Role of Free Nitrogen and Oxygen Radicals in the Pathogenesis of Lipopolysaccharide-Induced Endotoxemia

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We studied molecular mechanisms of changes in oxidative metabolism under conditions of experimental lipopolysaccharide-induced endotoxemia. Generation of reactive nitrogen and oxygen species in mice increased 18 h after treatment with lipopolysaccharide. These changes contributed to inactivation of enzymes and enzyme complexes (ribonucleotide reductase, NADH-ubiquinone oxidoreductase, and cytochrome *c* oxidase), dysfunction of the mitochondrial electron transport chain, and development of oxidative stress. Plaferon LB protected mice from the toxic effect of lipopolysaccharide.

Key Words: *nitric oxide; superoxide radical; oxidative stress; lipopolysaccharide; Plaferon LB*

Pathogenicity of gram-negative bacteria is determined by the presence of cell membrane component lipopolysaccharide (LPS) [9]. LPS activates transcription factors NFκB, NF-interleukin-6, and MAP kinase cascade (P38, JNK, *etc*.) via the TLR-4-CD14 receptor complex [7,10]. They increase expression of antiinflammatory cytokines, adhesion molecules, oxygenases, inducible nitric oxide synthase (iNOS), and other genes [9]. These molecules play a role in the development of uncontrolled inflammatory reactions mediated by the mechanisms of nonspecific protection (*e.g.*, macrophages and neutrophils). These cells produce considerable amounts of reactive oxygen (ROS) and nitrogen species (NO, ONOO, O_2^-, •OH, and H_2O_2), which contributes to oxygenic and nitrogenic stress. It manifests in sharp changes in activity of extracellular and intracellular antiradical enzymes, function of the electron transport chain, oxidative phosphorylation, and oxygen supply to tissues, which determine the development of metabolic disturbances. Treatment for endotoxemia of different genesis should include drugs modulating oxidative processes and redox status in the organism. Plaferon LB (PLB) was synthesized at the Institute of Medical Biotechnology (Georgian Academy of Sciences). This drug is a mixture of endogenous bioactive substances from the amniotic membrane of human placenta. The study of the mechanisms of pharmacological activity of PLB showed that this drug exhibits antioxidant and immunomodulatory properties.

Here we studied the molecular mechanisms of changes in oxidative metabolism under conditions of LPS-induced endotoxemia and evaluated the possibility of its correction with PLB.

MATERIALS AND METHODS

Experiments were performed on adult male ICR mice weighing 100±20 g. Control animals (n=20) were treated with physiological saline. For mode-

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ling experimental LPS-induced endotoxemia, group 1 mice (n=20) received intraperitoneal injection of 0.25 mg/kg LPS (*E. coli* serotype 0.55/85, Sigma). Group 2 animals (n=20) were injected with LPS and intraperitoneally received PLB in a dose of 2.5 mg/kg. The mice were decapitated under general anesthesia (40 mg/kg sodium ethaminal) 18 h after injection. The period of exposure to LPS *in vivo* and *in vitro* was selected taking into account the results of our previous electron paramagnetic resonance (EPR) studies. We showed that the intensity of LPS-induced NO synthesis by cultured mouse splenocytes and human peripheral blood monocytes reaches maximum 18 h after the start of incubation with LPS.

For obtaining splenocyte culture the spleen tissue from intact mice (n=20) was homogenized and the homogenate was centrifuged at 1000g for 10 min. The precipitate was diluted with medium 199 to a concentration of 1.5×10^6 cells/ml. The medium contained 10% fetal bovine serum, 1% solution of penicillin and streptomycin, and 0.03% glutamine. Splenocytes were divided into 3 groups. Physiological saline was added to group 1 cells (10 samples). LPS in a dose of 100 mg/ml was added to group 2 cells (10 samples). Group 3 splenocytes were treated with LPS and PLB in a dose of 100 ng/ml. The cells were incubated at 37°C for 18 h under constant supply of 10% CO_2.

EPR studies were performed on a RE 1307 radiospectrometer (microwave radiation frequency 9.77 GHz) equipped with a computer system of signal accumulation. EPR signals of oxidized ceruloplasmin (g=2.05), Fe^{3+}-transferrin (g=4.3), iron ions (Fe^{2+}, g=2.37, ΔH=350 G), and methemoglobin (g=6.0) were analyzed in the blood of animals [4]. The spectra were recorded in a Dewar quartz vessel (modulation amplitude 0.16 mT, microwave power 100 mW, liquid nitrogen temperature -196°C).

The amount of ROS in the blood was measured using spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, Sigma). The blood was incubated with DMPO in a concentration of 50 mM at room temperature for 3 min [15]. EPR spectra of ROS were recorded at room temperature and microwave power of 20 mW.

The concentration of free NO in the blood, spleen, and cell suspension from rats was estimated using a spin trap sodium diethyldithiocarbamate (DETC, Sigma). DETC (500 mg/kg) and Fe^{2+}-citrate (50 mg $FeSO_4\times6H_2O$ and 250 mg sodium citrate per kg body weight) in a dose of 50 mg/kg were infused into mouse caudal vein 10 min before decapitation [6]. EPR spectra of NO-Fe^{2+}-$(DETC)_2$ complexes were recorded at a temperature of liquid nitrogen and microwave power of 20 mW [1,12].

We measured the concentrations of free NO and ROS in the culture of splenocytes. Spin traps for free radicals of nitrogen and oxygen (50 mM DMPO and 10 mM DETC) were added to the cell culture (0.5 ml, 1.5×10^6 splenocytes/ml) by the end of incubation. The samples were centrifuged at 1000g for 10 min.

Biochemical studies were performed on a SF-46 spectrophotometer (LOMO). Catalase activity in blood plasma was measured by the method of Albi with modifications [2]. This method is based on the ability of H_2O_2 to form a stable colored complex with molybdenum salts. Optical density of the solution was measured at 410 nm. The amount of catalase degrading 1 μmol H_2O_2 over 1 min was taken as a unit of enzyme activity.

Superoxide dismutase (SOD) activity was measured in erythrocyte mass [3]. The erythrocyte mass was washed 2 times with physiological saline and hemolyzed (0.5 ml) with 0.5 Tris-HCl (pH 7.4). The hemolysate was mixed with 0.25 ml 96% ethanol and 0.15 ml chloroform to precipitate hemoglobin. The mixture was centrifuged at 5000g. For measuring SOD activity 0.02 ml supernatant was put to 3 ml incubation medium containing 0.41 mM nitroblue tetrazolium, 0.33 mM ethylenediaminetetraacetic acid, and 0.01 mM N-methylphenazonium methosulfate. Optical density of the solution was measured at 540 nm. NADH (0.1 ml, 0.8 mM) was added to a cuvette of the spectrophotometer. The mixture was agitated and kept in darkness for 10 min. Optical density was measured repeatedly. The reaction was verified by the difference between the first and second spectrophotometric values. The amount of SOD inhibiting the reduction of nitroblue tetrazolium by 50% was taken as a unit of enzyme activity. SOD activity was expressed in arb. units per ml erythrocytes.

Partial oxygen pressure (Po_2) and blood pH were estimated by the Astrup method on an OP 210/2 microanalyzer.

The results were analyzed by standard statistical methods. The significance of differences was evaluated by Student's t test.

RESULTS

The concentrations of ROS (O_2^-) and NO increased sharply 18 h after addition of LPS to the culture of mouse splenocytes. It manifested in intensification of EPR signals of spin adducts for these compounds and reflected activation of iNOS expression. Treatment with PLB was accompanied by inhibition of EPR signals of spin-labeled NO and O_2^- (Fig. 1).

The EPR signal of spin-labeled NO in mouse blood and spleen increased 18 h after administra-

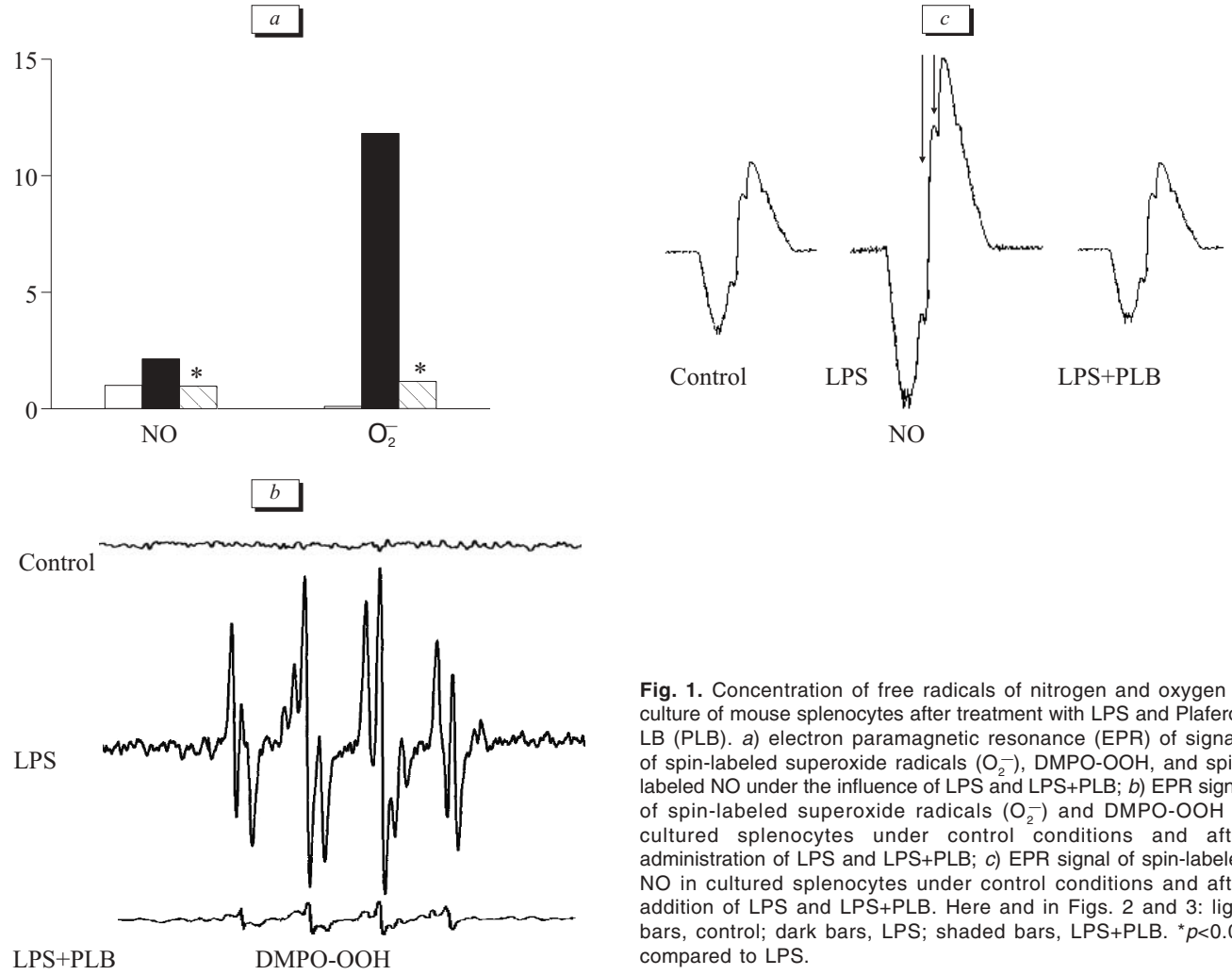

Fig. 1. Concentration of free radicals of nitrogen and oxygen in culture of mouse splenocytes after treatment with LPS and Plaferon LB (PLB). *a*) electron paramagnetic resonance (EPR) of signals of spin-labeled superoxide radicals (O_2^-), DMPO-OOH, and spin-labeled NO under the influence of LPS and LPS+PLB; *b*) EPR signal of spin-labeled superoxide radicals (O_2^-) and DMPO-OOH in cultured splenocytes under control conditions and after administration of LPS and LPS+PLB; *c*) EPR signal of spin-labeled NO in cultured splenocytes under control conditions and after addition of LPS and LPS+PLB. Here and in Figs. 2 and 3: light bars, control; dark bars, LPS; shaded bars, LPS+PLB. *$p<0.05$ compared to LPS.

tion of LPS. These data attest to increased NO synthesis associated with activation of iNOS in the test cells [14]. The concentration of NO in the blood and spleen decreased by 20 and 23%, respectively, after treatment with PLB (Table 1, Fig. 1).

NO specifically regulates activity of various enzymes, including cytochrome *c* oxidase, cytochrome P-450, catalase, NADH-ubiquinone oxidoreductase, succinate-ubiquinone oxidoreductase, *cis*-aconitase, lipoxygenase, and ribonucleotide reductase. The effects of NO depend on its concentration and redox potential of the surrounding tissue [11].

Administration of LPS sharply decreased EPR signal (duplet g=2.005, a=20 G) of ribonucleotide reductase in mouse spleen. These changes reflect a decrease in enzyme activity and inhibition of 2'-deoxyribonucleoside-5'-triphosphate synthesis (direct precursors of DNA). Ribonucleotide reductase activity depends strongly on the redox status of the organism. It determines the redox state of subunits in the catalytic site of enzyme (sulfhydryl groups, amino acid residues of tyrosine, and iron ions) [4]. Excess NO inhibits ribonucleotide reductase via nitrosylation of SH groups, which manifested in

TABLE 1. Paramagnetic Centers in the Blood of LPS-Treated Mice (mm/mg, $M\pm m$, n=20)

Group	NO	O_2^-	Fe^{2+}, g=2.2	Ceruloplasmin, g=2.5	Fe^{3+}-transferrin, g=4.2	Methemoglobin, g=6.0
Control	5.0±0.5	—	—	18.0±0.5	30.0±0.8	—
1	7.5±0.5**	7.0±0.8	25.0±2.0	23.8±1.5*	22.5±0.8*	13.3±0.8
2	6.0±0.5$^+$	2.8±0.5$^+$	10.0±2.5$^+$	20.0±1.2$^+$	32.0±1.1$^+$	8.0±0.9*

Note. *$p<0.001$ and **$p<0.05$ compared to the control; $^+p<0.001$ compared to group 1.

decreased EPR signal of this enzyme. Ribonucleotide reductase activity returned to normal after administration of PLB (Fig. 2).

The cytotoxic effect of NO is primarily associated with inhibition of mitochondrial respiration and oxidative phosphorylation. Mitochondria contain a considerable amount of proteins carrying heme and nonheme iron highly sensitive to NO. After administration of LPS, the EPR spectrum of mouse spleen included signals from nitrosyl complexes of heme (HbNO) and nonheme iron (FeSNO). The appearance of FeSNO and HbNO sites is related to nitrosylation of NADH dehydrogenase and heme group of cytochrome *c* oxidase, respectively (Fig. 2) [6]. Nitrosylation of mitochondrial electron-carrying proteins contributes to inactivation of the mitochondrial electron transport chain and decrease in respiration, oxidative phosphorylation, and synthesis of macroergic compounds in mitochondria. These changes result in activation of the hypoxanthine-xanthine oxidase system and increase in xanthine oxidase formation, which contribute to generation of superoxide radicals in the blood. We revealed an increase in the EPR signal of DMPO-OOH adduct in the blood (Table 1). Under conditions of L-arginine or tetrahydrobiopterin deficiency, NOS generates a considerable amount of superoxide radicals (O_2^-) and serves as an additional source of reactive oxygen [15].

PLB sharply decreased the EPR signal from nitrosyl complexes of heme and nonheme iron in the spectrum of animal spleen (Fig. 2). Probably, this drug has an inhibitory effect in iNOS activity.

The increase in reactive oxygen generation was accompanied by significant changes in activity of the antioxidant system. Activities of catalase and SOD in mouse blood increased sharply 18 h after LPS treatment (Fig. 3). These changes reflect a compensatory reaction of the organism to oxidative stress at the early stage of toxemia. Administration of LPS led to inactivation of the ceruloplasmin-Fe^{3+}-transferrin system in mouse blood (Table 1). This system neutralizes ROS and removes Fe^{2+} from the plasma (strong promoter of free radical oxidation). The increase in the concentration of oxidized ceruloplasmin and decrease in the content of Fe^{2+}-transferrin determine accumulation of Fe^{2+}, intensification of free radical processes, damage to membrane structures in formed elements of the blood, hemolysis of erythrocytes, and accumulation of methemoglobin (Table 1). Methemoglobin serves as a source of free iron ions. Fe^{2+} ions increase generation of reactive oxygen due to enzymatic oxidation. Activities of catalase and SOD decreased by 29 and 19%, respectively, in PLB-treated animals with

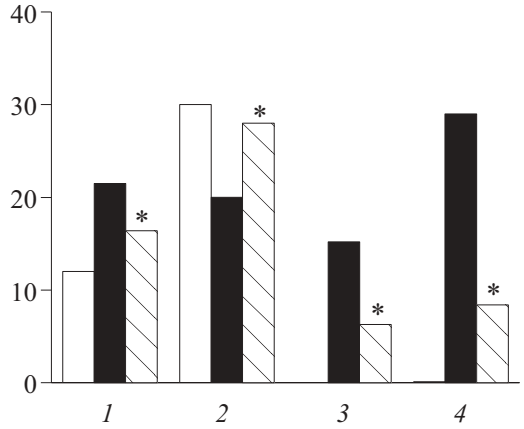

Fig. 2. EPR signals of NO (*1*), ribonucleotide reductase (*2*), FeSNO (*3*), and oxyhemoglobin (*4*) in mouse splenocytes after treatment with LPS and PLB.

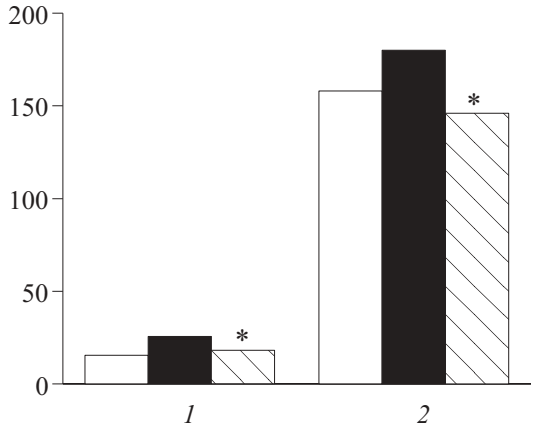

Fig. 3. Activity of antioxidant enzymes catalase (*1*, mcat/liter) and SOD (*2*, per ml erythrocytes) in mouse blood after treatment with LPS and PLB.

LPS-induced toxemia. EPR signals of ceruloplasmin and transferrin in these mice reached the control level (Table 1, Fig. 3).

pH of the blood in LPS-treated animals was lower compared to the control (6.99±0.08 and 7.35±0.05, respectively). These changes determine the decrease in hemoglobin oxygen affinity (Bore effect) and increased dissociation of oxyhemoglobin. Metabolic disturbances result in decreased oxygen consumption in tissues. The observed changes lead to an increase in blood Po_2 in LPS-treated mice (80.0±5.0 *vs*. 48.0±5.0 in the control). Accumulation of free radical oxidation promoters and iron ions and decrease in activity of the antioxidant system contribute to overproduction of superoxide radicals and development of oxidative stress. pH of the blood returned to normal (pH 7.12), while Po_2 remained high (Po_2=86) after treatment with PLB. These data illustrate low-intensity consumption of oxygen in tissues.

Our results show that the intensity of NO production increases 18 h after administration of LPS, which contributes to inactivation of enzymes and enzyme complexes (ribonucleotide reductase, NADH-ubiquinone oxidoreductase, and cytochrome *c* oxidase), dysfunction of the mitochondrial electron transport chain, development of oxidative stress, and inhibition of proliferative and reparative processes in the organism. PLB suppresses NO generation, which determines stabilization of mitochondrial respiration in tissues, decrease in production of ROS, and recovery of antioxidant activity and redox status in the organism. These changes provide normal function of enzyme systems (*e.g.*, ribonucleotide reductase) and improve proliferative and reparative processes in the organism.

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