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Influence of Platelet-Activating Factor, Its Cell Analogs, and Antagonist on the Production of Superoxide Radicals by Blood Leukocytes of Healthy and Hypercholesterolemic Individuals

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Abstract—The influence of the phospholipid platelet-activating factor (PAF), its cell analogs, and lipid PAF antagonist on the production of superoxide radicals by leukocytes isolated from the blood of healthy and hypercholesterolemia IIA individuals was studied. It was found that endogenous superoxide production level in the leukocytes of hypercholesterolemic individuals more than 4-5 times higher than in the leukocytes of healthy individuals. Exogenous PAF stimulates the superoxide production in the leukocytes of healthy individuals but significantly inhibits the superoxide production in the leukocytes of hypercholesterolemic individuals. The compounds 1-acyl-2-acetyl-sn-glycero-3-phosphocholine (1-acyl-PAF) and 1-alk-1′-enyl-2-acetyl-sn-glycero-3-phosphocholine (1-alkenyl-PAF) only slightly inhibited the endogenous superoxide production in the leukocytes of hypercholesterolemia individuals. However, pretreatment of leukocytes by 1-alkenyl-PAF or PAF-antagonist (1-O-alk-1′-enyl-2-(2′-acetoxybenzoyl)-sn-glycero-3-phosphocholine) results in a 50% inhibition of the PAF-induced superoxide production by leukocytes of healthy individuals. This PAF-antagonist alone or in combination with PAF induces a substantial (65-70%) inhibition of superoxide production in the leukocytes of hypercholesterolemic individuals. It is concluded that superoxide production by leukocytes of healthy individuals and especially by leukocytes of hypercholesterolemic individuals is process that depends on PAF or PAF-like lipids.

Key words: platelet-activating factor analogs, leukocytes, superoxide radicals, hypercholesterolemia

It is currently accepted that the oxidation of low-density lipoproteins (LDL) plays a leading role in cardiovascular pathology [1]. Lipid peroxides were first detected in atherosclerotic human aorta in 1952 [2], and subsequent studies revealed lipid peroxides in blood plasma in hyperlipidemia [3], essential hypertension [4], myocardial infarction [5], stroke [6], and ischemic heart disease [7]. The elevated content of lipid peroxides in atherosclerotic injury of arteries are associated with its appearance in oxidized LDL [1]. The oxidation of LDL occurs mainly in the circulation by the influence of neutrophils, monocytes, and monocyte-derived macrophages [8]. It is proposed that in bloodstream LDL is oxidized by superoxide radicals and H_2O_2 produced by activated neutrophils and monocytes [9]. The oxidation of LDL first results in the formation of the primary oxidation products (lipid peroxides) and further the formation of secondary oxidation products—unsaturated aldehydes (mainly 4-oxynonenal), epoxyaldehydes, and short-chain fatty acids—was observed [10]. Besides the low-molecular-weight substances in oxidized LDL, modified phospholipids containing epoxyisoprostane residues [11] and acyl PAF analog 1-acyl-PAF (1-acyl-2-acetyl-sn-glycero-3-phosphocholine) were found [12]. It is known that in blood serum in atherosclerosis the level of lysophosphatidylcholine, a metabolite of phosphatidylcholine and 1-acyl-PAF, bound with oxidized LDL is significantly increased [13]. It has been proposed that lysophosphatidylcholine is one of the most active LDL components responsible for the disturbance of endothelium-dependent artery relaxation [14].

Abbreviations: PAF) platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine); 1-acyl-PAF) 1-acyl-2-acetyl-sn-glycero-3-phosphocholine; 1-alkenyl-PAF) 1-O-alk-1′-enyl-2-acetyl-sn-glycero-3-phosphocholine; LDL) low density lipoproteins.

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On the other hand, it was shown that smoking, a well-known risk factor of cardiovascular diseases, results in oxidation of plasma lipoproteins and the formation of PAF or PAF-like lipids in plasma [15-17]. It was shown that smoke inhalation results in increase of erythrocyte and leukocyte count in the blood stream and to significant increase of malonic dialdehyde level in blood, lung, bronchoalveolar fluid [15], and LDL fraction [16]. At the same time the preliminary injection of PAF antagonist CV-3988 result in inhibition of lipid oxidation in the bloodstream and prevented the changes of blood that are induced by smoke inhalation [15]. In study [17] it was shown for the first time that smoking of chronic smokers or nonsmoking individuals results in the appearance in plasma of PAF or PAF-like lipids. These lipids were identified by TLC and their proaggregatory activity toward neutrophils and comparison of its activity with the activity of PAF-antagonist CV-3988 were studied [17]. These data show that oxidation of lipoproteins leading in the first stage to the formation of lipid peroxides in the end result in formation of the secondary oxidation products (aldehydes, epoxyaldehydes) that modify lysine amino groups of apoproteins [10] and to formation of a number of lipid metabolites—PAF, 1-acyl-PAF, lysophosphatidylcholine, and phospholipids containing fatty acid residues modified by oxidation. Taking into account a high biological activity of PAF, 1-acyl-PAF, lysophosphatidylcholine (see reviews [18-20]), and oxidized phospholipids [21], it can be assumed that these lipid metabolites can influence the interaction of oxidized LDL with endothelial cells and to be the induces of subsequent activation of neutrophils, monocytes, and macrophages that in turn lead to a "permanent" inflammatory reaction which may result in LDL oxidation on a significantly high level.

In this work, we studied the ability of PAF, 1-acyl-PAF, and 1-alkenyl-PAF to induce oxidative metabolism in neutrophils and by means of PAF antagonist we studied the role of PAF in the activation of blood leukocytes of hypercholesterolemic individuals.

MATERIALS AND METHODS

Dextran T-500 and Ficoll-hypaque from Pharmacia (Sweden), bovine heart cytochrome *c* and acetylsalicylic acid from Sigma (USA), purified human erythrocyte superoxide dismutase (specific activity 3000 ± 500 units per mg protein) from NPO Rostepidcomplex (Rostov-on-Don, Russia), and Hanks' solution without phenol red from NPO Virion (Tomsk, Russia) were used in this study.

PAF was obtained from beef heart choline plasmalogens as described previously [22].

1-Acyl-PAF (1-acyl-2-acetyl-sn-glycero-3-phosphocholine) was obtained by acetylation of 1-acyllyso-sn-glycero-3-phosphocholine with acetic anhydride and purified by column chromatography on silica gel as described previously [23].

1-Alkenyl-PAF (1-O-alk-1′-enyl-2-acetyl-sn-glycero-3-phosphocholine) was obtained by acetylation of 1-O-alk-1′-enyllysoglycero-3-phosphocholine with acetic anhydride, purified, and characterized as described previously [24].

1-O-alk-1′-enyl-2-(2′-acetoxybenzoyl)-sn-glycero-3-phosphocholine was obtained by acylation of 1-O-alk-1′-enyllyso-sn-glycero-3-phosphocholine with chloroanhydride of 2-acetoxybenzoic acid as described previously [25].

Bioactive amide of prostaglandin E_1 and ethanolamine plasmalogen PAF analog 1-O-alk-1′-enyl-2-acetyl-sn-glycero-3-phospho-(N-11α,15α-dioxy-9-keto-13-prostenoyl)ethanolamine was obtained by condensation of prostaglandin E_1 with 1-O-alk-1′-enyl-2-acetyl-sn-glycero-3-phosphoethanolamine in the presence of N,N-dicyclohexylcarbodiimide and purified by TLC as described previously [26].

Leukocyte-rich cell suspension was isolated from fresh human donor blood by precipitation of erythrocytes by dextran T-500 following by the centrifugation in one-step Ficoll-hypaque gradient as describe previously [27]. Blood of individuals with hypercholesterolemia type IIA was obtained in the Myasnikov Institute of Clinical Cardiology. The hypercholesterolemic individuals used in this study has the following biochemical indexes: total cholesterol, 8.3-14.3 mmol/liter; LDL cholesterol, 6.6-13.0 mmol/liter; high-density lipoprotein cholesterol, 0.68-1.22 mmol/liter; triglycerides, 1.3-2.3 mmol/liter.

For analysis of superoxide radical production isolated leukocytes ($4 \cdot 10^7$ cells per ml) were suspended in Hanks' solution containing cytochrome *c* (0.4 mg per ml) in absence or presence of PAF, cell PAF analogs, and PAF antagonist at 37°C for 15 min. After incubation, cells were centrifuged at 600*g* for 10 min. Absorption spectra of cytochrome *c* in the 450-600 nm region were measured in these supernatants with a Specord M40 spectrophotometer. The level of superoxide radical production was determined by the difference of optical density at 550 nm between experiment and control samples containing superoxide dismutase (0.4 mg per ml), the molar extinction coefficient 21 $mM^{-1} \cdot cm^{-1}$ being used [28].

Results given in the table are the means of three parallel measurements ± the standard error of the means. Similar results were obtained in 4-5 independent experiments.

RESULTS AND DISCUSSION

The results of the study on the influence of PAF, 1-acyl-PAF, and 1-alkenyl-PAF on the formation of superoxide radicals by leukocytes of the blood of healthy donors and hypercholesterolemic individuals are present-

Table 1. Influence of PAF on the superoxide radical production by isolated blood leukocytes of healthy donors and hypercholesterolemic individuals

Additives	Superoxide radical production, nmol/h per 10^7 cells	
	healthy donors	hypercholesterolemic individuals
Without additives	0.87 ± 0.20	4.3 ± 0.4
PAF	1.35 ± 0.06	1.55 ± 0.2
1-Acyl-PAF	1.16 ± 0.17	4.1 ± 0.35
1-Alkenyl-PAF	0.94 ± 0.20	3.9 ± 0.27

Note: Concentration of PAF and its analogs in medium was 1 μM.

ed in Table 1. The data in Table 1 show that leukocytes of blood of healthy donors has a definite level of oxidative metabolism that is monitored by superoxide radical production. The endogenous level of superoxide radical production in the leukocytes of hypercholesterolemic individuals was more than 4-5 times higher than in leukocytes of healthy donors. This difference in the superoxide production may indicate that leukocytes of hypercholesterolemic individuals are in more "activated" state than leukocytes of healthy donors. The addition of PAF to the incubation medium of leukocytes of healthy donors results in the stimulation of superoxide radical production. In contrast, addition of PAF to leukocytes of hypercholesterolemic individuals results in the opposite effect: PAF induced more than 60%-inhibition of superoxide radical production. 1-Acyl-PAF has a stimulating effect on the superoxide radical formation in leukocytes of healthy donors but to a lesser extent than PAF. The influence of 1-alkenyl-PAF on the oxidative metabolism of leukocytes of blood of healthy donors was negligible. 1-Acyl-PAF and 1-alkenyl-PAF practically has no influence on the superoxide radical production in the leukocytes of blood of hypercholesterolemic individuals. These data suggest that superoxide radical production in the leukocytes of both types significantly depend on PAF. However, in the leukocytes of blood of healthy donors the level of production of endogenous PAF as well as their activation by endogenous PAF may be relatively low but in leukocytes of hypercholesterolemic individuals endogenous PAF production may be high, this resulting in the increased activated state of the leukocytes. In this case addition of exogenous PAF appears to result in desensitization of PAF-receptors and decrease in superoxide radical production, respectively. To evaluate the role of PAF in stimulation of oxidative metabolism in leukocytes, we used a number of inhibitors and PAF antagonists. Human erythrocyte superoxide dismutase at concentration of 12 μM completely inhibits the PAF-induced superoxide radical production in leukocytes (Table 2). These data show that measured changes in cytochrome *c* spectrum at 550 nm in the absence or presence of PAF depend on superoxide radical production in the course of endogenous activation of leukocytes and after addition of exogenous PAF to leukocytes.

Table 2. Influence of PAF cell analogs, PAF antagonist, and other inhibitors on PAF-induced superoxide production by isolated leukocytes of healthy donors

Inhibitor	Concentration of compounds in the incubation medium, μM	Inhibition of superoxide radical production, %
Superoxide dismutase	6.0 12.0	74.0 ± 5.0 100.0 ± 4.5
1-Akenyl-PAF	1.0	49.8 ± 6.3
1-Acyl-PAF	1.0	7.0 ± 0.4
1-O-alk-1′-enyl-2-acetyl-sn-phospho-(N-11α,15α-dioxy-9-keto-13-prostenoyl) ethanolamine	1.0	22.3 ± 0.7
1-O-alk-1′-enyl-2-(2′-acetoxybenzoyl)-sn-glycero-3-phosphocholine	1.0 10.0	50.7 ± 4.6 76.7 ± 6.3
Acetylsalicylic acid	1.0	0.5 ± 0.2

The PAF analog 1-alkenyl-PAF is a specific inhibitor of PAF-induced platelet aggregation and does not influence ADP- or thrombin-induced platelet aggregation [24]. Addition of 1-alkenyl-PAF to isolated leukocytes resulted in 50% inhibition of PAF-induced superoxide radical production in leukocytes of blood of healthy donors (Table 2). At the same time, 1-acyl-PAF did not influence the PAF-induced superoxide radical production.

Recently we obtained plasmalogenic PAF analog containing a prostaglandin E_1 residue (1-O-alk-1′-enyl-2-acetyl-sn-glycero-3-phospho-(N-11α,15α-dioxy-9-keto-13-prostenoyl)ethanolamine) that is an broad spectrum inhibitor of platelet aggregation and inhibits PAF-, ADP-, or thrombin-induced platelet aggregation [26]. In the study of its activity toward leukocytes of blood of healthy donors, it was found that this analog induced only small (no more than 20-22%) inhibition of PAF-induced superoxide radical production (Table 2).

Table 3. Influence of PAF and PAF antagonist containing acetylsalicylic acid residue on superoxide radical production by isolated blood leukocytes of hypercholesterolemic individuals

Additives	Inhibition of superoxide radical production, %
PAF	49.6 ± 17.8
PAF antagonist containing acetylsalicylic acid residue	67.7 ± 12.2
PAF and PAF antagonist	71.0 ± 4.3

Note: The final concentration of PAF and PAF antagonist in incubation medium were 1 μM.

A PAF antagonist containing an acetylsalicylic acid residue (1-O-alk-1′-enyl-2-(2′-acetoxybenzoyl)-sn-glycero-3-phosphocholine) at concentration 1 μM induced a 50%-inhibition of PAF-induced superoxide production and at concentration 10 μM the extent of inhibition was up to 70-80% (Table 2).

The study of the influence of PAF and PAF antagonist on the superoxide radical production in isolated leukocytes of blood of hypercholesterolemic individuals revealed that exogenous PAF at concentration 1 μM induced a 50%-inhibition of the oxidative metabolism (Table 3). PAF antagonist containing an acetylsalicylic acid residue at the same concentration (1 μM) inhibits the superoxide radical production by 67.7 ± 12.2%. A joint action of PAF and PAF antagonist result in further decrease of superoxide radical production but in this case the extent of inhibition does not exceed 70%. At the same time, the acetylsalicylic acid itself is not able to inhibit superoxide radical production in leukocytes, consistent with published data [8].

The data show that superoxide radical production in leukocytes of healthy donors and hypercholesterolemic individuals to a great extent is a PAF-dependent process or process dependent on formation of PAF-like lipids. However, there are substantial differences between healthy donors and hypercholesterolemic individuals. The endogenous level of oxidative metabolism in the blood leukocytes of hypercholesterolemic individuals more than 4-5 times exceeds that of leukocytes of healthy donors. What is the reason for the increased activation of the leukocytes of hypercholesterolemic individuals in comparison to leukocytes of healthy donors? Since the addition of exogenous PAF in this case does not result in stimulation of superoxide production, it can be assumed that leukocytes of hypercholesterolemic individuals is activated by endogenous PAF as the result of its increased synthesis in comparison to leukocytes of healthy donors. This assumption may be supported by the data of study [29] that show that in leukocytes of hypercholesterolemic (IIA) individuals ionophore A23187-, chemotactic peptide fMet-Leu-Phe-, or zymosan-induced PAF synthesis significantly increased in comparison to leukocytes of healthy donors and in the plasma of these individuals an increased level of lysoderivative of PAF (1-O-alkyllyso-sn-glycero-3-phosphocholine) was observed. It was shown that functional activity of monocytes of hypercholesterolemic individuals toward chemotaxis and adhesion to endothelial cells significantly increased in comparison to blood monocytes of healthy donors [30].

It is known that upon stimulation of blood leukocytes synthesis of PAF proceeds along with synthesis of 1-acyl-PAF (see review [18]). Increased level of lysophosphatidylcholine (1-acyllyso-sn-glycero-3-phosphocholine) that observed in blood serum of patients with atherosclerosis [13] may be an additional precursor pool for synthesis of 1-acyl-PAF in the leukocytes of hypercholesterolemic individuals.

The data presented here suggest that the formation of oxidized phospholipids and PAF-like lipids observed in LDL oxidation of neutrophils and monocytes and as in consequence the newly formed active forms of oxygen may initiate oxidation of "new" LDL. Thus, the mechanisms of LDL oxidation and neutrophil activation become interrelated and in the case of hypercholesterolemic individuals the "normal state" of leukocytes may be a constantly activated state accompanied by a constant production of superoxide radicals. Since the oxidized LDL inhibits activity of NO-synthetase in endothelial cells [31] and uptake of L-arginine, a substrate for NO synthesis [32], by these cells the superfluous generation of the oxidized LDL may, in turn, result in subsequent violation of normal regulation of blood vessels that depends on synthesis of NO by endothelial cells.

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