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Effects of Free Fatty Acids on Fibrinolytic Activity

Toshiharu MURAOKA and Hiromichi OKUDA
Department of Medical Biochemistry, School of Medicine, Ehime University, Onsen-gun, Ehime 791-02

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A novel method for the estimation of fibrinolytic activity is proposed. In this method, a fibrin clot suspension is used as a substrate (fibrin is known to be a physiological substrate of plasmin). The fibrin clot suspension was prepared by homogenization of human fibrin clots. With this method, we found that free fatty acids inhibited the plasmin activity, and long-chain, unsaturated free fatty acids had a particularly strong inhibitory action on plasmin. As regards the mechanism of the inhibitory action, free fatty acids may not inhibit complex formation between plasmin and fibrin, but may make it impossible for plasmin to act on fibrin due to deformation of the surface of the fibrin clot.

It is well-known that thrombosis might be involved in the pathogenesis of atherosclerosis (*1–4*), and fibrinolysis has a significant role in the resolution and organization of thrombi on vessel walls (*5*). Astrup reported that films of fibrin might be formed on the arterial walls, especially where strain had caused tissue damage with local release of thromboplastic agents, and that in an organism with an unbalanced fibrinolytic system, where lysis was delayed, a sequence of atherosclerotic events was initiated (*6*). Onoyama and Tanaka found that the fibrinolytic activity of macroscopically normal areas seemed to decrease with age in the intima and media of the thoracic aorta and seemed to be low in cases with a high atherosclerotic index (*5*). In the course of our experiments to clarify the relationship between fibrinolysis and formation of thrombi, we devised a new method for the estimation of fibrinolytic activity. In this method, fibrin (a physiological substrate of plasmin) was used as a substrate. We found that free fatty acids markedly inhibited plasmin activity, using this new method. The present report describes this work and discusses the significance of free fatty acids in the genesis of atherosclerosis.

MATERIALS AND METHODS

Chemicals—Stearic, oleic, palmitic, myristic, and lauric acids were obtained from Nakarai Chemicals, Ltd. (Tokyo, Japan). Linoleic, linolenic, capric, and caprylic acids were from P-L Biochemicals, Inc. (Milwaukee, U.S.A.). The purity of each fatty acid was examined by thin layer chromatography (99% pure). Casein, prepared from milk, was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Bovine albumin (Fraction V) was from Armour Pharmaceutical Co. (Arizona, U.S.A.). Streptokinase (100,000 U) was purchased from Takeda Chemical Industries, Ltd. (Tokyo, Japan). Tosyl-L-arginine methyl ester (TAME) was from the Protein Research Foundation, Osaka University (Osaka, Japan).

Preparation of Plasmin—Plasmin was prepared from human serum. All the procedures were carried out at 4°C. Human serum was diluted 20-

fold with distilled water, adjusted to pH 5.3 with 5% acetic acid and then left to stand at 4°C overnight. The solution was centrifuged at 1,600 × *g* for 10 min. The precipitate (englobulin fraction) was dissolved in 0.03 M EDTA (pH 4.8) and stirred at 4°C for 3 h. The pH of the solution was adjusted to 4.8 and it was left to stand at 4°C overnight. The resulting precipitate was collected by centrifugation at 1,600 × *g* for 20 min and dissolved in 1/15 M phosphate buffer (pH 7.4). This solution was stored in a refrigerator as plasminogen solution.

An aliquot (1 ml) of plasminogen solution was diluted with 3 ml of 1/15 M phosphate buffer (pH 7.4) and 5,000 units of streptokinase was added. Incubation was carried out at 37°C for 10 min and the resulting solution was used as plasmin solution.

Estimation of Plasmin Activity—(a) Casein method (7): Casein solution was prepared as follows: 8 g of casein was suspended in 60 ml of 1/15 M phosphate buffer (pH 7.4) and 4 ml of 1 N sodium hydroxide was added to the suspension, which was then stirred for 1 h at room temperature. The solution was adjusted to pH 7.4 and the final volume was made up to 100 ml with distilled water (8% casein solution).

To 0.1 ml of the plasmin solution, 0.25 ml of casein solution was added and the mixture was incubated at 37°C for 30 min. After incubation, 1.5 ml of 10% perchloric acid was added to the solution and it was left to stand for 30 min at room temperature. Then, the solution was centrifuged at 1,600 × *g* for 10 min. The protein concentration of the resulting supernatant (0.2 ml) was estimated by the method of Lowry *et al.* (*8*).

(b) Hestrin method (9): The plasmin solution, 0.1 ml, was added to 0.3 ml of substrate solution containing 10 μmol of TAME and 0.6 ml of 0.1 M borate buffer containing 10 mM calcium chloride, pH 8.6. The resulting mixture was incubated at 37°C for 30 min. After incubation, 1.5 ml of alkaline hydroxylamine solution (equal volumes of 3.5 N sodium hydroxide and 2 N hydroxylamine) was added to the reaction mixture and it was left to stand for 15 min at room temperature. Subsequently, 1 ml of 18% trichloroacetic acid, 1 ml of 4 N hydrochloric acid and 1 ml of 10% ferric chloride were added. The solution was centrifuged at 1,600 × *g* for 10 min. The remaining TAME in the supernatant was followed at 530 nm with a Hitachi 200 spectrophotometer.

(c) Fibrin clot suspension method: Fibrin clot suspension was prepared as follows. Human plasma was separated from stored human blood by centrifugation (1,600 × *g*, 30 min). Calcium chloride was added up to 25 mM to the human plasma. The mixture was incubated at 37°C for 30 min and left to stand at 4°C overnight. The resulting fibrin clot was separated from serum by centrifugation at 1,600 × *g* for 10 min and washed several times with 1/15 M phosphate buffer, pH 7.4. The fibrin clot was suspended in 3 volumes of 1/15 M phosphate buffer then subjected to homogenization (PCU-2 unit, Kinematica GmbH, Switzerland). The resulting fibrin suspension was boiled for 10 min in boiling water to inactivate the contaminating plasminogen. The final concentration of the fibrin suspension thus obtained was adjusted to 8% (w/v) by addition of 1/15 M phosphate buffer, pH 7.4, and it was used as a substrate.

The estimation of plasmin activity with the fibrin suspension as a substrate was carried out as follows. The fibrin suspension (0.25 ml) was mixed with 0.1 ml of enzyme solution (0.1–1.0 units) and incubated at 37°C. After incubation for 30 min, the reaction mixture was boiled for 5 min and centrifuged at 1,600 × *g* for 10 min. The protein concentration of the resulting supernatant (0.2 ml) was estimated by the method of Lowry *et al.* (*8*).

RESULTS

We have proposed a novel method to estimate plasmin activity. As shown in Fig. 1, the protein content in the supernatant increased linearly with increase of plasmin concentration.

In the course of the experiment, free fatty acids were found to inhibit the plasmin activity. As shown in Table I, long-chain unsaturated free fatty acids, namely oleic, linoleic, and linolenic acids, were found to inhibit plasmin activity strongly.

It was of interest that the inhibitory action of oleic acid was dependent on the substrate used in the estimation of plasmin activity. As shown in Table II, when casein or TAME was used as a substrate, no inhibitory effect of oleic acid was observed. On the other hand, it markedly inhibited the plasmin activity with fibrin as a substrate.

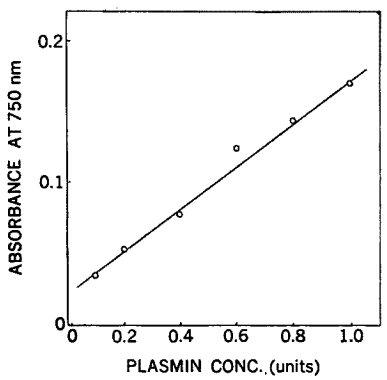

Fig. 1. Estimation of plasmin activity with fibrin clot suspension as a substrate. The fibrin clot suspension (0.25 ml) was mixed with various amounts of plasmin solution and incubated at 37°C for 30 min. The reaction was stopped by boiling for 10 min and the reaction mixture was centrifuged at 1,600 × *g* for 10 min. The protein in 0.2 ml of the resultant supernatant was estimated by the method of Lowry *et al.* (*8*). 1 unit was defined as the activity hydrolyzing 100 μmol of TAME per 1 mg of protein.

TABLE I. Effects of various fatty acids on plasmin activity with fibrin as a substrate. The fibrin clot suspension, 0.25 ml, was preincubated with 0.1 ml of each fatty acid at 37°C for 5 min. 0.1 ml of plasmin solution (containing 1 unit) was added and the mixture was incubated at 37°C for 30 min. The final concentration of each fatty acid was 2 mM in the assay system. In the control experiment, 0.1 ml of oleic acid was replaced with the same volume of 1/15 M phosphate buffer.

Addition		% inhibition
Oleic acid	(C18. 1)	74. 5
Linoleic acid	(C18. 2)	60. 8
Linolenic acid	(C18. 3)	70. 5
Stearic acid	(C18. 0)	15. 7
Palmitic acid	(C16. 0)	20. 3
Myristic acid	(C14. 0)	34. 0
Lauric acid	(C12. 0)	23. 5
Capric acid	(C10. 0)	9. 8
Caprylic acid	(C 8. 0)	12. 4

Next, the relationship between the inhibitory action and the quantity of free fatty acid bound to fibrin was examined, as described in the legend to Fig. 2. As shown in the figure, the amount of free fatty acid bound to fibrin increased with increase of the inhibition of plasmin activity. This result suggested that the inhibition of plasmin activity

TABLE II. Effects of various substrates on the inhibitory action of oleic acid. The substrate solution, 0.25 ml, was preincubated with 0.1 ml of oleic acid at 37°C for 5 min and enzyme solution, 0.1 ml, was added to the mixture. The final concentration of oleic acid was 5 mM in the reaction mixture. In the control experiment, 0.1 ml of oleic acid was replaced with the same volume of 1/15 M phosphate buffer.

Substrate	% inhibition
Fibrin clot suspension	79
Casein	0
TAME	−46

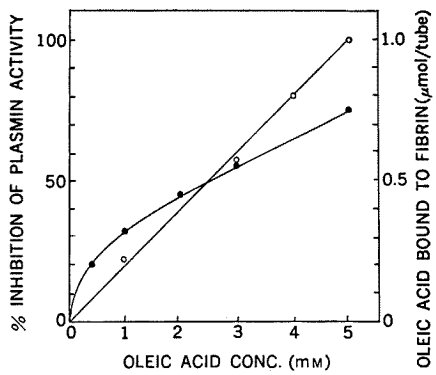

Fig. 2. Effect of oleic acid on plasmin activity with fibrin as a substrate. The fibrin clot suspension, 0.25 ml, was preincubated with 0.1 ml of various concentrations of oleic acid at 37°C for 5 min. 0.1 ml of plasmin solution (containing 1 unit) was added to the mixture, which was then incubated at 37°C for 30 min. The incubation mixture was centrifuged at 1,600 × *g* for 10 min and the protein concentration in 0.2 ml of the supernatant was estimated according to Lowry *et al.* (*8*). In the control experiment, 0.1 ml of oleic acid was replaced with the same volume of 1/15 M phosphate buffer, pH 7.4. Oleic acid bound to fibrin was determined as follows: 0.25 ml of fibrin clot suspension was incubated with 0.1 ml of various amounts of oleic acid at 37°C for 5 min. The incubation mixture was centrifuged at 1,600 × *g* for 5 min. The pellet (fibrin clot) was collected and used for estimation of free fatty acid according to Dole *et al.* (*15*). ●, % inhibition of plasmin activity; ○, oleic acid bound to fibrin (μmoles/tube).

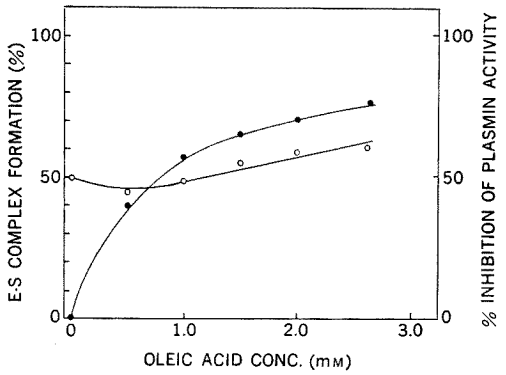

Fig. 3. Effect of oleic acid on the formation of plasmin-fibrin complex. The fibrin clot suspension, 0.25 ml, was preincubated with 0.1 ml of various concentrations of oleic acid at 37°C for 5 min. The plasmin solution, 0.1 ml (containing 1 unit), was added to the mixture, which was then incubated at 37°C for 5 min and centrifuged at 4°C (1,600 × *g*) for 5 min. 0.05 ml of the resulting supernatant and the original plasmin solution were used for estimation of plasmin activity with TAME as a substrate. The activity of plasmin bound to the fibrin clot was estimated from the difference in activities between the supernatant and the original plasmin solution. Inhibition by oleic acid of the plasmin activity was estimated as described in the legend to Fig. 2. ●, % inhibition of plasmin activity; ○, E-S complex formation (%).

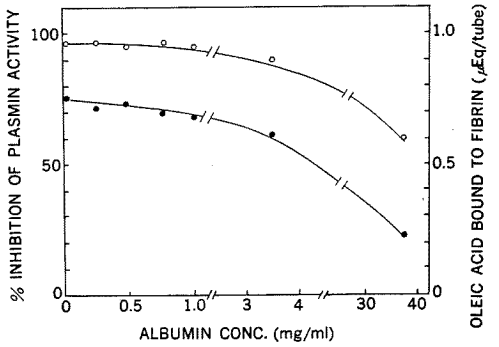

Fig. 4. Effect of albumin on the inhibitory action of oleic acid. 0.25 ml of the fibrin clot suspension containing various concentrations of albumin was preincubated with 0.1 ml of oleic acid at 37°C for 5 min. 0.1 ml of plasmin solution (containing 1 unit) was added to the mixture. The final concentration of oleic acid was 5 mM in the assay system. Inhibition by oleic acid of the plasmin activity was estimated as described in the legend to Fig. 2. ●, oleic acid bound to fibrin (μEq/tube); ○, % inhibition of plasmin activity.

was elicited as a result of binding of free fatty acid to the surface of the fibrin clot.

Moreover, the relationship between the inhibitory action of free fatty acids and the complex formation of plasmin with fibrin was examined. Figure 3 shows that, as the quantity of free fatty acid was increased, complex formation between fibrin and plasmin was not affected, but the inhibitory action of the free fatty acid increased. This suggests that the plasmin bound in the presence of free fatty acid could not exert fibrinolytic action on fibrin.

It is well-known that fatty acid in the blood exists in a bound form with albumin. Thus, an experiment was designed to examine the inhibitory action of free fatty acid on plasmin activity in the presence of albumin. As shown in Fig. 4, the amount of oleic acid bound to fibrin decreased with increase of albumin concentration. The rate of inhibition by oleic acid also decreased slightly as the albumin content increased. However, 60% inhibition was observed even in the presence of 3.8 mg/ml of albumin, which is the same concentration as that in blood. These results suggest that the inhibitory action of free fatty acids might have some physiological significance *in vivo*.

DISCUSSION

In the early stage of inflammation, plasmin is known to have important functions, such as the formation of kinin from kininogen, fibrinolysis, and so on. Various methods have been reported for the estimation of plasmin activity (*7, 9–12*). In most cases, a soluble substrate such as TAME or casein was used. However, these substrates are artificial ones and the physiological substrate of plasmin is known to be insoluble fibrin. Clearly a physiological substrate is preferable to an artificial one, especially to study the physiological significance of enzyme reactions. In the fibrin plate method (*12*), which employs fibrin as a substrate, it is difficult to analyze the kinetics of plasmin activity, because a long incubation time (about 24 h) is required.

Fujii (*13*) reported a new method, employing a complex of fibrin clot and Blue Dextran 2,000 as

a substrate. This method is very simple, but due to the differences in properties between the fibrin complex and native fibrin, this method may not be suitable to estimate the physiological activity of plasmin.

Our method provides a new approach to study the behaviour of plasmin under physiological conditions. With this method, we found that free fatty acids have a marked inhibitory action on plasmin. When an artificial substrate such as TAME or casein was used, such an inhibitory action of free fatty acids on plasmin was not observed. As regards the mechanism of inhibition of plasmin activity, free fatty acids may not inhibit complex formation between plasmin and fibrin, but may prevent plasmin from acting on fibrin due to deformation of the surface of the fibrin clot.

Shio *et al.* (*14*) suggested a possibility that free fatty acid accelerates fibrin clot formation by releasing coagulation factors from blood platelets.

Based on these findings, the effects of free fatty acids on fibrin formation can be summarized as follows. If free fatty acid levels are increased at intima of blood vessels, the fatty acids might act on the blood platelets to release coagulation fractors, resulting in acceleration of fibrin formation from fibrinogen. Then, the free fatty acids bind to fibrin at the surface and inhibit the fibrinolytic action of plasmin. Thus, free fatty acids induce deposition in two ways. One is acceleration of fibrin formation and the other is inhibition of fibrinolysis. It can be postulated that the resulting fibrin deposited at the surface of the intima causes thrombosis and initiates the progress of atherosclerosis.

Experiments are now in progress to clarify the mechanism of free fatty acid accumulation at intima of blood vessels and to investigate further the phenomena induced by free fatty acids.

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