Triterpenoid Miliacin Inhibits Stress-Induced Lipid Peroxidation

T. V. Panfilova, A. A. Shtil', and B. A. Frolov

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A plant triterpenoid miliacin prevented stress-induced activation of LPO and accumulation of LPO products in the blood. The inhibitory effect of miliacin was not related to direct inhibition of reactive oxygen species generation.

Key Words: *triterpenoids; miliacin; stress; lipid peroxidation; lymphocytes*

Plant triterpenoid miliacin exhibits protective activity under conditions of stress-induced changes in the humoral and cellular immune response and cortisol-induced apoptosis in lymphocytes [4,5]. Lipid peroxidation (LPO) is the major mechanism of stress-induced damage to cells [3,6]. We hypothesized that this triterpenoid can prevent stress-induced activation of LPO.

This work was designed to test this hypothesis. We studied the mechanisms underlying the action of miliacin.

MATERIALS AND METHODS

The ability of miliacin to modulate stress-induced LPO was tested on (CBA×C57Bl/6)F_1 mice. The animals were divided into 6 groups: group 1 comprised intact controls receiving 3 intraperitoneal injections of miliacin (single dose 2 mg/kg, days 1, 5, and 9); group 2 animals received 3 intraperitoneal injections of solvent Tween 21 (final concentration of 160 nmol/kg); group 3 animals were exposed to stress without pretreatment, and groups 4 and 5 animals were exposed to stress after pretreatment with miliacin and solvent, respectively.

The mice were exposed to combined stress (immobilization) for 4-16 h: over the first 2 h at 4°C and than at 18-20°C. LPO in blood serum was studied using the model system. Generation of reactive oxygen species (ROS) was induced by 50 mM solution of Fe^{2+}. $FeSO_4 \times 7H_2O$ (1 ml) was added to blood serum. The serum was diluted 1:40 with phosphate buffered saline (20 mM KH_2PO_4 and 105 mM KCl, pH 7.45) and put into a cuvette of a KhLM-003 chemiluminometer. Induced oxidation of unsaturated fatty acids was studied by recording chemiluminescence. The amplitude of maximum luminescence (A_{CL}, arb. units) and total yield of fluorescence (Σ_{CL}, arb. units) reflected the maximum level of LPO and number of branched lateral chains (amount of $ROO^{\bullet}$ peroxide radicals) per 1 Fe^{2+} ion [2].

Antioxidant activity of miliacin was estimated by the ability of this compound to suppress generation of ROS, LPO inductors [1]. H_2O_2 (50-100 μM) was added to the incubation medium of cultured lymphocytes to stimulate ROS generation [7]. Fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCFDA, Sigma) was used for the detection of oxygen burst. Acetyl ester of this substance can permeate the cytoplasm. This probe underwent deesterification in the cytoplasm, which prevented its transport from the cell. The probe fluoresced green at the excitation and emission wavelengths of 540 and 600 nm, respectively. Fluorescence significantly increased during activation of intracellular oxidation, which manifested in an increase in the relative number of bright cells in the population of lymphocytes. Lymphocytes were isolated from 3

Department of Pathophysiology, Orenburg State Medical Academy; *Institute of Carcinogenesis, N. N. Blokhin Russian Cancer Research Center, Russian Academy of Medical Sciences, Moscow. ***Address for correspondence:*** shtilaa@yahoo.com. A. A. Shtil'

groups of (CBA×C57Bl/6)F_1 mice not exposed to any treatment (intact animals) or receiving miliacin (treatment) and solvent as described above (control). Splenocytes were sampled 24 h after the end of treatment with miliacin or solvent. Lymphocytes were isolated by the standard method on a Ficoll-Verografin density gradient (1.090 g/cm^3) and centrifuged at 400*g* for 10 min. The cells were washed with Hanks solution and resuspended in 1 ml medium 199 containing 0.5% fetal bovine serum (culture medium). The cell suspension (0.2 ml, 4×10^6 cells) was put in a 12-well plate with medium 199 containing 5 μM DCFDA. Incubation was performed at 37°C and 5% CO_2 for 30 min. Lymphocytes were pelleted by centrifugation and suspended in 0.6 ml culture medium; 0.2 ml lymphocyte suspension (1.3×10^6 cells) was put in each of 3 wells with 1.8 ml culture medium. H_2O_2 in final concentrations of 50 and 100 μM was added to the 1st and 2nd samples of lymphocytes. The 3rd sample of cells did not include H_2O_2 (control). Incubation was performed at 37°C and 5% CO_2 for 30 min. The cells were pelleted by high-speed centrifugation and suspended in 0.5 ml Hanks solution. Fluorescence was measured on a FACS Calibur flow cytofluorometer (Becton Dickinson). Intracellular oxidation was studied by a cell shift toward the bright region of the scale (relative to control cells in the 3rd sample) and expressed in percents of the total cell number. The results of cytofluorometry (not less than 10,000 cells in each sample) were analyzed by means of CellQuest™ software (Becton Dickinson).

The results were analyzed by Student's *t* test. The differences were significant at $p<0.05$.

RESULTS

Stress exposure significantly modulated LPO. The intensity of LPO strongly depended on the duration of stress (Table 1). A_{CL} and Σ_{CL} decreased after 4-h stress, which reflects mobilization of the antioxidant protective mechanisms under these conditions. The intensity of LPO increased 8 h after the start of stress and reached maximum by the end of immobilization (16 h). LPO activation persisted for a long time, since parameters of chemiluminescence did not return to normal even 24 h after the end of immobilization.

Triple injections of miliacin or solvent to unstressed animals had no effect on parameters of chemiluminescence (compared to intact mice, Table 2). However, injections of the triterpenoid before stress exposure decreased LPO intensity. Pretreatment with the solvent produced no effects.

TABLE 1. Dependence of LPO in the Plasma from (CBA×C57Bl6)F_1 Mice on the Duration of Stress (*M*±*m*)

Group	A_{CL}	Σ_{CL}
Intact animals (*n*=13)	2.54±0.10	8.07±0.45
Stress, 4 h (*n*=7)	2.28±0.08*	6.54±0.26*
Stress, 8 h (*n*=6)	3.11±0.14*	11.62±0.47*
Stress, 16 h (*n*=5)	6.78±1.2*	19.30±1.78*
24 h after 16-h stress (*n*=6)	3.99±0.16*	14.88±1.26*

Note. *n*, number of animals. *$p<0.05$ compared to intact animals.

TABLE 2. Effect of Miliacin on LPO in the Plasma from Unstressed (CBA×C57Bl6)F_1 Mice and Animals Exposed to 16-h Stress (*M*±*m*)

Group	A_{CL}	Σ_{CL}
Intact animals (*n*=14)	2.80±0.18	7.78±0.44
Stress (*n*=17)	5.71±0.49*	20.46±1.13*
Miliacin (*n*=12)	2.97±0.07	8.85±0.41
Miliacin+stress (*n*=13)	4.17±0.14^{abc}	16.67±0.68^{abc}
Solvent (*n*=6)	2.78±0.10	8.47±0.55
Solvent+stress (*n*=6)	6.21±0.66^{cd}	23.04±2.12^{cd}

Note. $p<0.05$: *compared to intact mice; [a]compared to stressed mice; [b]compared to the miliacin group; [c]compared to the miliacin+stress group; [d]compared to solvent-receiving mice.

Our findings suggest that stress against the background of miliacin treatment was associated with less pronounced activation of LPO and lower accumulation of blood LPO products that can be involved in *in vitro* Fe^{2+}-induced oxidation. It was important to answer the question whether miliacin can suppress ROS generation. Experiments with DCFDA showed that the relative number of bright cells in a H_2O_2-free culture medium did not exceed 0.5% total lymphocyte count (for each group of animals). Fluorescence of splenocytes significantly increased after addition of H_2O_2 to the culture medium. We revealed an increase in cell number in the bright region of the scale (Table 3).

Increasing the concentration of H_2O_2 was accompanied by an increase in fluorescence. It was most pronounced in experiments with lymphocytes from mice receiving miliacin or solvent. However, we found no intergroup differences in fluorescence of lymphocytes. The shape and size of cells (direct and lateral light scattering) remained unchanged under these conditions.

Our study failed to find evidence that miliacin directly suppresses ROS generation. The inhibition of LPO under the influence of this triterpenoid is probably mediated by other mechanisms. Miliacin

TABLE 3. Effect of Miliacin on DCFDA Fluorescence in Lymphocytes from (CBA×C57Bl6)F_1 Mice Induced by H_2O_2 in Various Concentrations (*M*±*m*)*

Group of animals (lymphocyte donors)	H_2O_2 concentration in the culture medium, μM	
	50	100
Intact animals	40.07±1.61 (9)	45.68±4.78 (9)
Solvent treatment (control)	34.78±2.71 (8)	43.80±1.12 (8)
Miliacin treatment (experiment)	35.00±1.74 (15)	45.20±0.71 (13)

Note. *Ratio of cells (%) in the bright region of the scale. Number of animals is shown in brackets.

produces a membrane-stabilizing effect, which determines the resistance of membranes to ROS-induced LPO. The inhibition of LPO at a system level probably contributes to antistress activity of miliacin.

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