Effect of Oxidation-Modified Fibrinogen on the Formation and Lysis of Fibrin Clot in the Plasma

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Turbidimetry studies showed that after addition of thrombin to fresh donor plasma light scatter in the sample increases and slowly attains a plateau. The process of fibrin formation was less intensive in the presence of oxidized fibrinogen. The formation of fibrin clot in lyophilized plasma was characterized by a biphasic kinetic of light scatter, oxidized fibrinogen inhibited both phases of the process. In the presence of streptokinase, oxidized fibrinogen did not modify the kinetics of fibrin clot lysis. Addition of oxidized fibrinogen to plasma reduced optical density of fibrin clot the more intensely the higher was the degree of oxidative modification of fibrinogen.

Key Words: *oxidative stress; turbidimetry; fibrinogen*

Oxidative stress and intensification of free radical generation play the key role in the development of ischemia, inflammation, and many diseases associated with these conditions, for example, hypertension, diabetes, cancer, and cardiovascular diseases [5]. The effects of oxidative stress can be direct (modification of cells by free radicals) and indirect (through cell components modified by oxidation, *e.g.* oxidized lipids and proteins). Oxidized LDL play an important role at all stages of the development of atherosclerosis and its complications, starting from the appearance of foamy cells in the vascular walls with subsequent formation of atherosclerotic plaques and their rupture, which leads to strokes and infarctions. Other proteins, *e.g.* fibrinogen, can also underwent oxidative modification. Fibrinogen is involved in clotting and fibrinolysis, is an independent risk factor of atherosclerosis, strokes, and infarctions. The mechanism of processes underlying the development of atherosclerosis with participation of fibrinogen is little studied. In addition, fibrinogen is most sensitive to oxidative modification among plasma proteins. This fact suggests that oxidatively modified fibrinogen (OF) modulates atherogenesis and clotting.

We previously showed that highly oxidized fibrinogen by obtained UV exposure markedly disregulated blood rheology and clotting: it stimulates ADP-induced platelet aggregation, increases blood viscosity, and promotes accumulation of fibrin-monomer complexes [2,3].

Using the method of turbidimetry we studied the effects of OF on the formation and lysis of fibrin clot (FC) under the effect of exogenous thrombin.

MATERIALS AND METHODS

Fibrinogen (Sigma) was dissolved in buffered physiological saline (5 mM NaH_2PO_4, 140 mM NaCl, pH 7.4) in a concentration of 2 mg/ml. Streptokinase (Sigma) was dissolved in buffered saline to a concentration of 2000 U/ml. Fibrinogen solution was incubated with different concentrations of $FeSO_4 \times 7H_2O$—H_2O_2 (5×10^{-5} M, 10^{-4} M, 3×10^{-4} M, 5×10^{-4} M) for 1 h at 37°C. Desferrioxamine mesylate (100 mM) was then added (10 μl/ml fibrinogen solution) for binding Fe^{2+} and Fe^{3+} ions. Thus oxidized protein was dialysed for 15 h in phosphate buffer (pH 7.4). Human lyophilized

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thrombin with activity of about 100 U/ml was used. Distilled water (1 ml) and stabilizer (0.2 ml; Thrombin test kit, Renam) were added to the flask with thrombin. The stock solution of thrombin was diluted 20-fold with saline before use.

The kinetics of FC formation was recorded in a solution containing 40 mM KH_2PO_4 and 10 mM KCl (pH 7.4); $CaCl_2$ was used as 15 mM solution. Donor venous blood was collected into tubes with 3.8% (0.11 mol/liter) sodium citrate (9:1). The plasma was separated by 15-min centrifugation at 2500 rpm on a CM 6M centrifuge. Normal pooled plasma collected from 20 donors aging 20-40 years, stabilized with HEPES-citrate buffer, and lyophilized was also used. Before the study, 1 ml distilled water was added to a flask with dry plasma.

Light absorpion of the incubation medium was measured on a KFK-2 photoelectrocalorimeter with a 364 nm photofilter in a 0.5-cm cuvette at 37°C and recorded with using a KSP-4 recorder. The recording solution (2 ml), $CaCl_2$ (150 μl), plasma (0.2 ml), fibrinogen (0.1 ml), and thrombin (0.1 ml) were added to the cuvette; then 0.1 ml streptokinase was added. The degree of oxidative modification of proteins was evaluated by accumulation of carbonyl derivatives in the reaction with 2,4-dinitrophenylhydrasine [1].

The results were processed using Student—Fisher test.

RESULTS

The turbidimetric method for registration of FC formation under the effect of exogenous thrombin allows evaluation of quantitative parameters of two stages of this process: formation of protofibrils (medium turbidity does not change during this stage) and their lateral aggregation (this stage is characterized by the appearance of solid phase). The initial phase is evaluated by the time during which optical parameters of the incubation medium do not change (T_{lag}; Fig. 1). Phase 2 starts when a sufficient level of protofibrils is attained and is associated with an increase in optical density [4]. Optical density of FC can be evaluated by the maximum values of the light scatter kinetic curves. We hypothesized that optical density of FC determined its mechanical compactness. The process of fibrinolysis under the effect of streptokinase was evaluated by the time of optical density decrease from its peak to its initial value (T_{lysis}; Fig. 1).

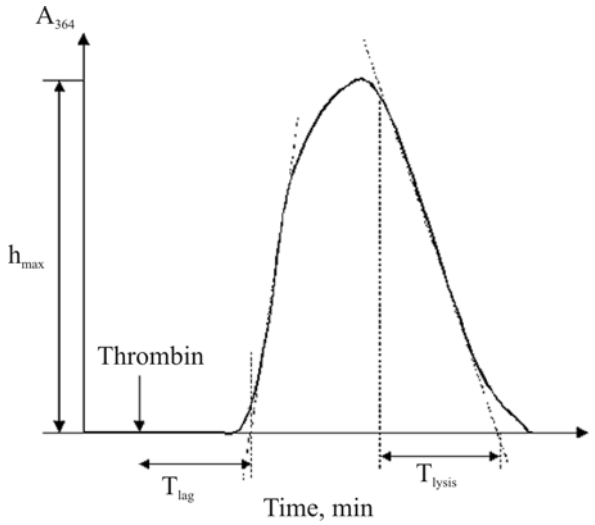

Fig. 1. Typical clotting and fibrinolysis curve obtained by the turbidimetric method.

OF was added to fresh or lyophilized donor plasma and the kinetics of its clotting in the presence of thrombin was recorded. After addition of thrombin to fresh plasma, light scatter increased and slowly reached a plateau (Fig. 2, *a*). After addition of OF, fibrin formation was slower, which was seen from smoother slope of the light scatter kinetic curve (Fig. 2, *a*). The percent of light scatter decrease 10 min after thrombin addition increased with increasing fibrinogen oxidation degree (Table 1). Addition of native (non-oxidized) fibrinogen (NF) in the studied concentrations did not change clotting parameters. These results suggest that mechanical compactness of FC decreases in the presence of OF.

The increase of light transmission during FC formation in lyophilized plasma was biphasic (Fig. 2, *b*). Oxidized fibrinogen also inhibited fibrin formation. The inhibition of fibrin formation process increases

TABLE 1. Relationship between the Percentage of Inhibition of Light Scatter Intensity Maximum and the Degree of Oxidative Modification of Added Fibrinogen in Different Clotting Systems (*M*±*m*)

Fibrinogen oxidation degree, nmol/mg protein	Donor plasma	Lyophilized plasma	Donor plasma+ streptokinase	Lyophilized plasma+ streptokinase
0.8	38±1	38±2	0	38±1
2.43	44±5	44±4	20±4	40±3
3.4	46±2	47±5	23±2	47±4
11.5	49±3	60±7	27±5	56±6

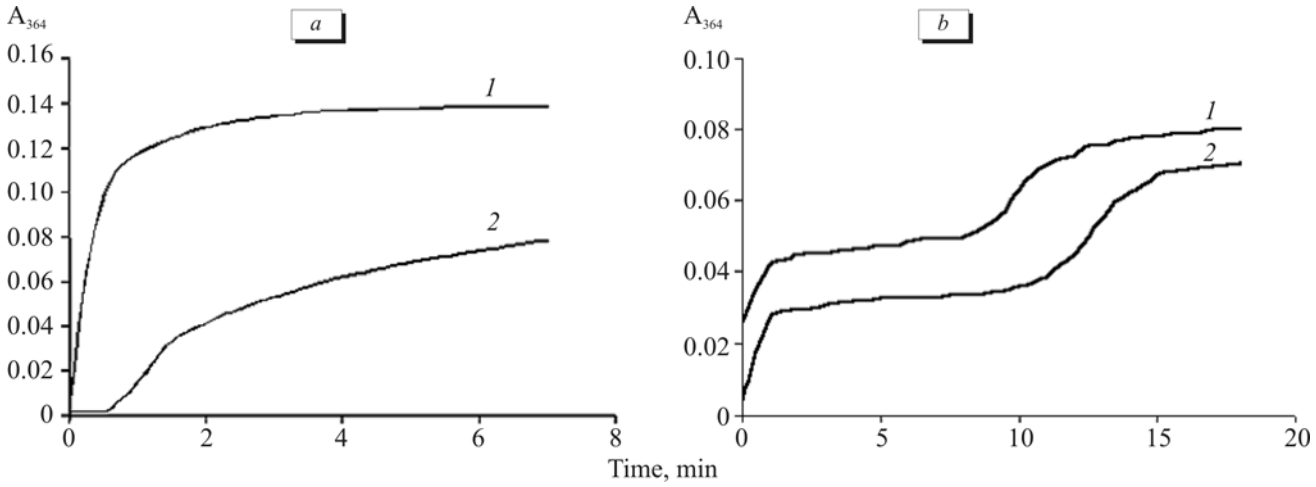

Fig. 2. Clotting curves after addition of NF (*1*) and OF (11.5 nmol/mg, *2*) to fresh (*a*) and lyophilized (*b*) plasma.

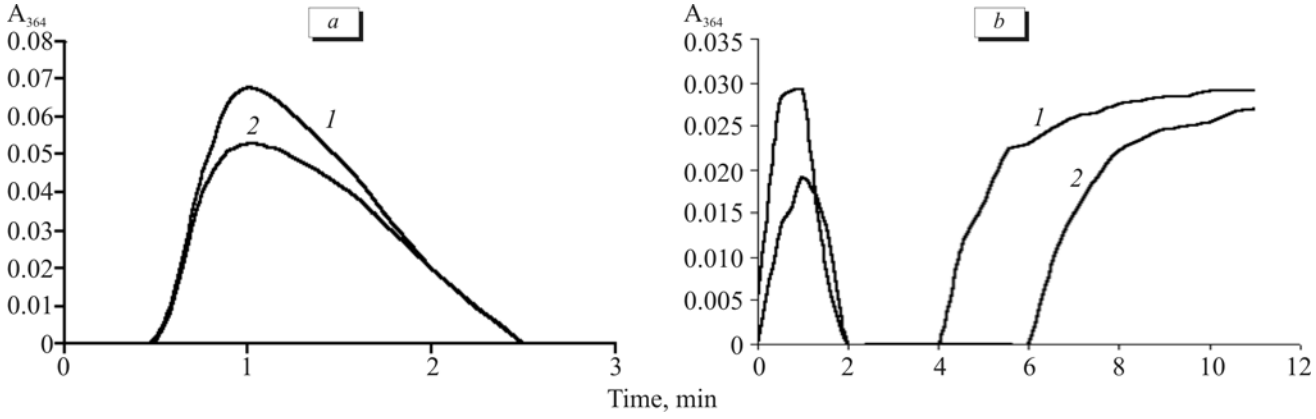

Fig. 3. Kinetics of FC formation and lysis in the presence of streptokinase after addition of NF (*1*) and OF (11.5 nmol/mg, *2*) to fresh (*a*) and lyophilized (*b*) plasma.

with increasing the degree of oxidative modification of fibrinogen. The presence of two light scatter peaks suggests that FC formation in lyophilized plasma was a two-staged process.

After addition of streptokinase to fresh plasma, the kinetic curve of clotting and fibrinolysis attained the maximum values of light scatter returned to its initial level (Fig. 3, *a*). This indicated that the formed FC was dissolved by streptokinase. The maximum intensity of light scatter decreased with increasing fibrinogen oxidation degree. Our experiments revealed no effect of OF on the kinetics of FC lysis.

In the presence of streptokinase, the clotting curve for lyophilized plasma had two light scatter peaks with a latent period between them (Fig. 3, *b*). The duration of this latent period increased 2-3-fold with increasing the degree of fibrinogen oxidative modification.

Addition of OF reduced optical density of FC in all test systems (Table 1). We hypothesize that mechanical compactness of FC and hence, its mechanical strength also decrease in the presence of OF. The decrease in mechanical strength of FC is probably responsible for detachment of a clot from the vascular wall leading to ischemia of this or that organ; for example, it can provoke stroke or infarction.

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