Lipase Activities in Post-heparin Plasma and Tissues, and Susceptibilities of Lipoproteins in Experimental Diabetic Rats

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Heparin administration to diabetic rats caused no change in VLDL, an increase in IDL and a decrease in LDL on electrophoretic analysis of plasma lipoproteins, while the administration to control rats markedly decreased VLDL and increased IDL and LDL. Both hepatic triglyceride lipase (HTGL) and lipoprotein lipase (LPL) activities in the postheparin plasma were lower in the diabetic rats than in the controls, and the reduction of HTGL activity was greater than that of LPL activity in the diabetic rats. The LPL activity in the adipose tissue was lower in the diabetic rats than in the controls, but the activities in the cardiac and skeletal muscles were similar in the two rats. The HTGL-catalyzed fatty acid (FA) releases from the diabetic VLDL and IDL were lower than those from the normal rat VLDL and IDL, while the LPL-catalyzed FA release in the diabetic rats was not different from those in the controls. The decreases in LPL and HTGL activities and the markedly impaired susceptibility of IDL to HTGL coincide well with the postheparin changes in plasma lipoproteins in diabetic rats, an increase in IDL and a decrease in LDL.
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Cholesterol (Ch) levels as well as LDL levels are low in rat plasma and hardly affected by dietary Ch intake (1-3). Therefore, it is difficult to produce hypercholesterolemia and atherosclerosis in this species by feeding them a high Ch diet even for a long period (4, 5). However, in rats with diabetes induced by chemical agents such as alloxan and streptozotocin, hypercholesterolemia can be easily induced by Ch feeding (6-8). A long term feeding of even a relatively low Ch diet (eg. 0.25 percent) could induce severe hyperlipidemia and atheromatous changes in their aortas (9). Such hyperlipidemia in diabetic rats has been considered to

be caused from hyperphagia (10), increased intestinal absorption of dietary Ch (10-12), increased biosynthesis of lipids in the intestine (12-15) and delayed lipid removal from the circulation due to decreased LPL and HTGL activities (16-20). Lipase activities which affect plasma lipoproteins are found mainly in adipose tissues, liver, cardiac and skeletal muscles. Although the LPL activity in adipose tissue has been proven to decrease in insulin-depleted diabetes in humans as well as experimental animals, that in the muscles is controversial; increased or unchanged or decreased (21, 22).

Herein, plasma postheparin lipolytic activities, tissue LPL and HTGL activities and the susceptibilities of TG-rich lipoproteins to lipases were determined in normal and diabetic rats to examine the relationship between lipoprotein profiles and lipolytic activities in these rats.

Materials and Methods

Male Sprague-Dawley rats weighing about 400 g were kept in an air-conditioned room (25±1°C, 50-60 percent of

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Abbreviations : VLDL ; very low density lipoprotein, IDL ; intermediate density lipoprotein, LDL ; low density lipoprotein, HDL ; high density lipoprotein, LPL ; lipoprotein lipase, HTGL ; hepatic triglyceride lipase, Ch ; cholesterol, TG ; triglyceride, PL ; phospholipids, FA ; fatty acid.

humidity) with lighting from 7:00 a.m. to 7:00 p.m., and fed an ordinary stock diet (type CA-1, Japan Clea Co. Ltd., Tokyo) *ad libitum*. The rats were injected 40 mg/kg body weight of alloxan monohydrate (Tokyo Kasei Co. Ltd., Tokyo) into the tail vein. One month later, after a small portion of blood was drawn from the subclavicular vein, 20 IU/kg body weight of heparin (NOVO, Nordisk A/S, Denmark) was injected into the tail vein and 5 min later the postheparin blood was obtained by cardiac puncture. The lipid concentrations, total and free Ch, TG, PL and free FA in the pre- and postheparin plasma were measured by enzymatic colorimetric methods using commercially available kits (23). Plasma HDL Ch was also estimated by the enzymatic colorimetry after VLDL and LDL were removed by the precipitation with dextran sulfate and $MgCl_2$ (23). Lipoprotein profiles were determined by 3 percent polyacrylamide gel disc electrophoresis using Sudan Black B-prestained plasma (24), followed by scanning densitometry (Johko Sangyo Co. Ltd., Tokyo).

LPL and HTGL were prepared from the postheparin plasma by heparin-sepharose column chromatography (25). In brief, 0.2 ml of heparin-sepharose suspended in buffer A consisting of 20 mmol/l Tris-HCl buffer (pH 7.0), 0.1 percent Triton X-100 and 30 percent glycerol was poured into a column 5 mm in diameter. The column was loaded with 2 ml of the postheparin plasma and washed with 5 ml of a 0.4 mol/l NaCl solution in buffer A and thereafter 0.1 ml of a 0.75 mol/l NaCl solution in the same buffer. The HTGL fraction was eluted from the column with 2 ml of a 0.75 mol/l NaCl solution in buffer A. Then, the LPL fraction was eluted with 2 ml of a 1.5 mol/l NaCl solution in buffer A after washing with 0.1 ml of a 1.5 mol/l NaCl solution in buffer A. To remove the high concentrations of NaCl and other additives from the eluates, 6 volumes of distilled water were added to the eluate which was then ultrafiltrated (Ultra Cent-30, Tosoh Co. Ltd., Tokyo). The ultrafiltration tube was centrifuged at 3,000 rpm for 20 min at 4°C. The procedure was repeated again and finally 6 volumes of saline were added to the tube. After centrifugation, the volume of the enzyme solution was adjusted with saline to the same volume as the original eluate. The enzyme solution was stored at -80°C until use.

Lipolytic activity was determined nonradioisotopically as follows (26); 10 ml of the LPL or HTGL solution prepared by the column chromatography from the sera of rats in both groups were added to 30 ml of normal rat VLDL separated by ultracentrifugation, the TG concentration of which was adjusted to 500 mg/dl, and 10 ml of 120 mg/ml FA free bovine serum albumin (Sigma Chemical Co. Ltd., #A-6003, St. Louis, Mo.) in 0.2 M Tris-HCl buffer (pH 8.2) and 20 mM $CaCl_2$. The mixtures were incubated at 37°C for 20 min with constant shaking at 60 cycles/min. To exclude the non-enzymatic release of FA from VLDL, the solution mixtures omitting the enzyme solutions were used as the controls. FA concentrations in these incubated solutions were measured by the enzymatic method. The net release of FA by the enzyme was calculated by subtracting the FA concentra-

tion in the controls from those in the complete incubation solutions.

In another experiment, adipose tissue, and cardiac and skeletal muscles (psoas) were obtained from the other control and diabetic rats. The tissue was stored at -20°C until use. The tissue was minced to small pieces and homogenized with 10 volumes of the detergent solution reported by Iverius *et al.* (27). The extract was centrifuged at 4°C at 10,000 rpm for 10 min to remove fatty layers and sediments, and the LPL activity in the extracts was determined as described above. The FA values measured in the incubation mixtures were corrected by subtracting the FA values in the tissue extracts.

The plasma VLDL and IDL from control and diabetic rats were separated by ultracentrifugation described by Havel *et al.* (28). The lipid concentrations were determined by the enzymatic colorimetric methods and the protein concentrations by the method using Coomassie Brilliant Blue G-250 (29). The FA release experiments from these VLDL and IDL by LPL and HTGL were carried out as described above (26). Statistical analyses were done with paired and unpaired Students' *t* tests.

Results

Figure 1 shows the plasma lipid concentrations of the control and diabetic rats before and after heparin administration. The TG and PL levels decreased, but the FA level increased significantly after heparin injection in both groups. The plasma Ch level was not affected. Although the net reduction in plasma TG level after heparin administration was 107.0 ± 9.64 mg/dl in the control rats and 109.8 ± 9.72 mg/dl in the diabetic rats, there being no significant difference between the two groups, the percent reduction in TG level was calculated as 47.2 in the former and as 14.4 in the latter. The net reduction in plasma PL level was larger in the diabetic rats (49.2 ± 6.73 mg/dl) than in the control rats (18.3 ± 3.25 mg/dl) and the percent reduction of PL was 11.9 in the diabetics and 14.4 in the controls, there being no significant difference between the two groups. The net increase of FA level was 3.56 ± 0.37 mEq/l in the controls and 5.92 ± 0.166 mEq/l in the diabetics, which was significantly larger than that in the controls. However, the FA level increased $14.0 \times$ fold from the preheparin plasma level after the heparin administration in the controls, while $8.2 \times$ fold in the diabetics. The HDL Ch concentration was elevated from 25.0 to 45.5 mg/dl in the controls ($p < 0.05$), and from 17.4 to 30.1 mg/dl in the diabetic rats ($p < 0.05$).

Figure 2 shows the plasma lipoprotein profiles of the control and diabetic rats before and after heparin injection, and Table 1 the composition ratios of VLDL, IDL, LDL and HDL in percentages. In the control rats, VLDL and HDL were major constituents comprising about 90 percent of total serum lipoproteins. In the diabetic rats, VLDL, IDL and LDL increased, but HDL markedly decreased. After heparin injection, VLDL decreased, IDL and LDL increased, and HDL

Table 1. Changes of lipoprotein profiles in control and diabetic rats after heparin administration.

		VLDL	IDL	LDL	HDL
Control					
Before	(8) [†]	40.4±5.02 [#]	2.2±1.56	10.1±1.57	47.3±3.21
After	(12)	15.3±1.76	18.0±1.45	20.5±1.22	46.3±2.22
p		<0.001	<0.001	<0.001	ns
Diabetic					
Before	(4)	50.2±2.93	9.4±1.68	29.8±4.21	10.5±0.78
After	(4)	51.5±5.65	18.2±5.74	16.1±2.03	14.2±1.05
p		ns	ns	<0.05	ns

Plasma lipoprotein profiles of control and diabetic rats were analyzed by 3 percent polyacrylamide gel disc electrophoresis before and after heparin administration (20 IU/kg body weight). [†]: Number of rats, [#]: Mean±S.E.M. (percent to total).

Fig. 1. Plasma lipid concentrations in control and diabetic rats before and after heparin administration. Control and diabetic rats were fed an ordinary stock diet for one month. Before and after heparin administration (20 IU/kg body weight), plasma lipid concentrations were determined by enzymatic colorimetries.

remained unchanged in the control rats. However, in the diabetic rats, the responses of lipoproteins to heparin injection were different from those in the control rats; IDL increased, LDL decreased and VLDL and HDL were unchanged.

Table 2 shows the correlation coefficients between the fasting blood glucose level and each plasma level of lipoproteins or lipids in the diabetic rats. Significant correlations were noted for IDL, LDL, total lipids, Ch and PL. Above all, the closest relationship was found for IDL, which was significant under a 0.1 percent level.

Both HTGL and LPL activities in the postheparin plasma decreased in the diabetic rats. The HTGL activity in the diabetic rats was 35.1 percent of that in the control ($t=13.324$, $p<0.001$) and the LPL was 48.0 percent ($t=5.986$, $p<0.001$), when FA releases were expressed by the net masses of FA released by lipase contained in 1 ml postheparin plasma (on the left panel in Fig. 3). When FA releases were expressed per mg protein in the lipase solutions, the similar tendencies were observed as shown on the right

Table 2. Correlation coefficients between fasting blood glucose and plasma lipoproteins in diabetic rats.

Lipoprotein and lipids	Correlation coefficients	p
Chylomicrons	0.558	0.1188
VLDL	0.653	0.0565
IDL	0.899	0.0010
LDL	0.848	0.0038
HDL	-0.562	0.1150
Total lipids	0.700	0.0359
Total cholesterol	0.771	0.0150
Free cholesterol	0.718	0.0294
Esterified cholesterol	0.789	0.0155
Triglycerides	0.564	0.1136
Phospholipids	0.692	0.0388

(n=9)

Lipoproteins were obtained by ultracentrifugation as described in the text.

Fig. 2. Lipoprotein profiles in control and diabetic rats before and after heparin administration. Control and diabetic rats were fed an ordinary diet for one month. Before and after heparin administration (20 IU/kg body weight), plasma lipoprotein profiles were analyzed by 3 percent polyacrylamide disc gel electrophoresis.

Table 3. VLDL and IDL compositions in control and diabetic rats.

	Prot	PL	FC	EC	TG	Core [¶]	TG/EC	PL/FC
VLDL								
Control	9.5 [#]	16.0 [#]	4.3 [#]	3.8 [#]	66.4 [#]	70.2 [#]	18.0 [§]	3.72 [§]
(5) [†]	±0.25	±0.43	±0.28	±0.42	±1.29	±0.87	±2.74	±0.133
Diabetic	9.1	15.1	8.7 ^{**}	13.5 ^{**}	53.7 [*]	67.2	5.0 ^{**}	1.74 ^{***}
(5)	±0.36	±0.67	±0.93	±2.34	±3.90	±1.48	±1.27	±0.111
IDL								
Control	16.0	14.6	3.7	6.0	59.7	65.7	11.46	4.01
(5)	±2.31	±0.73	±0.28	±1.11	±3.16	±2.73	±2.07	±0.183
Diabetic	13.7	22.9 ^{***}	10.7 ^{***}	33.5 ^{***}	19.2 ^{***}	52.7 ^{**}	0.58 ^{***}	2.15 ^{***}
(5)	±0.86	±0.34	±0.35	±1.25	±1.74	±0.69	±0.07	±0.064

VLDL and IDL were prepared from control and diabetic rat plasma and their protein and lipid concentrations were determined by CBB G-250 and enzymatic colorimetries.

[†] : Number of rats, [†] : Mean ± S.E.M. (percent to total), [¶] : Core = TG + EC, [§] : Ratio.

Significantly different to control; * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$.

panel of Fig. 3. The HTGL activity in the diabetic rats was 29.8 percent of the control ($t=11.694$, $p < 0.001$) and the LPL activity was 38.1 percent of the control ($t=8.023$, $p < 0.001$).

The LPL activities in the cardiac and skeletal muscles in the diabetic rats were not different from those in the control rats, while only the LPL activity of the adipose tissue in the diabetic rats decreased significantly (Fig. 4).

Table 3 shows the percent compositions of protein and lipid in VLDL and IDL. When the VLDL composition of the diabetic group was compared with that of the control, the proteins and PL contents were similar, free and esterified Ch contents increased and TG contents decreased. The change in IDL composition in diabetes was similar to that in

VLDL, but the increase in esterified Ch and the decrease in TG were prominent in IDL. In addition, PL increased in this lipoprotein fraction in diabetes. The core components (TG plus esterified Ch) in VLDL and IDL decreased or tended to decrease in the diabetic group, and esterified Ch in the core components as well as free Ch in the surface components increased significantly.

The effects of HTGL and LPL, isolated from normal rat postheparin plasma, on HTGL- and LPL-catalyzed FA release from lipoproteins were examined in the diabetic and control VLDL or IDL (Fig. 5). The FA release by HTGL was lower in both the diabetic VLDL and IDL than those in the controls. The FA release from VLDL was similar to that from

Fig. 3. HTGL and LPL activities in postheparin plasma from control and diabetic rats. HTGL and LPL were separated by heparin-sepharose column chromatography and their activities were determined non-radioisotopically as described in the text.

IDL in the control group, but in the diabetic group the FA release from IDL was lower than that from VLDL. The FA release by LPL was lower in IDL than in VLDL, but no significant difference was found between the controls and the diabetics.

Discussion

The metabolism of VLDL has been considered to be depressed in the patients with NIDDM and IDDM (30–38) as well as in the animals with diabetes induced by chemical agents such as alloxan (39, 41), although other factors such as lipid synthesis (10, 12–15, 32–34) or lipid absorption (10–12, 44) have been also suggested to contribute to the impaired lipoprotein metabolism in diabetes mellitus. Some investigators have reported a decrease of LPL activities in hypoin-sulinemic or insulin resistant diabetic states (30, 31, 33, 45, 46), while others have indicated a decrease of HTGL activities (47–49). LPL activity in adipose tissues has been regarded to decrease in diabetes (35, 45, 50–56) but not in skeletal muscles (45, 52, 55–57). The LPL activity of cardiac muscles has been reported to increase (56, 58) or

Fig. 4. Tissue LPL activities in control and diabetic rats. Adipose tissue (upper), cardiac (middle) and skeletal muscles (lower) were obtained from control and diabetic rats. LPL were extracted from the minced tissues and the LPL activities were determined as described in the text.

decrease (59–61) in diabetes, but other investigators found no remarkable changes in muscle LPL activity (56, 58). Recently, Tavanger *et al.* (54) reported that the LPL specific activity in the cardiac muscle was not changed in the diabetic rat, although the enzyme mass increased and the mRNA decreased in the tissue. In the present study, the LPL activity in the cardiac and skeletal muscles was not affected, but that in the adipose tissue decreased in a diabetic rat one month after alloxan injection. Since the total mass of adipose tissue in the whole body decreased in the diabetic rat (55), the total LPL activity in the whole body must be much further decreased. The mass of skeletal muscles occupies a major part of body constituents except in marked obesity and its total mass is not affected greatly by the diabetic state (55). Therefore, the reduction of LPL activity will be smaller than that of HTGL activity in a diabetic rat and a fairly large part of the total LPL activity in the postheparin plasma could be of muscular origin.

VLDL from rats fed a high Ch diet for 12 months increased their Ch contents and surface components of lipoproteins (9). The susceptibility of VLDL to LPL, which was estimated by FA release *in vitro*, decreased markedly in these Ch fed animals (9). The FA release from VLDL which had a high

Fig. 5. Susceptibilities of VLDL and IDL from control and diabetic rats to HTGL and LPL. VLDL and IDL were prepared from control and diabetic rat plasma and their TG concentrations were adjusted to 500 mg/dl by centrifugal ultrafiltration. FA releases from these VLDL and IDL by normal HTGL and LPL were measured as described in the text. Values are expressed in FA amounts released by lipase per mg protein.

surface/core component ratio was shown to be lowered (26). In this study, the surface/core component ratio in VLDL tended to be high in the diabetic rats, and furthermore, free Ch in the surface components and esterified Ch in the core component increased to 26.4 and 20.1 percent in the diabetic rats from 14.1 and 5.4 percent in the control rats, respectively. Especially, the decrease in a PL/free Ch ratio suggests that the membrane fluidity of lipoprotein particles is impaired in diabetes. As the TG/esterified Ch ratio also markedly decreased in the diabetic lipoproteins, especially in IDL, the content of TG or lipase-substrate in the core parts, was also diminished in the diabetic lipoproteins. When these VLDL and IDL were used as substrates for the estimation of their responses to LPL and HTGL, the FA release from the diabetic lipoproteins by HTGL decreased significantly compared to that from the control lipoproteins, but the FA release from VLDL and IDL by LPL was not affected. FA release from VLDL by LPL *in vitro* decreased significantly in the diabetic rats in the previous experiment (9). In that

experiment the diabetic rats were used 12 months after alloxan administration and TG content in VLDL decreased to about 30 percent in contrast to 54 percent in the present experiment. Thus, VLDL, the composition of which was markedly affected by diabetic conditions, showed decreased susceptibilities even to LPL, although those with moderately altered compositions did not change the susceptibilities to lipase. In addition, the increased glycation of VLDL apo-protein decreased the susceptibility to LPL *in vivo* and *in vitro* (62, 63). Since FA release from diabetic VLDL, the TG content of which was 54 percent, decreased significantly by HTGL, HTGL activity seemed to be more strongly affected by the compositional changes or glycation in lipoproteins than LPL, and the susceptibility of IDL to HTGL is presumably more sensitive than that of VLDL.

In the present study, there was no significant difference in the net reduction of plasma TG concentration after heparin injection *in vivo* between the two groups of rats, and the net changes of PL and FA were larger in the diabetics than in the controls, although the lipase activity *in vitro* decreased and the susceptibility of lipoproteins to lipases was impaired markedly. The discrepancy can be explained by the extraordinary high concentrations of substrates in the diabetic rats more than 3 times of the controls.

LPL is considered to catalyze mainly the metabolic conversion from VLDL to IDL, and HTGL, from IDL to LDL (64). HTGL, however, is reported to catalyze the reaction on VLDL as well as IDL at a similar rate (64). Since HTGL activity was affected by the compositional change in lipoproteins in a larger extent than LPL activity, the metabolic conversion of IDL to LDL *in vivo* would be further reduced in the diabetic rats, in addition to the reduced HTGL activity. Consequently, the delay of IDL catabolism will accelerate the accumulation of plasma IDL in a diabetic state. Actually, LDL decreased after heparin administration in the diabetic rats, while IDL increased as shown in this study. The closest correlation was found between fasting blood glucose and IDL level in the diabetic rats. A significant correlation was observed between fasting blood glucose and IDL or Ch, but not VLDL or TG. These must be due to the increase of IDL and the increase of Ch content in IDL.

Atherogenicity of VLDL is regarded to be weak compared with that of IDL or LDL (65, 66). Previously, diabetic rats, fed a diet containing 0.25 percent Ch for 12 months, showed marked hyperlipidemias and severe atheromatous lesions in their aortas (9). Increased levels of VLDL and LDL in these rats were about 6 times greater than the normal control levels, but IDL increased up to 26 times of the control levels. The marked increase of IDL in these rats might contribute to the development of severe vascular lesions.

The plasma lipid levels except Ch changed significantly after heparin administration in both the control and diabetic rats. The percent changes in TG and FA from the initial levels were less in the diabetic rats than in the controls, but the changes in PL were similar in the two groups. More than 70 percent of PL is present in HDL in rat serum,

although the distribution in HDL is slightly lower in diabetic rats than in the controls (9). Phospholipase activity is demonstrated in both LPL and HTGL, and the activity in the former is considered to be one-third to one-half that in the latter (67). The TG content in HDL increases in hypertriglyceridemia or diabetic hyperlipidemia (9), and the PL hydrolysis is thought to proceed with the progression of TG hydrolysis. These findings together with the marked elevation of plasma PL levels in the diabetic rat imply that the decrease of serum PL might be mainly caused from hydrolysis of HDL-PL.

These characteristics of lipoprotein metabolism in diabetic rats must relate to the characteristic metabolic features reported in rats such as defects in cholesteryl ester transfer proteins (68) or accelerated hepatic uptakes of LDL (69, 70) or exaggerated feed back control of Ch-synthesis in the liver (71). Consequently, further investigations are necessary to elucidate these correlations with lipoprotein metabolism in diabetes mellitus.

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