Mechanism of Activation of ADP-Induced Platelet Aggregation under the Influence of Oxidatively Modified Fibrinogen

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For evaluation of the mechanisms underlying the effect of oxidized fibrinogen on platelet aggregation we studied ADP-induced platelet aggregation in the presence of UV-oxidized fibrinogen and inhibitors of major enzymes of platelet activation. Cyclooxygenase inhibitor acetylsalicylic acid, protein kinase C inhibitor H7, and to a lesser extent, protein tyrosine kinase inhibitor genistein suppressed ADP-induced platelet aggregation. In the presence of oxidized fibrinogen the degree of suppression was lower than in the presence of nonoxidized fibrinogen. Phospholipase C inhibitor U73122 markedly suppressed platelet aggregation in the presence of oxidized and nonoxidized fibrinogen. It can be hypothesized that oxidized fibrinogen activates platelets by modulating activity of the key signal component phospholipase C.

Key Words: *oxidized fibrinogen; inhibitory assay; platelet aggregation*

Fibrinogen is a plasma globular glycoprotein involved in cascade reactions of blood coagulation. Fibrinogen stimulates migration and proliferation of smooth muscle cells, promotes platelet aggregation, increases blood viscosity [6], and exhibits mitogenic and angiogenic activity [11]. The concentration of this acute phase protein increases during inflammation. Plasma fibrinogen level exceeding the reference limit is an independent risk factor for the development of thrombosis during various inflammatory processes [8,12]. In patients with cardiovascular diseases a relationship was found between fibrinogen content and severity of disorders [5], mortality rate [9], and risk of postsurgical complications [7].

Free radical processes resulting in oxidation of low-density lipoproteins play a major role in the pathogenesis of atherosclerosis [2,4]. Other blood proteins also serve as a target for oxidative modification with free radicals, which can cause thrombotic complications [3]. Fibrinogen is one of the most easily oxidized plasma proteins [10]. However, its role in damage associated with free radical processes is poorly understood. Probably, activation of free radical processes is accompanied by accumulation of oxidized fibrinogen in the blood and stimulation of thrombus formation. This assumption is confirmed by published data that oxidized fibrinogen acts as inductor of platelet aggregation [1].

Here we evaluated the mechanisms underlying the influence of oxidized human plasma fibrinogen on platelet aggregation. We studied the effects of inhibitors of major enzymes of platelet activation on ADP-induced platelet aggregation in the presence of UV-oxidized fibrinogen.

MATERIALS AND METHODS

Fibrinogen (Sigma) was dissolved in buffered physiological saline containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 10 mM Na_2HPO_4 (pH 7.4). Oxida-

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tive UV modification was carried out using a DRK-120 ultrahigh-pressure mercury arc lamp with natural cooling (operating current 1.2 A). Oxidative modification of fibrinogen was controlled by the decrease in tryptophan fluorescence [1]. Fibrinogen (100 μl) was placed in a cuvette, the volume was brought to 4 ml with phosphate buffer (50 mM, pH 7.4), and the intensity of fluorescence was measured on a Perkin-Elmer LS50 spectrofluorimeter at 280 nm. The emission wavelength was 340 nm. Oxidized fibrinogen (10% oxidation) was used to study platelet aggregation.

The blood was taken from 15 healthy donors. ACD buffer containing 3.8% sodium citrate and 10 mM D-glucose was added to the blood (1:10 v/v). Platelet-rich plasma (PRP) was obtained by centrifugation of the blood at 200*g* for 15 min.

The inhibitory assay was performed by incubation of PRP with various inhibitors of enzymes involved in intracellular signaling. Platelet aggregation in PRP was studied in the presence of oxidized and nonoxidized fibrinogen. Each inhibitor was studied in 3 independent experiments. We used cyclooxygenase inhibitor acetylsalicylic acid (AA), protein kinase C inhibitor 1-(5-isoquinoline sulfonyl)-2-methylpiperazine (H7), tyrosine kinase inhibitor genistein, antioxidant and lipoxygenase inhibitor α-tocopherol acetate, and phospholipase C inhibitor U73122 (Sigma). Stock solutions of inhibitors were prepared in distilled water (AA and H7), ethanol (U73122 and α-tocopherol acetate), and dimethylsulfoxide (DMSO, genistein).

Platelet aggregation was studied by kinetic turbidimetry on an automatic platelet aggregation analyzer (Biola). PRP ($1.5\text{-}3\times10^8$ cells/ml) was mixed 1:1 with fibrinogen solution (2 mg/ml). The sample was heated to 37°C. Aggregation curves were recorded after 2 min. ADP (5 μmol/liter) served as an inductor of aggregation.

The results were analyzed by nonparametric Wilcoxon test. The differences were significant at $p<0.05$.

RESULTS

Fifteen independent experiments showed that oxidized fibrinogen activates ADP-induced platelet aggregation by 1.33±0.22 times compared to nonoxidized fibrinogen. Light transmission (LT) was measured 3.5 min after addition of ADP and served as a major index during inhibitory assay.

Metabolism of arachidonic acid in platelets is catalyzed by cyclooxygenase and lipoxygenase. Metabolites of cyclooxygenase are involved in the regulation of platelet function. For example, the proaggregation compound thromboxane A_2 is synthesized from arachidonic acid by the cyclooxygenase pathway. Cyclooxygenase blockade with indomethacin suppresses phase II of platelet aggregation and secretion of dense granules induced by ADP and other weak agonists.

Our experiments confirmed that AA inhibits phase II of platelet aggregation (Fig. 1, *a*). After addition of oxidized fibrinogen to PRP, suppression of platelet aggregation with the inhibitor was less pronounced than in the presence of nonoxidized fibrinogen (Fig. 1, *b*).

After incubation with AA at concentrations of 1.5, 5, and 150 μmol/liter in the presence of nonoxidized fibrinogen, LT decreased by 1.41, 2.22, and 4.25 times, respectively, compared to the control. In the

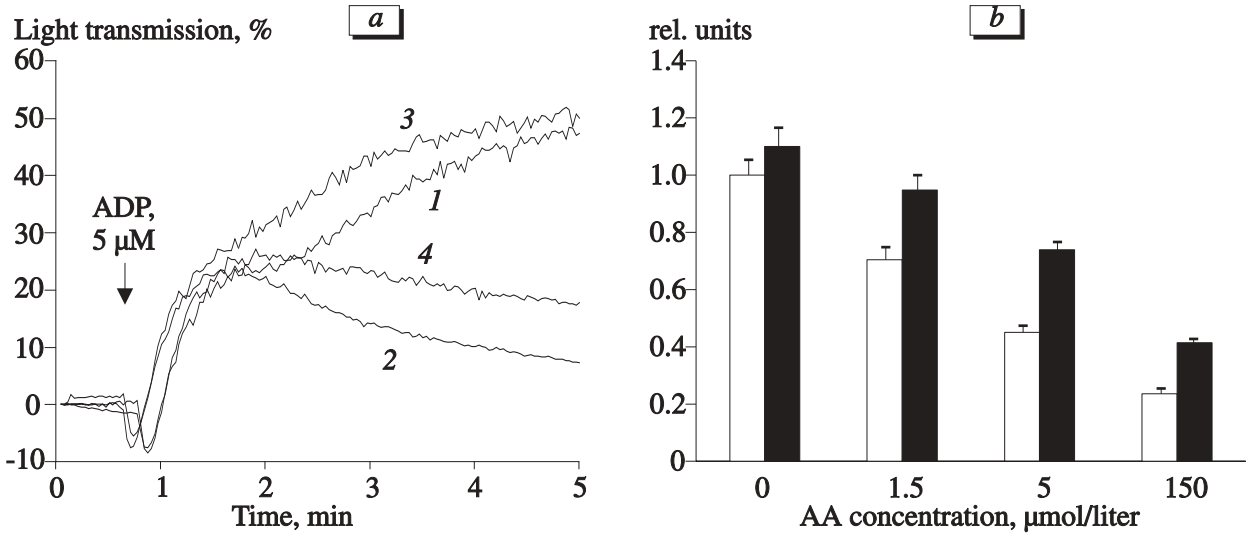

Fig. 1. Effect of acetylsalicylic acid (AA) on ADP-induced platelet aggregation in the presence of fibrinogen in a concentration of 1 mg/ml. Incubation: 20 min. *a*) Kinetic curves of changes in light transmission of ADP-induced platelet aggregation in the presence of nonoxidized (*1*, *2*) and 10% oxidized fibrinogen (*3*, *4*): without (*1*, *3*) and with 150 μmol/liter AA (*2*, *4*). *b*) light transmission in the presence of nonoxidized (light bars) and 10% oxidized fibrinogen (dark bars).

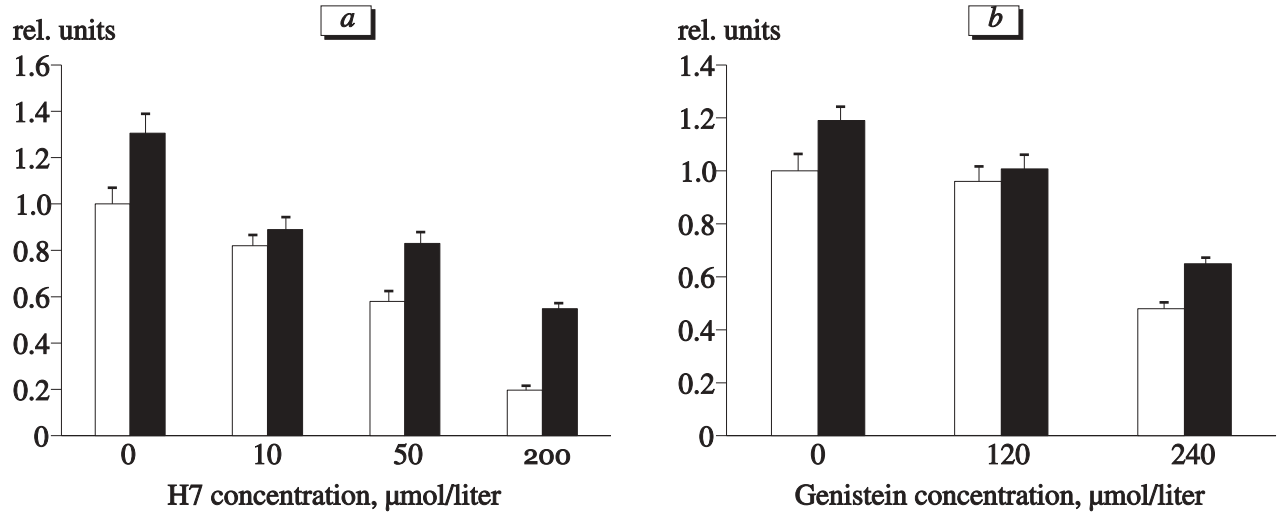

Fig. 2. Effects of inhibitors of protein kinase C (H7, *a*) and tyrosine protein kinase (genistein, *b*) on ADP-induced platelet aggregation in the presence of nonoxidized (light bars) and oxidized fibrinogen (dark bars) in a concentration of 1 mg/ml. Incubation with H7 and genistein for 60 and 20 min, respectively. Ordinate: changes in light transmission of the platelet suspension 3.5 min after the addition of 5 μM ADP.

presence of oxidized fibrinogen, LT decreased less significantly (by 1.16, 1.49, and 2.65 times, respectively, compared to the control). These data suggest that oxidized fibrinogen activates cyclooxygenase.

Our results are of practical importance, since AA is extensively used as the antiaggregation drug. Oxidative stress accompanying exacerbation of coronary heart disease and promoting oxidation of fibrinogen can reduce the effectiveness of AA therapy. These changes can be evaluated by studying fibrinogen oxidation in the blood.

Protein kinase C plays an important role in platelet activation. This enzyme is induced with diacylglycerol, and its activity depends on the presence of Ca^{2+}. Protein kinase C affects receptors on platelets (*e.g.*, α_1-adrenoceptor), modifies signal transduction by modulating the effect of agonists on adenylate cyclase and hydrolysis of PIP_2, and changes activity of Ca^{2+}-channels.

Inhibition of protein kinase C with H7 markedly suppressed platelet aggregation and changed the mean radius of aggregation and parameters of kinetic curves of LT (data not shown) in aggregation phases I and II. Oxidized fibrinogen counteracted the effect of this inhibitor (similarly to AA, Fig. 2, *a*). A considerable effect was observed at relatively high concentrations of H7 (above 200 μmol/liter). H7 in a concentration of 200 μmol/liter decreased LT in the presence of nonoxidized fibrinogen by 5.07 times compared to the

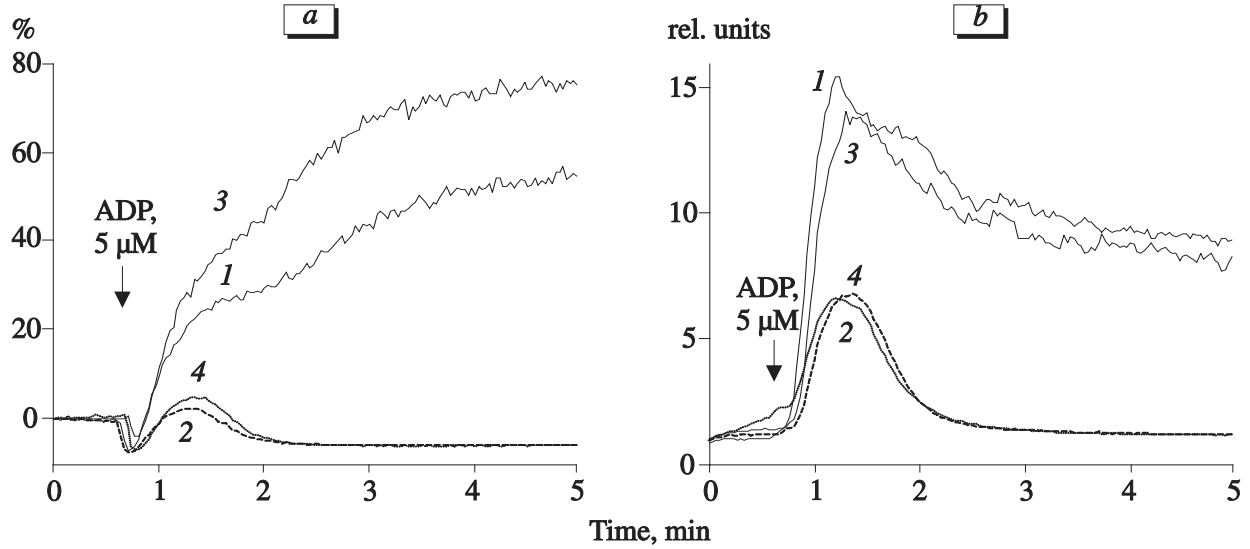

Fig. 3. Effect of the phospholipase C inhibitor U73122 on ADP-induced platelet aggregation in the presence of nonoxidized (*1*, *2*) and oxidized fibrinogen (*3*, *4*): without the inhibitor (*1*, *3*) and after 1-h incubation with 4 μM U73122 (*2*, *4*). Kinetic curves of changes in light transmission (*a*) and mean radius of aggregates (*b*).

control. However, LT decreased only by 2.38 times in the presence of oxidized fibrinogen.

Our results indicate that oxidized fibrinogen activates protein kinase C. It was interestingly to study the effects of inhibitors on another modulator of intracellular signaling (tyrosine kinase).

We found that genistein moderately suppressed ADP-induced platelet aggregation. It primarily concerned LT, but not the mean radius of aggregation. Oxidized fibrinogen insignificantly suppressed the effect of genistein even in the highest dose (240 μmol/liter, Fig. 2, *b*). In the presence of nonoxidized and oxidized fibrinogen, genistein decreased LT by 2.08 and 1.83 times, respectively.

We hypothesized that the effect of oxidized fibrinogen on platelets is mediated via activation of lipid peroxidation. It affects the pathways of intracellular regulation that involve free radical oxidation. We studied the effect of fat-soluble antioxidant α-tocopherol (50-300 μM) on ADP-induced platelet aggregation. Preincubation of PRP with α-tocopherol did not suppress ADP-induced platelet aggregation (independently on oxidation of fibrinogen). Probably, the effect of this inhibitor can be observed after treatment with other inductors of aggregation.

Phosphoinositide-specific phospholipase C is a key enzyme of intracellular signaling. Its product inositol triphosphate triggers the release of intracellular Ca^{2+}. Diacylglycerol (another product of phospholipase C) and Ca^{2+} activate protein kinase C.

We found that phospholipase C inhibitor U73122 markedly suppresses ADP-induced platelet aggregation. This effect was observed in the presence of nonoxidized and oxidized fibrinogen (Fig. 3).

Hence, inositol triphosphate-dependent increase in intracellular Ca^{2+} concentration is required for the action of oxidized fibrinogen. Activation of platelets by oxidized fibrinogen is probably associated with its influence on phospholipase C.

Arterial thrombosis results from the events that depend on the interaction between damaged vascular wall, blood cells, and coagulation factors with the involvement of specific mediators. Activation of this process is mediated by major coagulation mechanisms and associated with generation of free radicals (oxygen metabolites).

Published data show that free radicals stimulate production of tissue factor mRNA in cells of the vascular endothelium [3]. These changes are accompanied by a considerable increase in procoagulant activity of tissue factor in the blood.

Free radicals affect not only cells, but also blood proteins. Free radicals attack circulating lipoproteins, which results in the formation of oxidized lipoproteins inhibiting plasma antithrombin [3]. It increases the amount and activity of thrombin. Oxidation of a key antithrombotic factor α_2-antiplasmin, plasminogen activator, and thrombomodulin produces the same prothrombotic effect.

Plasma fibrinogen is easily oxidized [10]. Our results indicate that oxidation of fibrinogen has prothrombotic consequences. Phospholipase C of platelets is the most probable target for fibrinogen.

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