Tumor Necrosis Factor-α in Low Doses Preactivates and Activates Macrophages by Increasing Their Ability to Produce Reactive Oxygen Species and Oxidize Low-Density Lipoproteins: Protective Effect of Antioxidants

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Tumor necrosis factor-α in low doses activated rat peritoneal macrophages and intensified production of reactive oxygen species (zymosan-depended chemiluminescence). Single or 2-fold incubation with tumor necrosis factor-α activated and preactivated human blood macrophages and promoted oxidative modification of low-density lipoproteins (increased their mobility in agarose gel). Antioxidants (potassium phenosan, probucol, and desferal) suppressed oxidative modification of low-density lipoproteins induced by nonactivated, preactivated, and activated macrophages. Our results show that antioxidants hold much promise for the prevention and therapy of atherosclerosis.

Key Words: *macrophages; low-density lipoproteins; chemiluminescence; electrophoresis; antioxidants*

Our previous studies showed that the role of macrophages in the pathogenesis of atherosclerosis is not restricted by binding and metabolism of oxidized low-density lipoproteins (LDL). These cells also oxidize LDL and prepare them for further uptake [4]. The oxidizing capacity of macrophages depends on activation of the NADPH oxidase and myeloperoxidase systems [1]. Cytokines tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6, products of lipid peroxidation, and other factors act as specific and nonspecific stimulators of macrophages [3]. Transition of macrophages from the nonactive into active form proceeds via preactivation (priming), *i.e.* expression of surface receptors and production of reactive oxygen species, biologically active substances, and Ca^{2+} [3,5-7]. Doses of cytokines and time of incubation necessary for preactivation and activation of macrophages are poorly studied. Moreover, the ability of preactivated and activated human blood macrophages to induce oxidative modification of LDL and the effects of antioxidant preparations on this process were not studied.

Here we studied the ability of TNF-α-activated macrophages to intensify production of reactive oxygen species and stimulate oxidative modification of LDL. We also evaluated the protective effect of antioxidants potassium phenosan, probucol, and desferal in inhibiting oxidative modification of LDL by preactivated and activated macrophages.

MATERIALS AND METHODS

Peritoneal macrophages were obtained from outbred rats after intraperitoneal injection of 20 ml Hanks

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solution under ether anesthesia. Peritoneal exudate was centrifuged at 200*g* for 10 min. Cell count in the suspension was estimated in a Goryaev chamber. Luminol-dependent chemiluminescence (CL) was recorded in 3 ml dye-free Hanks solution (pH 6.7) containing 10^6 macrophages and 0.1 ml luminol at 37°C on a KhLM-3 chemiluminometer (Bikap). After recording spontaneous CL opsonized zymosan in a concentration of 10^{-5} M was added into a cuvette and zymosan-dependent CL was measured [2]. Human macrophages were obtained from donor blood. The blood (10 ml) was placed in a tube with 1 ml phosphate buffered saline (Sigma) and 50 U heparin. Macrophages were isolated by centrifugation on Ficoll Paque at 400*g* and 37°C for 20 min. The cells were placed in Petri dishes and incubated at 37°C for 18 h in a CO_2 incubator. LDL (d=1.019-1.065 g/cm^3) were isolated from the plasma of healthy donors by centrifugation in a density gradient. Protein content was measured by the method of Lowry.

Human and mouse macrophages were preactivated and activated with TNF-α, because its is one of the most potent cytokine secreted by monocytes and macrophages. Peritoneal macrophages were incubated with TNF-α (0.01-10.00 ng/10^6 macrophages) in Hanks solution at 37°C for 3 h, centrifuged, and washed. Spontaneous and zymosan-dependent CL was measured. For activation and preactivation human blood macrophages were incubated with TNF-α in the same dose for 10, 30, and 60 min or 3 h in a CO_2 incubator. After that LDL in a concentration of 150 μg/ml were added to wells without (control) and with TNF-α (experiment). The mixture was incubated in a CO_2 incubator for 3 or 24 h. Oxidative modification of LDL was evaluated by the increase in electrophoretic mobility of these particles in agarose gel. Electrophoretic mobility was measured by the method of Noble with modifications. The absolute value was determined by measuring the length of tracks (cm). Otherwise, images were scanned and digitized; the length of tracks was computed using special software (Delphi 6 programming language) and expressed in arb. units. The length of tracks for native non-incubated LDL was taken as 100%. Antioxidants potassium phenosan, probucol, and desferal (10^{-5} M) and LDL were added to nonactivated, preactivated, and activated human blood macrophages and incubated for 3 or 24 h. The significance of differences between the means in experimental and control (without TNF-α) series was determined by nonparametric Student's t test for small linked samples.

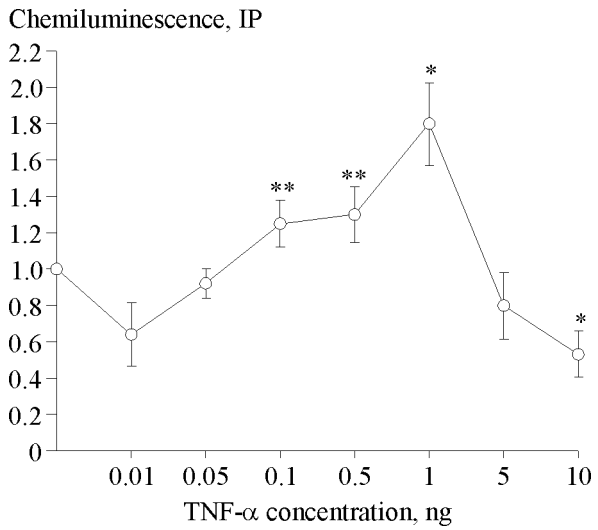

Fig. 1. Index of preactivation (IP) for rat peritoneal macrophages after 3-h incubation with TNF-α in various doses (*M*±*m*). *p<0.01 and **p<0.05 compared to the control (zero point).

RESULTS

TNF-α in doses of 0.1-1.0 ng produced a preactivating effect on zymosan-dependent CL of rat peritoneal macrophages. The index of preactivation was calculated as the difference between zymosan-dependent and spontaneous CL in experimental (with TNF-α) and control samples (without TNF-α) taken as 1. The preactivating effect was maximum after incubation with TNF-α in a dose of 1 ng: the index of preactivation

TABLE 1. Electrophoretic Mobility of LDL after 3-h Aerobic Incubation with Nonactivated (-TNF-α, Control) and Preactivated (+TNF-α) Macrophages (% of Native LDL, *M*±*m*)

Time of incubation of macrophages with TNF-α	Control	TNF-α concentration, ng/ml			
		0.5	1.0	5.0	10.0
10 min	108±3	109±6	109±7	109±4**	110±3*
30 min	103±5	113±4**	104±2	105±7	100±7
60 min	106±8	112±5**	104±4	104±12	101±8
180 min	96±7	104±7	100±4	92±5	98±8

Note. Preactivation: 10 and 30 min. Activation: 10 min. Here and in Table 2: *p<0.01 and **p<0.05 compared to native LDL. Each series (period of incubation) included 4 independent experiments.

TABLE 2. Electrophoretic Mobility of LDL after 3-h Aerobic Incubation with Nonactivated (-TNF-α, Control), Preactivated (+TNF-α), and Activated (++TNF-α) Macrophages (% of Native LDL, *M*±*m*)

Incubation of macrophages with TNF-α	Control	TNF-α concentration, ng/ml					
		scheme 1			scheme 2		
		0.1	0.5	0.1+0.5	0.5	5	0.5+5
10 min	116±1*	117±4*	120±2*	129±3*+	118±5*	116±4*	116±5**
30 min	110±4**	110±1*	110±3*	108±1*	109±6	109±4	109±3**

Note. +p<0.05: differences between single and 2-fold treatment with TNF-α. Control experiments were performed for each dose (0.1, 0.5 and 5 ng/ml).

for peritoneal macrophages surpassed the control by 1.8 times (Fig. 1). Lower doses were less effective, while TNF-α in a dose of 10 ng produced an inhibitory effect.

When studying the preactivating effect of TNF-α on the ability of human blood macrophages to induce oxidative modification of LDL, we found that incubation of cultured macrophages with TNF-α in various doses for 10, 30, 60, and 180 min increased electrophoretic mobility of LDL. However, these changes were statistically insignificant (compared to nonactivated macrophages, Table 1). TNF-α in a dose of 0.5 ng/ml was most potent, particularly after 30- and 60-min incubation. Incubation of macrophages with TNF-α in various doses for 3 h produced a moderate inhibitory effect and decreased electrophoretic mobility of LDL.

When evaluating preactivating (single treatment) and activating effects (2-fold treatment) of TNF-α in increasing doses on the ability of human blood macrophages to induce oxidative modification of LDL, we found that repeated addition of TNF-α in the activating dose to macrophages preactivated with TNF-α in low dose enhanced their ability to induce oxidative modification of LDL after 3-h incubation. The activating effect was significant after 10-min incubation with TNF-α in low preactivating and activating doses (scheme 1, Table 2).

These data suggest that short-term treatment of macrophages with TNF-α in low doses *in vivo* increases their sensitivity to the same or other activators, intensifies oxidative modification of LDL, and contributes to the development of atherosclerosis. The protective effect of antioxidants on the ability of preactivated or activated macrophages to induce oxidative modification of LDL was evaluated with TNF-α in low doses. Macrophages were incubated with LDL for 3 or 24 h to enhance the oxidizing effect on these particles.

When studying the effects of antioxidants on oxidative modification of LDL by nonactivated, preactivated, and activated macrophages we found that macrophages activated with TNF-α in a dose of 0.1±0.5 ng/ml for 10 min (scheme 1, Table 2) and incubated with LDL for 3 and 24 h caused most pronounced oxidative modification of LDL (compared to nonactivated and preactivated cells, Table 3). Antioxidants potassium phenosan, probucol, and desferal in a dose of 10^{-5} M were more potent in preventing oxidative modification of LDL by preactivated and activated macrophages (compared to nonactivated cells). Moreover, the inhibitory effect of antioxidants after 24-h

TABLE 3. Effect of Antioxidants on Electrophoretic Mobility of LDL after Incubation with Nonactivated, Preactivated, and Activated Macrophages for 3 or 24 h (% of Native LDL, *M*±*m*)

Time of incubation with TNF-α and state of macrophages		Control	Antioxidants, 10^{-5} M		
			potassium phenosan	probucol	desferal
3 h	nonactivated (0)	113±1*	112±1*	110±5	110±6
	preactivated (0.1 ng/ml)	115±5*	115±0.1*	115±1*	111±4**
	activated (0.1±0.5 ng/ml)	125±5*+	119±7**	118±5**	120±2*
24 h	nonactivated	116±4**	107±2**	113±3*	114±2*
	preactivated (0.1 ng/ml)	119±1*	104±4°	106±3*°	103±1*°
	activated (0.1±0.5 ng/ml)	126±1*+	104±1*°	104±1*°	104±2°

Note. +p<0.05: differences between single and 2-fold treatment with TNF-α. °p<0.01 compared to the control. Each series (period of incubation) included 3 independent experiments.

incubation with LDL was more pronounced compared to that observed after 3-h incubation. We revealed no significant differences in the effect of the test antioxidants after incubation with LDL and preactivated or activated macrophages for 3 and 24 h.

Our results indicate that single or 2-fold treatment of macrophages with TNF-α in low doses stimulates production of reactive oxygen species by macrophages and increases their ability to induce oxidative modification of LDL. We first showed that antioxidants are more potent in inhibiting oxidative modification of LDL by activated and activated macrophages (compared to nonactivated cells). These data show that cytokine-stimulated macrophages undoubtedly play a role in the pathogenesis of atherosclerosis.

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