Blood oxygen-carrying function during the oxidative stress induced by lipopolysaccharide with a modification of the L-arginine-NO pathway

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Abstract

Purpose: Our aim was to study the blood oxygen-carrying function during the oxidative stress with a modification of the L-arginine-NO pathway.

Material and methods: Oxidative stress was induced by intravenous administration of *Escherichia coli* lipopolysaccharide (LPS) to rabbits. To modify the L-arginine-NO pathway, animals were administered with N^G-nitro-L-arginine methyl ester intravenously 60 min after the LPS. Mixed venous blood was sampled for evaluation of blood oxygen transport before and at 120 and 240 min after the LPS administration; tissue samples (heart, lung, liver, kidney and muscle) were also prepared. The following parameters were measured hemoglobin-oxygen affinity, concentrations of conjugated dienes, Schiff bases, α-tocopherol and activity of catalase.

Results: During the NO synthase inhibition the oxidative stress was characterized by a shift of hemoglobin oxygen dissociation curve rightwards, more prominent activation of lipid peroxidation and decreased tissue levels of antioxidant defense factors.

Conclusions: The inhibition of NO generation induces a shift of prooxidant-antioxidant balance – obviously, not only due its potentially high levels and reactivity with the various target molecules (with a development of oxidative stress), but also because of the lower contribution of other factors including the hemoglobin-oxygen affinity change into the body antioxidant potential.

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Introduction

Under the tissue steady-state conditions, the excessive oxidant generation is counterbalanced by enzymatic and non-enzymatic antioxidants inside and outside the cells; thus the some optimal level of prooxidant-antioxidant balance is created [1]. If this balance is changed due to the excessive free radical production and/or antioxidant system damage, the so-called oxidative stress appears. Under this condition the excessive free radical generation and non-specific tissue impairment occur without a control by antioxidant mechanisms [2]. Experimental evidence suggests that reactive oxygen species may be important mediators of cellular injury during endotoxemia (induced by LPS), either as a result of macromolecular damage or by interfering with extracellular and intracellular regulatory processes [3].

Nitric oxide (NO) is thought to play a key role in the pathogenesis of sepsis. Bacterial endotoxin induces the release of many mediators, including NO, responsible for a late-phase hypotension, vasoplegy, acidosis, hypoxia and multiorgan failure [4,5]. NO is an important participant of this complex system of prooxidant-antioxidant balance. NO is a free radical capable both to ameliorate the oxidative injury (as a chain-terminating radical scavenger) and to be the source of reactive nitrogen species [6]. NO has the complex relationships with the free oxygen radicals – both interaction (NO can react with $O_2^{\bullet-}$ to generate a potent oxidant peroxynitrite) and competition (with a development of oxidative or nitrosative stress in the biological compartment) [7]. In a recent time the interactions between NO and hemoglobin (Hb) were extensively investigated. Three main NO-derivatives of Hb are known: methemoglobin, nitrosylhemoglobin ($HbFe^{2+}NO$), and S-nitrosohemoglobin (SNO-Hb) [8-10]. In a molecule of $HbFe^{2+}NO$ the NO moiety is a ligand to ferrous heme, and SNO-Hb is a result of NO interaction with

cysteine (93) of β-globin chain [11,12]. The presence of different hemoglobin-NO adducts can differently influence on the whole blood hemoglobin-oxygen affinity (HOA). Methemoglobin and SNO-Hb raise the HOA and $HbFe^{2+}NO$ decreases it. Such modulation of the blood oxygen-binding properties may be important for gas exchange and tissue oxygenation [13,14]. Our aim was to study the blood oxygen-carrying function during the oxidative stress with a modification of the L-arginine-NO pathway.

Material and methods

Animals

The adult male Chinchilla rabbits (n=21; body weight 2.5-3.1 kg) were kept for 2 weeks in a constant-climate environment with respect to temperature, humidity and daylight cycle. Animals were fed on a laboratory diet with water and food ad libitum until use and fasted overnight with free access to water before the operation. Operation procedures were performed between 8.00 and 12.00 to avoid the chronobiological variations. All the experimental procedures described in this paper are in accordance with the Guiding Principles for the Care and Use of Animals accepted by the Ethical Committee of Grodno Medical University.

Procedure

Animals were anesthetized with pentobarbital sodium (50 mg/kg). Sham-operated rabbits (1st group) received 1.0 mL saline intravenously (n=5) served as a control for mixed venous blood and tissue sampling. In rabbits of the 2nd group (n=9) the oxidative stress was induced by intravenous administration of 500 μg/kg *Escherichia coli* lipopolysaccharide (LPS) from Sigma Chemical. Animals of the 3rd group (n=7) intravenously received NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) from Sigma Chemical, 7.5 mg/kg, 60 min after the LPS. The catheters for mixed venous blood sampling were inserted into the right atrium through the external jugular vein. Blood was collected in heparinized syringes. Such sampling for estimation of the blood oxygen transport was performed before and at 120 and 240 min after the LPS administration. Tissue sampling (heart, lung, liver, kidney and muscle) was performed after 240 min of oxidative stress; tissues were washed with a cold phosphate-buffered saline to remove the blood traces, and then the samples were immediately frozen in liquid nitrogen and stored until analysis.

Lipid peroxidation products

Conjugated diene content was determined by the fluorescence intensity of UV absorption at 232-234 nm, characteristic for the conjugated diene structures [15]. Schiff base level was evaluated by the fluorescence intensity of chloroform extract at excitation and emission wavelengths of 344 and 440 nm, respectively [15] with a spectrofluorimeter "F-4010" (Hitachi).

Antioxidant system

α-tocopherol content was measured by the fluorescence intensity of heptane extract at excitation and emission wavelengths of 292 and 325 nm, respectively [16] with a spectrofluorimeter "F-4010" (Hitachi), using α-tocopherol (Sigma) as a reference. Catalase activity in the biological materials was estimated by the decrease of hydrogen peroxide capable to form a stable stained complex with molybdenum salts, with a spectrophotometer "СФ-46" at 410 nm [16].

Blood oxygen-carrying function

pO_2 were measured with micro gas analyzer ABL-330 (Radiometer) at 37°C with the following correction to actual temperature value. HOA was evaluated by p50 (blood pO_2 at its 50% oxygen saturation) determined by the "mixing" method at 37°C, pH 7.4 and pCO_2 40 mm Hg ($p50_{stand}$) [17]. p50 at actual pH, pCO_2 and temperature ($p50_{act}$) was calculated from $p50_{stand}$ with Severinghaus' formulas [18] using the temperature coefficient of 0.024. Oxygen dissociation curves of Hb (ODCs) were calculated with Hill's equation using n=2.8. The amounts of Hb and methemoglobin were determined spectrophotometrically.

Measurement of plasma NO_3^-/NO_2^-

Plasma samples (50 μl) were deproteinized by incubation with 140 μl of deionized H_2O and 10 μl of 30% $ZnSO_4$ at room temperature for 15 min. Samples were then centrifuged at 2000 g for 10 min. Nitrate was converted to nitrite using cadmium beads, and nitrite was measured spectrophotometrically [19].

Statistical analysis

The data were statistically evaluated by Student's t-test with a significance level of $p<0.05$. The results are presented as mean ± standard error of mean (SE). The analyses and graphs were performed using computer software packages.

Results

LPS administration resulted into the prominent activation of lipid peroxidation processes, with a creation of oxidative stress. Tissue lipid peroxidation activity increased after LPS administration – *Fig. 1* (conjugated dienes in heart, lung, liver, kidney and muscle: by 38.8, 39.2, 48.9, 16.4 and 20.0%, respectively; Schiff bases: by 11.7, 60.1, 309.2, 69.5 and 60.7%, respectively; all $p<0.05$). The largest rises in conjugated diene and Schiff base content under oxidative stress (LPS + L-NAME) comparing with only LPS injection were noted in lungs (16.2 and 14.7%, respectively; both $p<0.05$) and liver (54.3 and 20.6%, respectively; both $p<0.05$). This indicates a higher activity of the free radical lipid oxidation during NO synthase inhibition.

The lowering of the antioxidant defense factors was observed in tissues. Catalase activity and α-tocopherol content in the lung of rabbits with oxidative stress (LPS) were significantly lower than in control – by 24.6 and 18.3%, respectively (all $p<0.05$); in liver the catalase activity decreased only by 6.9% ($p<0.05$). Under NO synthase inhibition such fall of antioxidant defense factors comparing with LPS group was even more significant in all tissues tested (*Fig. 2*).

The content of NO utilization products (NO_3^-/NO_2^-) after LPS administration was larger than at baseline level; and

Figure 1. **Indices of lipid peroxidation in rabbit tissues under the oxidative stress induced by LPS combined with administration of N^G-nitro-L-arginine methyl ester (L-NAME): conjugated dienes (A), Schiff bases (B). The values are means ±SE. * – significant difference from the control group ($p<0.05$); # – significant difference from the group of rabbits received LPS ($p<0.05$)**

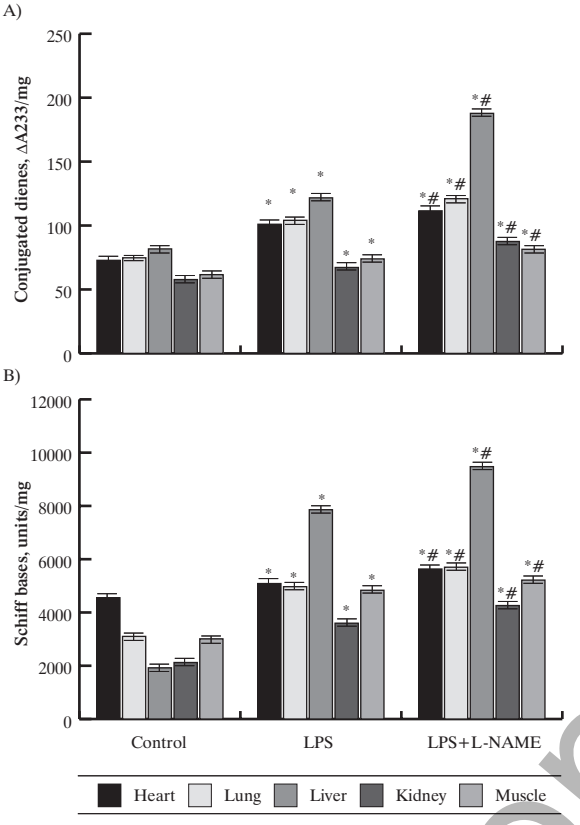
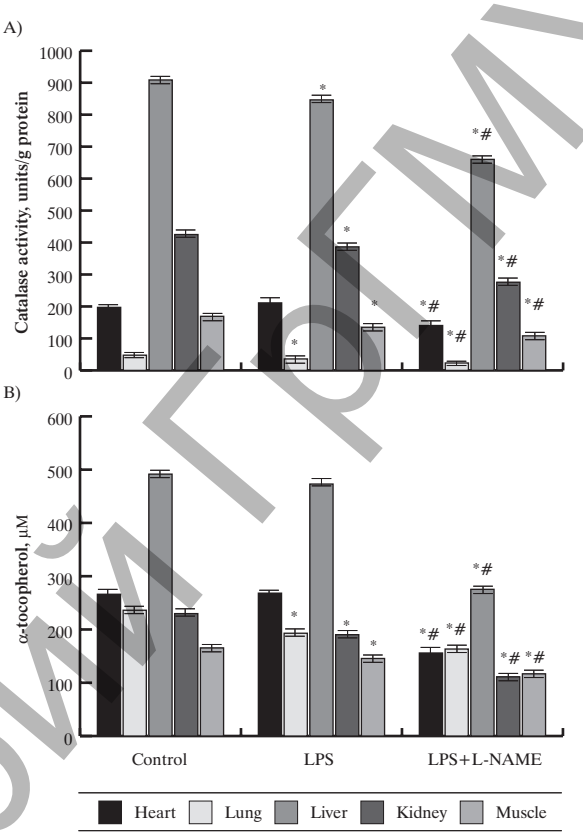


Figure 2. **The changes in catalase activity (A) and α-tocopherol content (B) in rabbit tissues under the oxidative stress induced by LPS combined with administration of N^G-nitro-L-arginine methyl ester (L-NAME). The values are means ±SE. * – significant difference from the control group ($p<0.05$); # – significant difference from the group of rabbits received LPS ($p<0.05$)**



administration of L-NAME after LPS increased it at 120 min (less than after LPS only) and at 240 min (*Tab. 1*).

The blood oxygen-binding properties considerably changed after the endotoxin administration (*Tab. 1*). Oxidative stress was accompanied by a development of hypoxia, even more marked during the NO synthase inhibition. The value of $p50_{stand}$ decreased at 120 and 240 min by 9.7 ($p>0.05$) and 10.6% ($p<0.05$), respectively. Meanwhile, $p50_{act}$ rose at 240 min after LPS by 9.3% ($p<0.05$) because of the changes in pH, pCO_2 and body temperature. After the injection of L-NAME the values of $p50_{act}$ increased by 31.3 and 29.5% (both $p<0.001$) after 120 and 240 min of oxidative stress, respectively, reflecting the more prominent shift of actual ODCs rightwards (*Fig. 3*).

Discussion

One can see that oxidative stress combined with NO synthase inhibition was characterized by the actual ODC shift rightwards, more marked lipid peroxidation activation and lowering of antioxidant defense factors in blood and tissues. Hb is an allosterically regulated protein and therefore has many binding sites capable to form the reversible non-covalent bonds with a primary ligands that can result in quaternary conformational changes and their modulation by the secondary effectors [13]. In our investigations the ODC position is dictated by effects of pH, pCO_2 and other factors; but one should take in account also NO and its interactions with Hb. NO is considered as the ligand determining the Hb oxygen-binding properties [14].

Intraerythrocyte interaction between NO and Hb is important for regulation of the both molecules *in vivo*. The red cell properties do not limit such interaction under the physiologic conditions [20]. In arterial blood the reaction between NO and oxyhemoglobin produces nitrate and methemoglobin, and in venous blood $HbFe^{2+}NO$ is generated; under high pO_2 it can be oxidated to met-Hb and NO_3^- [21,22]. The NO-binding site at β-globin chain was also found; such binding results in SNO-Hb [11]. The value of p50 for extracellular SNO-Hb is less than 10 mm Hg [23], and p50 for $HbFe^{2+}NO$ is 39.6±1.5 mm Hg [24]. In our experiments the lowest ODC shift leftwards was observed in animals received L-arginine and exposed to hypothermia [1].

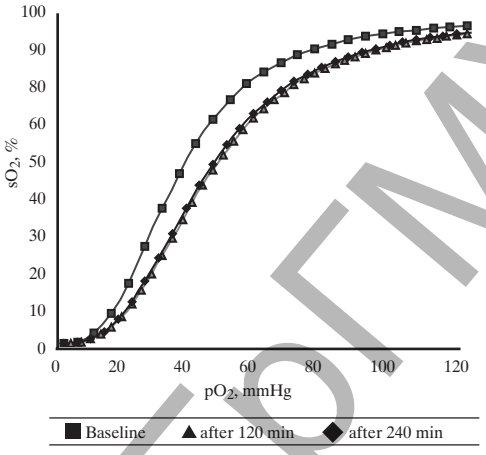
NO can affect tissue oxygenation through its influence on HOA and blood flow regulation. Simultaneously the mechanisms of O_2 transport (including the blood oxygen-binding properties) can modify the activity of the L-arginine-NO pathway. NO can modify HOA through the intraerythrocytic regulatory

Table 1. **The changes NO_3^-/NO_2^- and indices of blood oxygen-carrying function in rabbits during the oxidative stress induced by LPS combined with administration of N^G-nitro-L-arginine methyl ester (L-NAME)**

Index	Baseline	After	
		120 min	240 min
oxidative stress (LPS)			
n	9	9	9
NO_3^-/NO_2^-, μM	6.11±0.36	11.41±0.52*	14.27±0.44*#
$p50_{act}$, mm Hg	35.5±0.77	37.1±1.48	38.8±1.13*
$p50_{stand}$, mm Hg	31.0±1.14	28.0±0.53*	27.7±0.91*
Hb, g/dL	9.72±0.20	9.33±0.20	9.30±0.21
pO_2, mm Hg	33.78±2.23	30.94±2.50	28.12±1.34*
methemoglobin, %	0.28±0.09	0.88±0.11*	0.72±0.07*
pH, units	7.322±0.015	7.191±0.035*	7.137±0.037*
pCO_2, mm Hg	47.56±3.12	38.21±2.47*	43.39±1.33
HCO_3^-, mM	24.11±1.08	14.20±1.03*	14.28±0.94*
TCO_2, mM	25.43±1.09	15.37±1.04*	15.51±0.89*
ABE, mM	-1.97±1.37	-12.83±1.43*	-13.71±1.47*
SBC, mM	22.46±1.11	13.81±1.01*	13.14±1.22*
oxidative stress (LPS+L-NAME)			
n	8	8	7
NO_3^-/NO_2^-, μM	6.11±0.26	8.95±0.52*	13.83±0.67*#
$p50_{act}$, mm Hg	33.9±0.95	44.5±2.14*	43.9±1.32*
$p50_{stand}$, mm Hg	31.4±0.74	31.3±0.90	29.8±0.74
Hb, g/dL	10.25±0.25	10.05±0.20	9.99±0.27
pO_2, mm Hg	31.60±2.01	26.15±2.21	20.89±3.04*
methemoglobin, %	0.28±0.08	0.56±0.07*	1.07±0.10*#
pH, units	7.353±0.020	7.103±0.040*	7.029±0.023*
pCO_2, mm Hg	48.65±3.87	47.21±3.36	61.54±2.80*#
HCO_3^-, mM	26.63±1.80	14.27±1.95*	14.75±1.73*
TCO_2, mM	28.11±1.85	15.53±1.93*	16.37±1.59*
ABE, mM	0.98±1.73	-14.98±2.69*	-16.46±1.90*
SBC, mM	24.13±1.65	12.88±2.11*	9.06±1.69*

Note: Data are expressed as mean ±SE.
Significant difference ($p<0.05$) from the baseline level (*) and after the 120 min of received LPS (#)

Figure 3. **Actual oxyhemoglobin dissociation curves for mixed venous blood in rabbits during the oxidative stress induced by LPS combined with administration of N^G-nitro-L-arginine methyl ester**



mechanisms, oxygen-dependent nature of NO production, generation of different NO-Hb derivatives [14]. The concentrations of NO and its derivatives in the microcirculatory part of vascular bed must be much higher than in larger vessels (100-fold and more). NO fraction directly interacting with Hb must also be higher. At the microcirculatory level this may be very important for the change of oxyhemoglobin binding and ultimately for the tissue oxygenation. NO can react with $O_2^{\cdot-}$ to generate a potent oxidant peroxynitrite that can modify the Hb properties [6,7]. Hb may also defend against peroxynitrite, thereby functioning as an intracellular antioxidant. This may also be important for the modification of Hb function and its involvement in formation of O_2 flux to tissues and in maintenance of the body prooxidant-antioxidant balance [1].

Oxidative stress may be considered as the defect of aerobic metabolism – the stochastic process of a free radical production and non-specific tissue damage without the regulation by antioxidant defense mechanisms [2]. Interactions between NO and free oxygen radicals creates the definite balance ($O_2^{\cdot-}$ scavenger system competes with NO for the $ONOO^-$ generation) resulting in the oxidative stress development in a biological object [7]. The HOA change can regulate the oxygen flux to tissues according to requirements, thus preventing its excessive use for the free radical oxidation; therefore, HOA may be considered as one of the factors participating in maintenance of body prooxidant-antioxidant balance. During the oxidative stress the HOA changes mediated by NO-dependent mechanisms (in first turn, endothelial) can affect the oxygen flux to tissues and body prooxidant-antioxidant balance as a whole. Inhibition of NO synthesis induces a shift of this balance – obviously, not only because of potentially high NO levels and reactions with the diverse target molecules, but also due to the lower contribution of other factors including HOA in the antioxidant defense of body. These data support the notion that HOA may alter tissue oxygen supply and may be involved in the pathogenesis of oxidative stress induced by administration of LPS.

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