Regulatory Role of Nitric Oxide in Neutrophil Apoptosis

Ye. A. Stepovaya, T. V. Zhavoronok, Yu. V. Starikov, V. A. Bychkov, N. Yu. Chasovskih, Ye. G. Starikova, G. V. Petina, V. V. Novitsky, and N. V. Ryazantseva

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Apoptosis of blood neutrophils from healthy donors was studied under conditions of cell culturing with different concentrations of H_2O_2, selective NO synthase inhibitor, and inductor of NO synthesis (L-arginine). *In vitro* incubation of neutrophilic leukocytes with 5 mM H_2O_2 led to activation of the apoptotic program in neutrophils, which was seen from increased content of Bax protein in the cells and increased number of apoptotic cells in the culture. Increased content of annexin-positive cells after incubation of neutrophil culture with NO synthase inhibitor suggests involvement of NO in the regulation of neutrophil apoptosis under conditions of oxidative stress, while L-arginine prevented H_2O_2-induced programmed cell death.

Key Words: *nitric oxide; apoptosis; neutrophils; active oxygen forms; oxidative stress*

Modulation of intracellular redox-dependent regulation of apoptosis is associated with increased level of reactive oxygen species (ROS) in cells leading to the formation of oxidative stress (OS) [3,4]. The development of OS is associated with oxidative modification of cell macromolecules, including regulatory enzymes, impairment of membrane structure and Ca^{2+} metabolism [6]. NO is an active radicals involved in OS development.

In cells, NO is formed from L-arginine under the effect of enzymes of the NO synthase family and by its chemical nature is a stable radical with one single electron [1]. Due to this property NO acts as a physiological regulator and as a damaging agent of OS in various diseases (tumor process, neurodegenerative diseases, inflammation, *etc.*), associated with dysregulation of programmed cell death. NO radical can exhibit pro- and antiapoptotic effects [8,9].

Department of Fundamentals of Clinical Medicine, Department of Pathophysiology, Department of Biochemistry and Molecular Biology, Laboratory of Molecular Medicine, Siberian State Medical University, Tomsk, Russia. ***Address for correspondence:*** tavaza@ngs.ru. T. V. Zhavoronok

We studied molecular mechanisms of NO involvement in apoptosis regulation. Neutrophils (well-differentiated cells with short life span, dying by apoptosis in tissues soon after their release from the bone marrow) were selected as the model cell system.

MATERIALS AND METHODS

Venous blood was collected from 32 donors (18 men and 14 women) aged 18-40 years (mean age 25.0±5.4 years). The blood was collected using standard vacuum systems BD VACUTAINER™ with lithium heparin.

Neutrophilic leukocytes were isolated from the blood under sterile conditions by gradient centrifugation in a double Ficoll-Paque density gradient (1.077 and 1.093 g/cm^3, Pharmacia). Viability of isolated neutrophils was evaluated by trypan blue exclusion. To this end, 0.1 ml cell suspension was mixed with an equal volume of 0.5% trypan blue (Serva) and dead (stained blue) cells were counted in a Goryaev's chamber. The percentage of viable cells containing no dye was 95%. The cells were cultured in 96-well plates (2×10^6/ml) in complete nutrient medium containing 90% RPMI-1640 (Vec-

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tor-Best), 10% FCS (Biolot) inactivated at 56°C for 30 min, 0.3 mg/ml L-glutamine, 100 μg/ml gentamicin, and 2 mmol/ml HEPES (Flow).

Oxidative stress was induced by adding H_2O_2 solution in final concentrations of 1, 5, 10, and 50 mM to the cell cultures. The cells were then cultured for 18 h at 37°C and 5% CO_2. In order to study the molecular mechanisms of NO involvement in the regulation of neutrophil apoptosis under conditions of OS, L-NAME (N^{ω}-nitro-L-arginine methyl ether; Fluka) and L-arginine (MP) in final concentrations of 500 μM were added to cell cultures and incubated with 5 mM H_2O_2.

The content of annexin-positive cells in the culture, content of ROS and Ca^{2+} in neutrophils were measured by laser flow cytometry on an Epics XL cytometer (Beckman Coulter). Apoptosis of neutrophilic leukocytes was evaluated by recording phosphatidylserine expression on the outer surface of the cell membrane using FITC-labeled annexin V (Caltag) [13]. The result was expressed in percent of apoptotic cells. The content of ROS in neutrophils was evaluated using a dye with blocked fluorescence (dichlorofluorescein diacetate; Sigma Aldrich). The result was equal to the proportion of dichlorofluorescein diacetate fluorescence intensity to total cell count and was expressed in arbitrary units. The content of Ca^{2+} in neutrophilic leukocytes was measured by the method based on evaluation of the fluorescence intensity of lipophilic probe Fluo 3 AM [11] (MP Biomedicals™) binding Ca ions. The result was equal to Fluo 3 AM fluorescence intensity to total cell count and was expressed in arbitrary units.

The content of NO metabolites (nitrites) in water solutions was measured by the spectrophotometric method based on color reaction with Griess' reagent (1% sulfanilamide and 0.1% naphthylene diamine (MP) diluted in 12% acetic acid) [2,5].

The content of Bax protein in neutrophils was measured by Western blot analysis [6]. Cell extracts were obtained by cell lysis in PBS containing 50 mM Tris-HCl buffer (pH 6.5), 100 mM dithiotreitol, 2% sodium dodecylsulfate (Helikon), 0.1% bromophenol blue, 15% glycerol (Helikon), and a mixture of protease inhibitors (Sigma). The proteins were separated by molecular weights at 12 V voltage for 60 min and transferred onto a nitrocellulose membrane (Bio-Rad) as described previously [12]. The proteins were transferred electrophoretically at 60 mA current for 90 min. Glycerol-3-phosphate dehydrogenase protein served as the reference and internal control (antibodies; Chemicon); the content of Bax protein was expressed as the proportion of the studied protein signal to the signal of glycero-3-phosphate dehydrogenase protein in the studied samples.

The data were processed by methods of variation statistics. The data are presented in Table 1 as the median (Me), upper and lower quartiles (Q_1-Q_3). The normality of distribution of the results was evaluated by the Kolmogorov—Smirnov test. The significance of differences between the means was evaluated using Mann—Whitney nonparametric test. The differences were considered significant at $p<0.05$.

RESULTS

Culturing of normal human neutrophils with different concentrations of H_2O_2 made it possible to create an experimental model of OS activating programmed death of the maximum number of these cells. The concentration of intracellular ROS and the

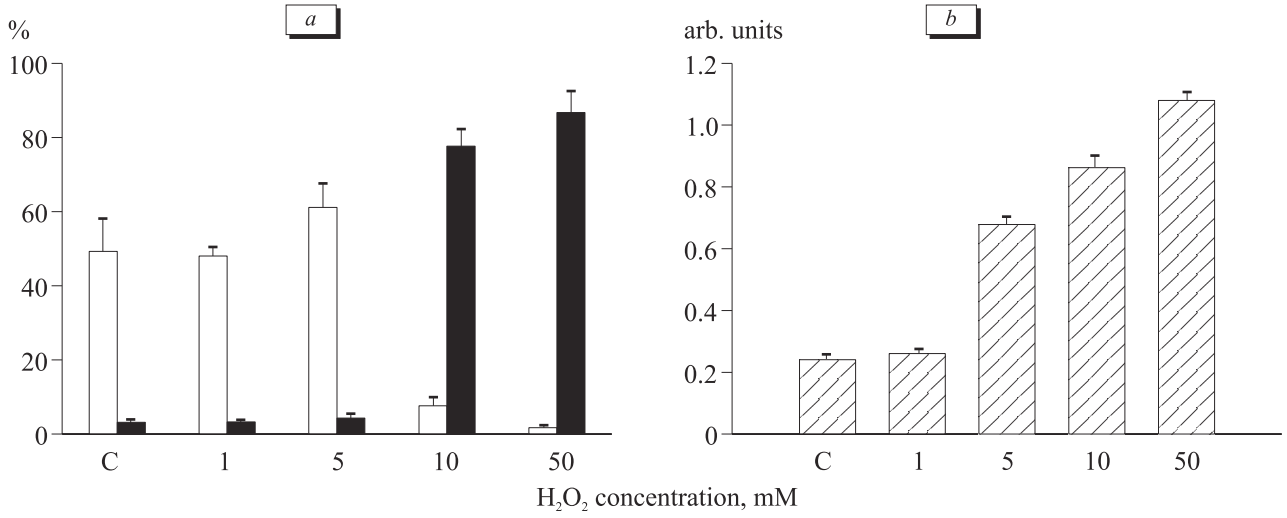

Fig. 1. Dynamics of annexin-positive (light bars) and necrotic (dark bars) cells (*a*) and the content of intracellular ROS in neutrophilic leukocyte culture (*b*) at different concentrations of H_2O_2.

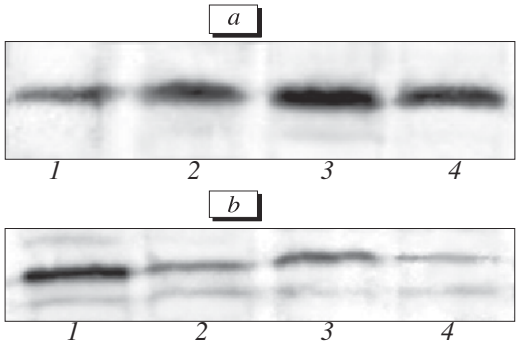

Fig. 2. Western blot analysis of Bax protein in normal human neutrophil cultures. *a*) Bax protein; *b*) glycero-3-phosphate dehydrogenase (reference and internal control). Here and in Fig. 3: *1*) intact culture; *2*) culturing with 5 mM H_2O_2; *3*) culturing with 5 mM H_2O_2 and L-NAME; *4*) culturing with 5 mM H_2O_2 and L-arginine.

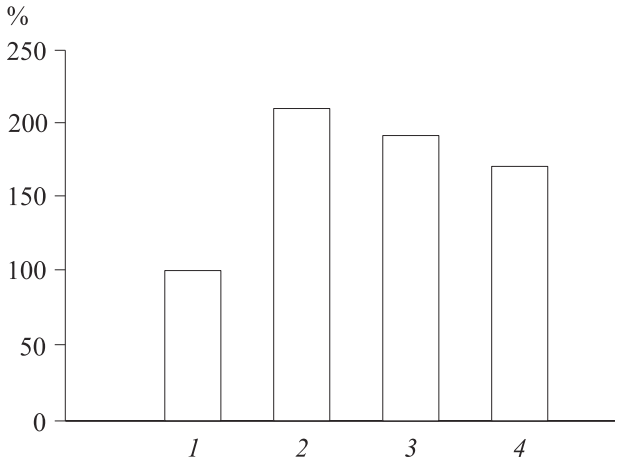

Fig. 3. Content of Bax protein in normal human neutrophil cultures (Western blot results). The content of Bax protein in intact culture of donor cells was taken as 100%.

content of annexin-positive neutrophils increased during incubation with 5 mM H_2O_2 (Fig. 1). The content of proapoptotic protein Bax increased in neutrophils incubated with H_2O_2 in a concentration of 5 mM (Figs. 2, 3).

In high concentrations, H_2O_2 (a medium-strong oxidant) affects intracellular organelles, *e.g.* mitochondria. This leads to uncoupling of oxidative phosphorylation and uncontrolled increase of intracellular ROS content [10]. Since mitochondria are Ca reservoirs in cells, changes in mitochondrial membrane permeability are critical for the maintenance of Ca homeostasis. Analysis of Ca content in neutrophils revealed its increase ($p<0.05$) under the effect of 5 mM H_2O_2 in comparison with the control (Table 1).

Addition of H_2O_2 in a final concentrations of 10.0 mM and higher to the neutrophil culture *in vitro* led to cell death via the necrotic pathway (Fig. 1).

In order to clear out the regulatory role of NO in the realization of programmed cell death, neutrophilic leukocytes were incubated with H_2O_2 and L-NAME (NO synthase selective inhibitor) or L-arginine (NO synthase substrate).

The content of NO metabolites in supernatants of neutrophil culture under conditions of experimental oxidative stress significantly surpassed the control level ($p<0.05$). Inhibition of NO synthase in neutrophils incubated with H_2O_2 significantly reduced the content of NO metabolites compared to neutrophil culture incubated with 5 mM H_2O_2 alone ($p<0.05$). Analysis of NO metabolite production after addition of L-arginine to neutrophil culture with induced oxidative stress revealed a significant increase in the studied parameter in comparison with the control and with neutrophil culture incubated with 5 mM H_2O_2 ($p<0.05$; Table 1).

Addition of L-NAME (NO synthase inhibitor) to the culture of neutrophilic leukocytes under conditions of experimental OS (high ROS content in the cells) significantly increased the number of apoptotic cells and Ca^{2+} content in neutrophils in comparison with the control ($p<0.05$). Study of the effects of L-arginine (NO synthase inductor) on neutrophil culture under conditions of experimental OS showed that the content of Ca^{2+} in cells and the number of apoptotic neutrophils did not differ from the control, ROS level significantly surpassed the normal ($p<0.05$) and remained at the level observed in cells cultured with 5 mM H_2O_2 (Table 1). Treatment of neutrophil culture with NO synthase inhibitor and L-arginine under conditions of OS induction had no effect on the level of Bax in comparison with the cells cultured with 5 mM H_2O_2 (Figs. 2, 3).

Hence, addition of 5 mM H_2O_2 to the culture of neutrophilic leukocytes increases the content of annexin-positive cells and elevats ROS content in the cells, which indicates the development of OS. ROS are involved in permeabilization of mitochondrial membranes and activation of the internal pathway of programmed death, which is seen from increased concentration of intracellular Ca^{2+} and Bax level.

The results confirm the NO involvement in apoptosis of 18-h culture of neutrophilic leukocytes exposed to OS *in vitro*. Addition of NO synthase inductor increased the content of NO metabolites in incubation medium and prevented the development of programmed cell death of neutrophilic leukocytes under conditions of experimental OS. The content of annexin-positive cells was normal, presumably due to the stabilizing effect of NO on the realization of the internal apoptosis pathway, which was seen from normalization of intracellular Ca^{2+}

TABLE 1. Effects of L-NAME and L-Arginine on Apoptosis, ROS and Ca^{2+} Levels in Neutrophils, NO Metabolite Concentrations in Incubation Medium under Conditions of OS *In Vitro*

Experiment conditions	Annexin-positive cells, %	ROS content in a cell, arb. units	Ca^{2+} content, arb. units	Concentrations of NO metabolites in incubation medium, μmol/liter
Control	49.25 (45.03-58.13)	0.240 (0.214-0.259)	0.220 (0.114-0.234)	0.108 (0.106-0.110)
Incubation with 5 mM H_2O_2	61.10 (57.80-67.50)*	0.678 (0.596-0.705)*	0.444 (0.403-0.489)*	0.121 (0.120-0.132)*
Incubation with H_2O_2 (5 mM) and L-NAME (500 μM)	61.90 (59.75-64. 05)*	0.740 (0.720-0.751)*+	0.384 (0.365-0.403)*	0.109 (0.106-0.110)+
Incubation with H_2O_2 (5 mM) and L-arginine (500 μM)	54.90 (47.10-60. 25)	0.676 (0.656-0.700)*	0.193 (0.178-0.214)+	0.142 (0.140-0.146)*+

Note. Data are presented as the medium, upper and lower quartiles. $p<0.05$ compared to: *control, +culture with 5 mM H_2O_2.

content. Changes in calcium content in neutrophils under the effect of NO could result from NO binding to cytochrome oxidase, leading to hyperpolarization of the mitochondrial membrane and blockade of the release of proapoptotic factors from the mitochondria [7].

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