of GSH and MDA activity in total blood of guinea pigs with immunologically-induced bronchial asthma, treated with NO donor

Groups of guinea pigs. No. guinea pigs/group	GSH activity (γ/g Hb)	Stimulation (+) or inhibition (-) %, compared to L_x (100 %)	MDA activity (μmols/g Hb)	Stimulation (+) or inhibition (-) %, compared to L_x (100 %)
L1. n = 6	1018+/-102.7 P < 0,0015	+ 62.59 (L4)	11.36+/-2.17	- 3.07 (L4); -11.94 (L2) + 6.94 (L3)
L2. n = 6	1101.3+/-82.13 P<0,001	+ 75.84 (L4) + 8.15 (L1)	12.90+/-1.05	+10.07 (L4) +13.56 (L1) +17.68 (L3)
L3. n = 5	856.86 +/- 178.72	+ 36.85 (L4) - 15.83 (L1) - 22.18 (L2)	10.67+/-2.01	- 9.40 (L4) - 6.52 (L1) - 17.64 (L2)
L4. n = 5	626.12+/-89.77	100 %	11.72+/-2.08	100 %

The obtained results (table 2) demonstrate that the level of GSH undergoes a statistically increase in groups L1 (+62.59 %) and L2 (+75.84 %), both compared to L4, contrary to the level in the pulmonary homogenate (17). In group L3, treated with NO donor, GSH concentration was higher (+36.85 %) than in group L4, but lower than in both L1 (-15.83 %) and to L2 (-22.18 %).

Our data are in agreement with other data in the literature (20) which prove that NO reacts with thiols to form a bioactive and stable intermediary, called S-NO-glutathione (3,14), less reactive with O_2 and $\overline{O}_2^{\bullet}$, thus the probability of toxic peroxynitrite ($ONOO^-$) (22) formation being reduced. S-NO-GSH may protect the cells against NO-dependent cytotoxicity (23).

Some authors also consider the possibility that the extrahepatic tissues release GSH when its level in the liver and plasma decreases. The elevated GSH level in groups L1 and L2 (erythrocyte GSH is synthesized in about 10 min.), might be explained by its increased synthesis and enhanced

liver export to blood, together with the inability of the lungs to capture it, although essential for annihilating the harmful free radicals (FR).

Lipid peroxidation at membrane level is one of the major events inducing the development of some inflammatory processes and enhancing vascular permeability. In this respect we followed the changes in malonat dialdehyde (MDA) concentration, a biomarker for lipid peroxidation (table 2), and we found that there is a statistically significant increase in group L2 (+13.56%) as compared to group L1, L3 (+17.68%), and L4 (+10,07%), demonstrating that most cell and basophil degranulation occur simultaneously with the initiation of lipoperoxidation oxidative process.

In group L3 , MDA concentration is lower than those measured in groups L1 (-6.52%), L2 (-17.64%), and L4 (-9.40 %), thus ascertaining a significant decrease in FR and lipid peroxidation. The lowering of MDA level in this group does not mean that lipid peroxidation occurs no more, the production of high amounts of conjugate dienes, found by V. Mohsenim (24) in the pacients exposed to NO_2 being likely. Other researchers (25) found that MDA was not present in the pulmonary fluid of the subjects exposed to NO_2, but was detected in those who inhaled air. NO, produced in high amounts in asthma patients (8,26), is also an lipoperoxidation inhibitor.

This MDA decrease in group L3 also occurs either due to less LP used as a substrate by GPx and GSH, or MDA involvement, soon after its formation, in certain reactions with the $–NH_2$ groups of the proteins (especially from xanthine oxidase, without affecting the –SH groups), resulting in the development of some Schiff bases (8,27,28).

According to these findings, we may say that the beneficial effect of the new NO donor at blood level, at the above mentioned concentration, is due to the stimulation of the synthesis of some enzymatic and non-enzymatic markers of oxidative stress with major antioxidant role in the early stage of bronchial asthma, immunologically induced by OA in guinea pigs.

CONCLUSIONS

1. A NO donor (ammonium salt of N-nitrosophenyl-hydroxylamine), was tested on an experimental model of immune inflammation induced in guinea pigs, with ovalbumin (OA), with the goal of understanding the mechanism of action of this donor, during the initial stage of bronchial asthma.
2. In view of characterizing the antioxidant potential of the immediate stage of asthma, we determined in total blood, the changes in the activity of some enzyme (SOD, GPx) and non-enzyme antioxidants (GSH) and malon-dialdehide (MDA), as an assessment of the presence of oxidative stress at blood level in the above mentioned experimental conditions.
3. By injecting the NO donor (0.1 mg/Kg guinea pig), for 2 weeks,

the biochemical parameters showed: the increased activity of SOD at series L3, compared to series L1 and L2, becoming closer to the activity of the control series L4; the increased activity of GPx at series L3, compared to series L1, L2 and L4; the decreased level of GSH at series L3, compared to series L2 and L1, but with an increased level compared to series L4.

4. The decreased concentration of MDA at series L3, compared to series L1, L2 and L4, proving a lower level of the lipid peroxides and damaging free radicals, at series L3, in total blood of guinea pigs, during the initial stage of bronchial asthma.
5. The positive effect of the NO donor, with the above specific concentration, is due to the stimulation of the synthesis of oxidative stress biomarkers, which have an important antioxidant role, during the initial stage of bronchial asthma.

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