GENERAL PATHOLOGY AND PATHOPHYSIOLOGY

Role of Recombinant Mitogen-Activated Protein Kinases JNK and p38 in the Regulation of Apoptosis in Blood Mononuclear Cells under Conditions of Oxidative Stress *In Vitro*

N. V. Ryazantseva, V. V. Novitsky, N. Yu. Chasovskih, E. V. Kaygorodova, E. G. Starikova, Yu. V. Starikov, and T. T. Radzivil

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 145, No. 5, pp. 505-508, May, 2008
Original article submitted September 5, 2007

Programmed death of peripheral blood mononuclear cells from healthy donors was studied during culturing with various concentrations of H_2O_2 and selective inhibitors of JNK (SP600125) and p38 MAPK (ML3403). *In vitro* incubation of mononuclear leukocytes with 1 mM H_2O_2 stimulated apoptotic cell death. Treatment with inhibitors (SP600125 and ML3403) during *in vitro* oxidative stress prevented the increase in the number of annexin-positive mononuclear cells. Our results indicate that MAP kinases JNK and p38 are involved in the mechanisms of oxidative dysregulation of apoptosis.

Key Words: *oxidative stress; reactive oxygen species; apoptosis; mitogen-activated protein kinases*

The pathogenesis of various diseases (oncology, cardiovascular, and neurodegenerative disorders; acute and chronic inflammation; and diabetes mellitus) is associated with cell damage due to oxidative stress under conditions of prooxidant/antioxidant imbalance [6]. The impairment of apoptosis dysregulation (excessive activation or inhibition of apoptosis) is an important pathogenetic stage of these diseases [4,6,8]. Reactive oxygen species (ROS) serve as an adverse factor. The local effect of ROS is related to high reactivity. The increase in the content of ROS is followed by oxidative modification of biomolecules, changes in activity of the key enzyme systems, and structural dysfunction of the membrane [3,6]. Oxidant-activated phospholipases stimulate a variety of kinases, including mitogen-activated protein kinases (MAP kinases) of the JNK and p38 families. Transcription factors (*e.g.*, c-Jun, ATF-2, Elk-1, p53, NFAT4, c-Myc, ANF-2, Max, and MEF2C), phospholipases, other protein kinases, cytoskeleton-associated proteins, and membrane receptors are the substrates for these enzymes [1,11,14]. JNK and p38 protein kinases phosphorylate target proteins involved into the regulation of programmed cell death and function of the corresponding transcription factors.

Previous studies showed that JNK can induce apoptosis via phosphorylation and activation of transcription factor P53 [1] and proapoptotic pro-

Department of Pathophysiology, Department for Fundamental Bases of Clinical Medicine, Siberian State Medical University, Federal Agency of Health Care and Social Development, Tomsk, Russia. ***Address for correspondence:*** cnil@mail.ru. N. Yu. Chasovskih

0007-4888/08/1455-0569

teins Bax and Bad (after translocation in mitochondria), as well as by phosphorylation and inactivation of antiapoptotic proteins of the Bcl-2 family [10-12,14]. The substrate of JNK (c-Myc) is also involved in apoptosis [8,10]. Activation of p38 protein kinase induces phosphorylation of Bad and increases expression of proapoptotic protein P53 [8].

Some authors reported that JNK and p38 exhibit antiapoptotic activity [12,13], which depends on the nature of inducing signals, combined pathways of signal transduction, and type of cells. The role of stress-activated protein kinases JNK and p38 in programmed cell death under conditions of oxidative stress requires further investigations. Oxidative stress is a general mechanism for pathological processes of different genesis. Treatment with selective inhibitors of these enzymes (SP600125 and ML3403) is one of the methodological approaches to study functions of protein kinases JNK and p38.

Here we studied molecular mechanisms of apoptosis dysregulation during oxidative stress. The knowledge of these mechanisms will allow us to develop a new strategy of modulation by pharmacological treatment of the corresponding molecular targets.

MATERIALS AND METHODS

Experiments were performed with blood samples from 28 healthy donors (15 men and 13 women, 18-55 years old) stabilized with 25 U/ml heparin. Mononuclear cells (MNC) were isolated from the blood by centrifugation on a Ficoll-Paque density gradient (ρ=1.077 g/cm^3, Pharmacia). The cells were cultured in 96-well plates (2×10^6 cells/ml) with complete nutrient medium, which contained 90% RPMI-1640 (Vektor-Best), 10% fetal bovine serum (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 addition of H_2O_2 in concentrations of 10, 50, 100, and 500 μM and 1 mM to cell cultures. The cells were incubated at 37°C and 5% CO_2 for 18 h. Selective inhibitor of JNK (4 μl 20 μM SP600125, Biosource) or p38 (ML3403, Biosource) was added to cell cultures to evaluate the role of JNK and p38 kinases. Incubation was performed at 37°C and 5% CO_2 for 18 h. Further culturing was conducted in the presence of 1 mM H_2O_2. Apoptotic activity of cells was studied in the annexin test after 18-h incubation.

MNC apoptosis was studied by recording the expression of phosphatidylserine on the outer surface of the cell membrane using FITC-labeled annexin V (Caltag) [15]. After incubation, the cells (2×10^6 cells/ml) were suspended in a buffer (Caltag) containing annexin V-FITC for 10 min. Flow cytofluorometry was performed on an Epics XL cytometer (Beckman Coulter) with an argon laser. Green fluorescence (530 nm FITC) was analyzed in MNC gates.

ROS production in cells was studied using a non-fluorescent dye dichlorofluorescin diacetate (DCF-DA, Sigma Aldrich). The suspension of MNC (initial concentration 2×10^6 cells/ml) was put in a sterile polystyrene tube with a working solution of DCF-DA. The cells were resuspended and incubated at 37°C for 20 min; 11 μl EDTA was added. Incubation was performed at 37°C for 30 min. After incubation, the cells were pelleted by centrifugation at 1000 rpm for 1 min. Erythrocytes were lysed by adding 200 μl lysing buffer, washed once in 200 μl phosphate buffered saline, and resuspended in 400 μl buffer. Conversion of DCF-DA into a fluorescent compound (green range) was studied by flow cytofluorometry. The intensity of fluorescence was expressed in arb. units per cell.

The results were analyzed by methods of variational statistics. The normality of data distribution was estimated by Kolmogorov—Smirnov test. The significance of differences between the means was determined by nonparametric Mann—Whitney test (independent samples) and Wilcoxon test (dependent samples). The differences were significant at $p<0.05$. The data are presented as the median and upper and lower quartiles.

RESULTS

Culturing of blood MNC from healthy donors with H_2O_2 in various concentrations provided conditions similar to oxidative stress. Oxidative stress is characterized by strong activation of free radical oxidation and/or impairment of antioxidant protection, which results in significant intracellular accumulation of ROS. The molecular targets for ROS are lipids, proteins, and nucleic acids. Oxidant-induced activation of destructive signal systems and transcription of the corresponding redox-sensitive genes determine the initiation of programmed cell death [3].

ROS concentration in MNC from healthy donors was measured by flow cytofluorometry. ROS concentration in the intact cell culture was 0.24 (0.19-0.33) arb. units/cell. Addition of H_2O_2 (in concentrations 10, 50, 100 and 500 μM) to the cell culture had little effect on ROS concentration in MNC, which is probably related to effective work of the antioxidant system. ROS concentration in MNC increased significantly only after culturing with 1 mM H_2O_2. These changes illustrate a shift

TABLE 1. Concentration of ROS and Percent of Apoptotic Cells in the Total Population of Peripheral Blood MNC from Healthy Donors during *in Vitro* Culturing with H_2O_2 in Various Concentrations

Experimental conditions	Intracellular ROS concentration, arb. units		Percent of annexin-positive cells	
	median	upper and lower quartiles	median	upper and lower quartiles
Intact culture	0.24	0.19-0.33	1.33	0.99-1.95
Incubation with H_2O_2				
10 μM	0.13	0.06-0.21	1.84	1.02-2.08
50 μM	0.19	0.17-0.26	1.51	1.07-2.17
100 μM	0.25	0.21-0.37	1.75	0.31-2.64
500 μM	0.28	0.27-0.33	1.20	0.41-2.06
1 mM	0.61*	0.54-0.68	13.11*	12.17-15.13

Note. *$p<0.001$ compared to intact culture.

of oxidative metabolism toward depletion of the antioxidant reserves (Table 1) [3,6].

ROS regulate cell proliferation, induce transcription of certain genes, produce a cytotoxic effect, or trigger apoptosis, which depends on the concentration of these substances [6,7,11]. The annexin test after incubation of cultured cells with H_2O_2 in concentrations of 10, 50, 100, and 500 μM revealed no changes in the number of apoptotic cells (as compared to that in the intact culture). The induction of MNC death was significant in a culture exposed to 1 mM H_2O_2 (13.11% [12.17-15.13%] *vs*. 1.33% [0.99-1.95%] in the control, Table 1).

The cytotoxic effect of H_2O_2 is mediated by various mechanisms. H_2O_2 *in vitro* causes single-strand DNA breaks leading to cell apoptosis [2,6]. H_2O_2 inhibits glycolysis in cells due to inactivation of aldehyde dehydrogenase and decrease in lactate concentration. The induction of free radical oxidation is followed by changes in physicochemical properties of the plasma membrane, which results in transmembrane anion transfer and increase in permeability for macromolecules. The increase in intracellular Ca^{2+} concentration is accompanied by activation of phospholipases and phosphoinositide metabolism. ATP depletion causes cell death [5].

TABLE 2. Percent of Annexin-Positive Cells in the Total Population of Peripheral Blood MNC from Healthy Donors during *in Vitro* Culturing with H_2O_2 and MAP Kinase Inhibitors

Experimental conditions	Percent of annexin-positive cells	
	median	upper and lower quartiles
Intact culture	1.33	0.99-1.95
Incubation with		
1 mM H_2O_2	13.11*	12.17-15.13
1 mM H_2O_2+ML3403	0.91$^+$	0.25-2.78
1 mM H_2O_2+SP600125	0.68$^+$	0.33-2.12

Note. *$p<0.001$ compared to intact culture; $^+p<0.001$ compared to culture with 1 mM H_2O_2.

The imbalance of oxidative metabolism contributes to activation of MAP kinase cascades, which serves as a redox-sensitive component of the apoptosis-inducing system [1,3,6,11]. ROS activate MAPK Kinase Kinase proteins, including ASK1 (apoptosis signal-regulating kinase 1 for activation of JNK and p38). These proteins trigger the signal cascade. The elevation of ROS concentration often correlates with the increase in phosphorylation of JNK and p38 [10,12]. Activated MAP kinases affect transcription factors (*e.g.*, Nf-kB and P53). Under certain conditions, Nf-kB stimulates the expression of apoptosis-inducing genes (Fas and death receptors). P53 plays an important role in stimulation of the internal program [6,8]. Besides this, JNK and p38 directly activate several proapoptotic proteins [1,10]. SP600125 prevented initiation of apoptosis, which was induced by oxidative stress. Similar results were obtained after incubation of MNC with 1 mM H_2O_2 in the presence of ML3404 (Table 2).

Our results indicate that protein kinases JNK and p38 play a proapoptotic role in redox-dependent variations of the regulation of MNC programmed death *in vitro*. The involvement of MAP kinases in dysregulation of cell death during oxidative stress is probably associated with activation of kinase cascade-initiating elements (under excessive ROS) or direct effect of ROS on JNK and p38 [1]. Phosphorylation of protein kinases is followed by posttranslational changes in target molecules and/or impaired expression of apoptotic genes [6]. ROS in low concentration can produce JNK- and p38-

mediated effects associated with cell adaptation (*e.g.*, proliferation and differentiation). The conditions of oxidative stress are accompanied by adaptive reserve depletion and dysregulation of signal transduction systems for cell survival or death.

This work was supported by the Federal Target Scientific-and-Technical Program "Researches and Projects on Priority Directions of Science and Technology" for 2002-2006 (State Contracts No. 02. 442.11.7276 and 02.445.11.7419) and Russian Foundation for Basic Research (grant No. 07-04-12150).

REFERENCES

1. S. Vlaopulos and V. S. Zumpurlis, *Biokhimiya*, **69**, No. 8, 1038-1050 (2004).
2. D. K. Das and N. Molik, *Ibid*., **69**, No. 1, 16-24 (2004).
3. E. E. Dubinina, *Vopr. Med. Khimii*, **47**, No. 6, 561-581 (2001).
4. O. B. Zhukova, N. V. Ryazantseva, V. V. Novitskii, *et al*., *Tsitologiya*, **49**, No. 1, 26-31 (2007).
5. E. F. Lushnikov and A. Yu. Abrosimov, *Cell Death (Apoptosis)* [in Russian], Moscow (2001).
6. E. B. Men'shikova, V. Z. Lankin, N. K. Zenkov, *et al*., *Oxidative Stress. Prooxidants and Antioxidants* [in Russian], Moscow (2006).
7. N. F. Shof and V. E. Kagan, *Biokhimiya*, **69**, No. 1, 48-56 (2004).
8. C. P. Baines and J. D. Molkentin, *J. Mol. Cell. Cardiol*., **38**, No. 1, 47-62 (2005).
9. D. E. Bredesen, *J. Neurotrauma*, **17**, No. 10, 801-810 (2000).
10. S. D. Cho, N. S. Ahn, J. W. Jung, *et al*., *Eur. J. Cancer Prev*., **15**, No. 1, 57-63 (2006).
11. J. J. Haddad, *Respir. Res*., **3**, 26 (2002).
12. C. Harada, K. Nakamura, K. Namekata, *et al*., *Am. J. Pathol*., **168**, No. 1, 261-269 (2006).
13. K. Sabapathy, Y. Hu, T. Kallunki, *et al*., *Curr. Biol*., **9**, No. 11, 16-25 (1999).
14. F. Teraishi, S. Wu, L. Zhang, *et al*., *Cancer Res.*, **65**, No. 14, 6380-6387 (2005).
15. M. Van Engeland, L. J. Nieland, F. C. Ramaekers, *et al*., *Cytometry*, **31**, No. 1, 1-9 (1998).