Bovine Lipoprotein Lipase and Fibrinolysis*

Keijiro SAKU**, Kikuo ARAKAWA**, Masato MIZOBUCHI***, Masood AHMAD****, Pia GLAS-GREENWALT**** and Moti L. KASHYAP****

Introduction

We have previously reported that hypertriglyceridemia was associated with depressed fibrinolysis (Xth International Congress on Thrombosis and Haemostasis, July 14–19, 1985, San Diego, California, USA); that is, in a series of 71 patients with primary type II, IV and V dyslipoproteinemias, the following components of fibrinolytic enzyme system, using a standardized fibrin plate method, were measured and compared with the values obtained in 240 normal donors (mean $\pm$ 2SD); inhibitor of plasminogen activation (IPA), endothelium derived vascular plasminogen activator (VPA) and alpha 2 plasmin inhibitor (PI). Table 1 shows abnormally elevated IPA was found in 27%, 40% and 68% of patients with type IIa, IIb and IV/V lipoprotein phenotype, respectively. The frequency of subnormal VPA was 33%, 20% and 27%, respectively. The correspondence values for elevated PI were 0%, 0% and 14%, respectively. Elevated IPA levels were found 100% (11/11) of patients with severe hypertriglyceridemia (TG > 1,000 mg/dl). Multiple regression analysis after adjustment for dependent variables (Table 2) revealed a significant correlation between plasma triglyceride (TG) and IPA ($r=0.37$, $p=0.002$). From above mentioned background, we speculate that TG-rich lipoproteins may transport the inhibitor of the fibrinolysis, however, such an inhibitor (IPA) is still not well defined. The present

study, therefore, were designed to assess whether lipoprotein lipase (LPL), which catabolize TG rich lipoproteins, might participate in fibrin clearance. The effects on fibrinolysis of purified bovine lipoprotein lipase were investigated in vitro system using both a chromogenic substrate S-2251 and a radiolabeled fibrin assay.

Materials and Methods

Chemicals: Sodium-¹²⁵I (2 mCi) and ¹⁴C-triolein (5 mCi) were obtained from New England Nuclear Corp. (Boston, Mass.). Fibrinogen (bovine plasminogen-free and plasminogen-rich) from Poviet (Organon Teknika, Oss, the Netherlands); bovine thrombin from Park Davis, 5,000 US units (NIH) (Morris Plains, N.J.); Urokinase (Abbott Lab., N. Chicago, Ill.) was diluted with EDTA-buffer to appropriate concentrations; plasminogen (plasmin-free) from Sigma Chemical Co. (St. Louis, Mo.). S-2251 (chromogenic substrate for plasmin) was obtained from KabiVitrum (Stockholm, Sweden).

Purification of bovine lipoprotein lipase (LPL): Bovine LPL was purified from skimmed bovine milk by affinity chromatography on heparin-Sepharose as described previously.¹⁾ Protein was determined by the method of Lowry et al.²⁾

Assay of fibrinolytic activity:

1) Radioactive fibrinolytic assay. The method for iodination of fibrinogen was essentially that described by Loskutoff et al.³⁾ One hundred μ l of ¹²⁵I-fibrinogen (12,000 cpm/ml) were immediately pipetted into each well of a 96-well immunoassay plate (Vanguard International Inc., Neptune, N.J.) using a 12-channel Titertek, then oven dried by incubation at 47°C overnight. Fibrinogen was converted to fibrin by adding 0.1 unit of thrombin per well. After washing the wells three times, plasmin, LPL (or buffer for controls) were applied to each well. The degree of lysis was determined by the release of radioactivity from each well into the

* Parts of this work were presented at the 18th Japan Atherosclerosis Society Meeting, Kyoto, Japan, May 1986

** Department of Internal Medicine, Fukuoka University School of Medicine, Fukuoka 814-01, Japan

*** Yokufuhkai Hospital, Tokyo 168, Japan

**** Department of Internal Medicine, University of Cincinnati Medical Center, Cincinnati, Ohio, USA 45267

assay buffer at various times. The radioactivity liberated increased recti-linearly with time for at least 6 hours at 37°C as described previously.⁴⁾ The coefficient variation of control sample was less than 5%.

2) Hydrolysis of S-2251. Plasmin activity was determined by using its amidolytic effect on the substrate (S-2251, H-D-Val-Leu-Lys-pNA). The rate of p-nitroanilline (pNA) released was meas-

ured photometrically at 405 nm using a Biochromatic Analyzer ABA-100 (Abott Laboratories, Rosemont, Ill.). The reaction mixtures of S-2251 (3.5 mmol/l, 70 μ l), plasmin (0.001 units/ml, 70 μ l), and LPL (or control buffer, 70 μ l) for plasmin mediated hydrolysis, S-2251 (3.5 mmol/l, 70 μ l), plasminogen (0.06 units/ml, 200 μ l), urokinase (50 CAT units/ml, 70 μ l) and LPL (or control

Table 1 Prevalence of abnormalities in parameters of fibrinolysis in patients with primary dyslipoproteinemia

Lipoprotein phenotype	IIa	IIb	IV/V
Elevated IPA	27%	40%	68%
Decreased VPA	33%	20%	27%
Elevated PI	0%	0%	14%

The data were compared with the reference range (mean $\pm$ 2 SD) of 240 normal healthy blood donors.

Table 2 Multiple regression analysis

	IPA vs. TG	IPA vs. HDL-C	IPA vs. BMI
Adjusted by	HDL-C & BMI	TG & BMI	HDL-C & TG
r-value	0.369	-0.203	-0.026
p-value	0.002	0.094	0.833

IPA: Inhibitor of plasminogen activation
HDL-C: High density lipoproteins cholesterol
BMI: Body mass index

Fig. 1 A: Effect of addition of LPL on the hydrolysis of S-2251 by plasmin.
B: Effect of addition of native LPL, reduction of caboxymethylated LPL and heat treated LPL on the hydrolysis of S-2251 by plasmin after 9 hours incubation at 37°C.

buffer, 70 μ l) for urokinase/plasminogen mediated hydrolysis, were incubated at 37°C throughout the study, and hydrolysis of S-2251 was monitored every hour up to 9 hours. Data shown are mean of 2-4 replicated values.

Assay of lipoprotein lipase activity: Thirty μ l of sample (LPL, or plasmin treated LPL) was incubated at 37°C for 20 minutes in duplicate with 140 μ l of PDB with heparin, 75 μ l of 5 mM triolein (14 C-triolein) and 5 μ l of apo C-II (0.5 mg/ml in 6 M guanidine-HCl). Incubation was terminated by the addition of 3.25 ml of MeOH: CHCl₃: Heptane (1.42:1.25:1). To it was added 1 ml of 140 mM K₂CO₃-H₃BO₄ (pH 10.4) and shaken. One ml of the supernatant was then counted for the radioactivity.

Results

Figure 1-A shows the effect of LPL on the hydrolysis of S-2251 by plasmin. Hydrolysis of S-2251 was enhanced as incubation time increased, but LPL alone did not act on S-2251. Figure 1-B

shows the effect of LPL, the reduction of carboxy-methylated LPL and heat treated LPL on the hydrolysis of S-2251 by plasmin. The formers enhanced this reaction, while heat treated LPL did not. This observation was also confirmed by a radioactive fibrinolytic assay; 2 μ g LPL protein increased plasmin mediated fibrinolysis by 20% of baseline and 4 μ g LPL protein increased it by 45%, and LPL alone did not lyse fibrin (data not tabulated).

Figure 2 shows the effect of LPL on the mixture of plasminogen, urokinase and S-2251. Urokinase converts plasminogen to plasmin and then hydrolyzes S-2251. LPL enhanced this reaction, while LPL or LPL mixed with plasminogen did not act on S-2251.

Figure 3 revealed SDS-polyacrylamide gel electrophoresis. LPL was cleaved by plasmin into several smaller proteins after one hour incubation at 37°C, however, triolein hydrolytic activity of both LPL and plasmin treated LPL were almost equal as shown in Table 3.

Fig. 2 Effect of addition of LPL on the hydrolysis of S-2251 (urokinase/plasminogen mediated hydrolysis) after 3, 6, 9 hours incubation at 37°C.

Fig. 3 Polyacrylamide gel electrophoresis. The mixture of LPL and plasmin (100 μ g and 2 μ g of protein, respectively) were applied after 0 and 1 hour incubation at 37°C.

Discussion

Our clinical data (Table 2) showed a significant correlation between plasma TG and IPA after adjustment for dependent variables (HDL-cholesterol and BMI), suggesting that TG-rich lipoproteins may transport the inhibitor of fibrinolysis (IPA).

Present study shows that LPL enhanced both plasmin and urokinase/plasminogen mediated fibrinolytic process. Reduction of carboxymethylated LPL but heat treated LPL enhanced both hydrolysis of S-2251 and radiolabeled fibrinolysis

Table 3 Effect of plasmin on triolein hydrolytic activity of LPL (nmol triolein/min/ μ g LPL protein)

	Incubation time (min)		
	0	30	60
LPL	212.5 (100%)	97.9 (46.0%)	30.8 (14.5%)
LPL + plasmin	198.5 (100%)	91.9 (46.3%)	23.7 (11.9%)

Protein (μ g) ratio of LPL: plasmin was 50: 1

by plasmin. These data indicates that LPL activated fibrinolysis. Plasmin cleaved LPL into several smaller proteins without affecting its triolein hydrolytic activity, however, it is unknown which protein fraction may have a central role in enhancing plasmin action.

Since LPL is found in vascular endothelium, our data suggest that LPL may have an important role in modulating fibrin clearance. The interaction between LPL and the inhibitor (IPA) are still remained unclear.

References

- 1) Taskinen, M. R., Johnson, J. D., Kashyap, M. L., Shirai, K., Glueck, C. J. and Jackson, R. L.: Catabolism of human very low density lipoproteins in vitro: a fluorescent phospholipid method for monitoring lipolysis. *J. Lipid Res.*, **22**: 382-386 (1981).
- 2) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J.: Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**: 265-275 (1951).
- 3) Loskutoff, D. J. and Edgington, T. S.: Synthesis of a fibrinolytic activator and inhibitor by endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **74**: 3903-3907 (1977).
- 4) Saku, K., Ahmad, M., Glas-Greenwalt, P. and Kashyap, M. L.: Activation of fibrinolysis by apolipoproteins of high density lipoproteins in man. *Thromb. Res.*, **39**: 1-8 (1985).

Summary

The role of lipoprotein lipase (LPL) in fibrinolysis was assessed using both a chromogenic substrate (S-2251, H-D-Val-Leu-Lys-pNA) specific to plasmin and a radioactive fibrinolytic assay. LPL was purified from skimmed bovine milk by affinity chromatography on heparin-Sepharose. LPL alone did not act on S-2251 but enhanced plasmin activity on it. The mixture of plasminogen, urokinase and LPL hydrolyzed S-2251 to a greater extent than the mixture of plasminogen and urokinase did. Heat treated LPL did not enhance this reaction, however, reduction of carboxymethyl-

ated LPL did, which was confirmed by a radioactive fibrinolytic assay. LPL was cleaved into several smaller proteins by plasmin as revealed by SDS-polyacrylamide gel electrophoresis, however, triolein hydrolytic activity was not affected. Since LPL is found in vascular endothelium, our data may suggest an important role of LPL in modulating fibrin clearance.

Key words: bovine lipoprotein lipase, chromogenic substrate (S-2251), fibrinolysis, plasmin, plasminogen, urokinase.