THE INHIBITORY EFFECT OF LIPID PEROXIDE ON THE ACTIVITY OF THE MEMBRANE-BOUND AND THE SOLUBILIZED LIPOPROTEIN LIPASE

KAZUNARI WADA, M.D., HIDEO MIKI, M.D., MASAHICO ETOH, M.D.
FUMIO OKUDA, M.D., TOSHIAKI KUMADA, M.D.
AND REIZO KUSUKAWA, M.D.

The effects of lipid peroxide (15-hydroperoxy arachidonic acid) on the activity of lipoprotein lipase was investigated. The activities were examined in 2 enzyme species: the lipoprotein lipase bound to the coronary vessels of the rat heart and that solubilized in the human post-heparin plasma. As a substrate, ^3H -labelled human chylomicron was used.

Lipid peroxide decreased the maximal velocity of the reaction between the chylomicron and lipoprotein lipase on the vascular surface. In the post-heparin plasma, the lipid peroxide decreased the maximal hydrolysis rate, but did not change the half life time in the unsaturated reaction.

It is suggested that lipid peroxide directly damages a part of the membrane-bound lipoprotein lipase, and that the degeneration of cell membranes and the vasoconstriction caused by lipid peroxide will additionally disturb the hydrolysis of the plasma triglyceride on the vascular surface.

THE lipoprotein lipase (LPL), which is bound to the vascular surface, hydrolyzes plasma triglycerides (TG) in the vascular bed and makes the generated fatty acid available to the peripheral tissues. The activity of this LPL has a key role in the regulation of plasma TG-rich lipoproteins. Disorders in the interaction of endothelial LPL with TG-rich lipoproteins induce an abnormal catabolism of these lipoproteins and possibly contribute to the progress of atherosclerosis.¹

Recently, the toxicity of peroxidized lipids has been investigated with particular reference to its clinical significance. Previous experiments²⁻⁶ have indicated that the lipid peroxides damage proteins, enzyme and cell membranes. It has been also reported^{7,8} that the lipid peroxides inhibit the activity of lecithin-cholesterol acyl

transferase which regulates cholesterol transport between high density lipoproteins and the other lipoproteins of tissues. However, the effect of lipid peroxides on the activity of LPL has been obscure.

This effect was examined by two different procedures. One is a method to measure membrane-bound LPL activity and the other is a method to measure solubilized LPL activity.

MATERIALS AND METHODS

Preparation of Lipoprotein Substrate

Human chylomicron obtained from healthy human subjects was used as a substrate. Four hours after eating 200g of commercial butter, plasma samples were collected. After layering saline solution on the plasma, chylomicron ($S_f > 400$) was isolated by ultracentrifugation at 0.61×10^6 g-min using Spinco 40.3 rotor. The centrifugation was repeated twice under the same

Key Words:

Chylomicron
Lipoprotein lipase
Lipid peroxide

(Received September 10, 1982; accepted January 29, 1983)

Department of Internal Medicine, Yamaguchi University School of Medicine, Ube, Japan

Present mailing address: Kazunari Wada, M.D., Onoda Red Cross Hospital, Sue-Higashi, Onoda 756, Japan

Fig.1. Removal of human chylomicron-TG by the perfused rat heart. A: saturated reaction; B: unsaturated reaction. Insert: The plot of $\ln(S_t/S_0)$ VS $(S_0 - S_t)$ has a slope K_m . (●) = perfusate contain 15-hydroperoxy arachidonic acid; (○) = perfusate contain arachidonic acid

Fig.2. Elevation of pressure of coronary vessels by 15-hydroperoxy arachidonic acid. Insert: Magnifying pressure curve of tubing. H.R.Flow Inducer (Type E 200, Watson-Marlow Ltd.) was used.

conditions. The obtained chylomicron-TG was labelled with ^3H according to an exogenous isotopic labelling procedure⁹ and the specific activity was determined.

Lipid Peroxide

In this series of experiments, 15-hydroperoxy arachidonic acid (15-HPAA), which was supplied from Eisai Co., Japan, was used. The 15-HPAA was dissolved in 0.15 M NaHCO_3 and then added

TABLE I KINETIC CONSTANTS OF MEMBRANE-BOUND LPL AND INHIBITION OF LPL ACTIVITY BY 15-HPAA

Medium	V_{max} (μ moles TG/ml/min)	K_m (mM)
Control ^a	0.06 ± 0.005	0.10 ± 0.02
15-HPAA ^b	$0.04 \pm 0.002^*$	

a: Arachidonic acid was added to the perfusion medium.

b: 15-hydroperoxy arachidonic acid (10 nmoles/ml in lipid peroxide value) was added to the perfusion medium. $n = 6$ (* $p < 0.01$)

to a solution of bovine serum albumin (Fract. V., Sigma) dialyzed against Krebs-Henseleit bicarbonate buffer solution (pH 7.4). This mixture was used to examine membrane-bound LPL activity. To examine the solubilized LPL activity, the 15-HPAA dissolved in an albumin-ammonium buffer solution (pH 8.6) was used. After determining lipid peroxide value by Yagi's method¹⁰ these solutions were added to the media. The final concentration of the 15-HPAA in the medium was made up to 10 nmoles/ml of lipid peroxide value. As the controls, medium containing the same amount in moles of arachidonic acid (Nakarai Chem., Ltd.) was prepared.

Determination of Membrane-bound LPL Activity

The procedure described by Fielding¹¹ was used with a slight modification: coronary vessels of rat hearts were perfused at a constant flow rate with a modified circulating Langendorff apparatus. All glassware and tubing were siliconized. The perfusate was Krebs-Henseleit bicarbonate buffer containing 3.5% (w/v) of the dialyzed bovine serum albumin, 9% (v/v) of plasma (which had been previously depleted of TG-rich lipoproteins by centrifugation for 1.65×10^8 g-min), 0.1% (w/v) of glucose and the requisite volume of chylomicron substrate gassed with 95% of O₂ and 5% of CO₂. The Krebs-Henseleit bicarbonate buffer solution incubated first with the 15-HPAA or arachidonic acid at 37°C was added to the perfusion medium. During the perfusion, the left ventricular pressure was kept at atmospheric pressure by cannulation of tubing. The pressure of coronary vessels was recorded using a VR-12 (Electronics for Medicine). Maximal reaction velocity (V_{max}) was determined with recirculating perfusion system at a constant flow rate of 6 ml/min. Total volume of the substrate solution was 12 ml. Duplicate 0.2 ml samples of the perfusion medium were taken every 4 min during the

perfusion. To determine Michaelis constant (K_m), non-recirculating perfusion system was used. The reservoirs contained 0.05, 0.07 and 0.13 mM of chylomicron-TG and these substrate were perfused at 4.5 ml/min for 2 min. Triplicate 0.2 ml samples were taken at one minute intervals for analysis of TG content together with the same number of samples of the reservoir medium. After extraction of lipids by Dole's mixture, neutral lipids were transferred into the heptane layer at pH 9.2–10. Then, ³H-TG were measured by liquid scintillation spectrometric scanning. Details of the experimental procedure and of the determination of kinetics are described elsewhere.^{11–13}

Determination of Solubilized LPL Activity

To determine the solubilized LPL activity, post-heparin plasma was used. The procedure is based on the method described by Boberg and Carlson.¹⁴ Thirty minutes after an intravenous injection of 0.5 mg of heparin per kg of body weight to a healthy human subject, blood samples were collected into tubes containing 0.1M trisodium citrate stored in ice water. Two ml of plasma was drawn off and added to 6 ml of the substrate mixture. At the beginning of the experiment, apparent K_m value of human chylomicron for post-heparin plasma was determined from a Lineweaver-Burk plot. Then, the initial concentration of ³H-TG ([S]) in the medium was set at saturated and unsaturated levels: $[S] \gg K_m$ and $[S] \ll K_m$. The media were first incubated with the 15-HPAA or arachidonic acid at 37°C for 20 min. Then, 0.2 ml of the samples were drawn at 2–4 min intervals for 20 min and the amount of ³H-TG was scanned. The TG levels were plotted against time and elimination curves were described. The maximal hydrolysis rate of TG in the saturated reaction and half life time in the unsaturated reaction were examined.

Fig. 3. Kinetics of lipoprotein lipase in plasma after a single injection of heparin. Substrate: ^3H labelled human chylomicron-TG. K_m : 0.7 mM

Fig. 4. Removal of human chylomicron-TG in plasma after a single injection of heparin. A: saturated reaction; B: unsaturated reaction. (●) = medium contain 15-hydroperoxy arachidonic acid; (○) = medium contain arachidonic acid

RESULTS

Membrane-bound LPL Activity

In the control experiment, the perfusion pressure of the coronary vessels was within 35–41 mmHg at a 6 ml/min flow rate. Hydrolysis of chylomicron-TG by the perfused rat heart was at zero-order with greater than 1.0 $\mu\text{moles/ml}$ of the substrate concentration (Fig. 1A). The maximal removal rate of chylomicron-TG was 4.5 ± 0.3 nmoles/ml/min. The concentrations of less than 0.01 $\mu\text{moles/ml}$ gave a first-order elimination curve (Fig. 1B). The calculated V_{max} and

K_m were shown in Table I. When the 15-HPAA was added to the perfusion medium, rapid elevation of the pressure of coronary vessels was observed (Fig. 2), although the flow rate was constant at 6 ml/min. In some cases, elevation of the pressure reached 70 mmHg. The V_{max} obtained in these conditions decreased significantly, as shown in Table I. Reperfusion of the control substrate did not restore the activity, and the pressure was held at a high level. On the other hand, it was difficult to calculate the K_m value after an addition of the 15-HPAA. The reason will be discussed later.

TABLE II INHIBITION OF SOLUBILIZED LPL ACTIVITY BY 15-HPAA

Medium	$[S] \gg K_m$	$[S] \ll K_m$
	Maximal hydrolysis rate ($\mu\text{moles TG/ml/min}$)	Half life time (min)
Control ^a	0.09 ± 0.01	8.5 ± 1.3
15-HPAA ^b	$0.07 \pm 0.01^{**}$	8.2 ± 1.5

a: Arachidonic acid was added to the medium.

b: 15-hydroperoxy arachidonic acid (10 nmoles/ml in lipid peroxide value) was added to the medium.

n = 6 (**p < 0.01)

Solubilized LPL Activity

The K_m value obtained in this experimental system was 0.7 mM (Fig. 3). When initial TG concentration was set at greater than 2.0 $\mu\text{moles/ml}$ ($[S] \gg K_m$), zero-order reaction was obtained (Fig. 4A). During 20 min incubation with the chylomicron substrate, the elimination of chylomicron-TG gave a linear function. Setting it at less than 0.2 $\mu\text{moles/ml}$ ($[S] \ll K_m$), a first-order reaction observed (Fig. 4B). Maximal hydrolysis rate of the chylomicron-TG and half life time are shown in Table II. The maximal hydrolysis rate of the control substrate was very close to the value calculated from the Lineweaver-Burk plots. The 15-HPAA did not change the half life time of the unsaturated reaction.

DISCUSSION

The perfusion system provides a means of study kinetic properties of the membrane-bound LPL under physiological conditions. Since human lipoprotein is a good substrate for rat LPL^{13,15} and the endothelial LPL of the rat heart react as a single population of high affinity substrate binding sites,¹² the data obtained in this study represents the activities of membrane-bound LPL for human chylomicron in the high-affinity enzyme site. In fact, the calculated kinetics of the control substrate are close to the values previously determined by the same perfusion experiments.¹¹⁻¹³

The 15-HPAA decreased the $V_{\max}$ with simultaneous elevation of the perfusion pressure. The flow rate was constant and the left ventricular pressure was kept at atmospheric pressure during the perfusion. The 15-HPAA appears to cause a constriction of the coronary vessels. A previous report has indicated that the 15-HPAA induces vasoconstriction of the canine basilar artery with degeneration of the endothelium.¹⁶ When vasoconstriction occurs, the amount of

enzyme active on the vascular bed should decrease. Degeneration of the endothelium probably changes the electrical charge of the cell membranes and decreases the affinity of LPL to substrate. In these conditions, $V_{\max}$ must be underestimated. If the 15-HPAA directly damages the LPL, $V_{\max}$ will be decrease additionally. The vasoconstriction made the K_m value uncertain. Since the perfusion pressure varied, the hydrolysis rate at very low concentrations of TG (unsaturated reaction) could not be accurately determined.

Intravenous injection of heparin releases many enzymes into the plasma: LPL, hepatic triglyceride lipase (H-TGL),¹⁷ phospholipase¹⁸ and elastase.¹⁹ The post-heparin plasma contains these enzymes at indefinite proportions. Furthermore, LPL have two enzyme species: high- and low-affinity enzymes and the kinetic properties of low-affinity enzyme are quite different from that of high-affinity enzyme.¹² Therefore, the properties of the LPL solubilized in the plasma is not similar to that of the LPL supported on the vascular surface of the heart. However, the post-heparin plasma is useful for investigating the activity of LPL in the absence of the indirect inhibitory factors: degeneration of cell membranes and vasoconstriction and so on. In the post-heparin plasma, the 15-HPAA decreases the maximal hydrolysis rate of the chylomicron-TG, but does not change the half life time in the unsaturated reaction. This type of inhibition appears to be non-competitive inhibition. However, it is obscure whether 15-HPAA inhibits the activity of LPL and/or H-TGL. In past studies of enzyme inhibition by lipid peroxides, it has been shown that sulfhydryl enzymes are most susceptible to peroxidative attack.^{3,4,20} Further works are necessary to clarify these questions.

As for the substrate, large doses of lipid peroxides and long-term incubation alter the physiological properties of lipoproteins and

apoprotein peptides *in vitro* (our unpublished work). However, 10 nmoles/ml of lipid peroxide (this level is detectable in pregnant women or patients with atherosclerosis) did not change the electrophoretic properties of lipoproteins and apoprotein C-II which is a cofactor protein activating LPL. A definite explanation for the inhibition mechanism in post-heparin plasma could not be deduced in the present study.

Lipid peroxides are involved with lipoproteins in plasma²¹. Although it is uncertain that the lipoproteins which contain a lot of peroxidized lipids inhibit the activity of membrane-bound LPL in blood stream, the 15-HPAA decreased the maximal hydrolysis rate of chylomicron-TG on the membrane surface and in the post-heparin plasma.

In conclusion, it is thought that the 15-HPAA attacks the cell membranes of blood vessels and damages directly at least a part of the membrane-bound LPL. The degenerated cell membranes and the ensuing change of electrical charge of the membrane surface should decrease the affinity of LPL and delay the reaction velocity. It is also suggested that the vasoconstriction caused by the lipid peroxides will additionally disturb the hydrolysis of the plasma TG on the vascular surface.

Acknowledgement

We wish to thank Drs R. J. Havel and C. J. Fielding for technical advice on the perfusion work and thank Dr K. Yagi and his collaborators for advice on the study of lipid peroxide. We also thank Eisai Co., Japan for supplying 15-hydroperoxy arachidonic acid for our experiments.

REFERENCES

1. ZILVERSMIT DB: A proposal linking atherosclerosis to the interaction of endothelial lipoprotein lipase with triglyceride-rich lipoproteins. *Circ Res* 33: 633, 1973
2. ROUBAL WT, TAPPEL AL: Polymerization of proteins induced by free radical lipid peroxidation. *Biophysics* 113: 5, 1966
3. CHIO KS, TAPPEL AL: Synthesis and characterization of the fluorescent products derived from malonaldehyde and amino acids. *Biochemistry* 8: 2821, 1969
4. CHIO KS, TAPPEL AL: Inactivation of ribonuclease and other enzymes by peroxidizing lipids and by malonaldehyde. *Biochemistry* 8: 2827, 1969
5. HIRAMATSU T, HASEGAWA Y, HIRATA K, NISHIGAKI I, YAGI K: Formation of lipid peroxide in the retina of rabbit exposed to high concentration of oxygen. *Experimentia* 32: 622, 1976
6. YAGI K, MATSUOKA S, OHKAWA H, OHISHI N, TAKEUCHI Y, SAKAI H: Lipoperoxide level of the retina of chick embryo exposed to high concentration of oxygen. *Clin Chem Acta* 80: 335, 1977
7. TAKATORI T, PREVETT OS: Studies of serum lecithin-cholesterol acyl transferase activity in rat: Effect of vitamin E deficiency, oxidized dietary fat or intravenous administration of ozonides or hydroperoxides. *Lipids* 9: 1018, 1974
8. MICHEL HS, HILL PL, HYES KS: Depression of lecithin-cholesterol acyl transferase esterification in vitamin E deficient monkeys. *Am J Clin Nutr* 28: 995, 1975
9. FIELDING CJ: Validation of a procedure for exogenous isotopic labeling of lipoprotein triglyceride with radioactive triolein. *Biochim Biophys Acta* 573: 255, 1979
10. YAGI K: A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem Med* 15: 212, 1976
11. FIELDING CJ, HIGGINS JM: Lipoprotein lipase: Comparative properties of the membrane-supported and solubilized enzyme species. *Biochemistry* 13: 4324, 1973
12. FIELDING CJ: Lipoprotein lipase: Evidence for high- and low-affinity enzyme sites. *Biochemistry* 15: 879, 1976
13. WADA K, MIKI H, OKUDA F, ETOH M, KUSUKAWA R: Hydrolysis of human very low density lipoprotein triglycerides in rat heart. *Bull Yamaguchi Med Sch* 28: 105, 1981
14. BOBERG J, CARLSON LA: Determination of heparin-induced lipoprotein lipase activity in human plasma. *Clin Chim Acta* 10: 420, 1964
15. HAVEL RJ, FIELDING CJ, OLIVERCRONA T, SHORE VG, FIELDING PE, EGELRUD T: Cofactor activity of protein components of human very low density lipoprotein in the hydrolysis of triglycerides by lipoprotein lipase from different sources. *Biochemistry* 12: 1828, 1973
16. SASAKI T, WAKAI S, ASANO T, WATANABE T, KIRINO T, SANO K: The effect of a lipid hydroperoxide of arachidonic acid on the canine basilar artery: An experimental study on cerebral vasospasm. *J Neurosurg* 54: 357, 1981
17. KRAUS RM, WINDMULLER HG, LEVY RI, FREDRICKSON DS: Selective measurement of two different triglyceride lipase activities in rat postheparin plasma. *J Lipid Res* 14: 286, 1973
18. VOGEL WC, ZIEVE L: Post-heparin phospholipase. *J Lipid Res* 5: 177, 1964
19. SKOREPA J, PROCHAZKA B, TODOROVICOVA H: Differentiation of post-heparin esterase and heparin clearing factor by means of adsorption on calcium phosphate gel. *Clin Chim Acta* 5: 296, 1960
20. DASAI ID, TAPPEL AL: Damage to proteins by peroxidized lipid. *J Lipid Res* 4: 204, 1963
21. HAMADA N, WADA K, MIKI H, OKUDA F, FUKUTA S, IWAMOTO S, KIMURA Y, HONDA M, SHIBATA M, YAMAKAWA T, KUSUKAWA R: Plasma lipoperoxides and carrier lipoproteins. *Lipid Peroxide Res* 3: 97, 1979 (in Japanese)