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Glycation of very low density lipoprotein from rat plasma impairs its catabolism

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Summary. Rat VLDL were glycated in vitro in the presence or absence of a reducing agent. Prior to glycation, the VLDL triglyceride was endogenously radiolabelled with [^{3}H]-oleic acid. Post glycation the VLDL B-apoprotein was exogenously radiolabelled with [^{131}]I. The double labelled VLDL was then injected into normal rats and the decline in plasma radioactivity of the two isotopes was used as a measure of triglyceride and particle clearance. VLDL glycated in either the presence or absence of reducing agent exhibited a significantly slower removal of triglyceride and apoprotein B compared to normal VLDL. The ability of glycated VLDL triglyceride to act as substrate for lipoprotein lipase and hepatic lipase was examined. Increasing concentrations of normal and glycated VLDL triglyceride were incubated with post-heparin plasma. The kinetics of triglyceride hydrolysis were determined in a manner analagous to Michaelis-Menten analysis. Glycated VLDL was found to be poorer than normal VLDL as a substrate for lipoprotein lipase. Glycation of VLDL appears to interfere with the lipolysis of its triglyceride. This may explain the delayed clearance of glycated VLDL triglyceride in vivo. Glycation also extended the mean plasma residence time of the VLDL particle. These factors may, in part, contribute to the hypertriglyceridaemia observed in subjects with diabetes mellitus.

Key words: VLDL, glycation, triglyceride, kinetics, lipase, rat.

Atherosclerosis is the major cause of morbidity and mortalitiy among diabetic patients [1]. The mechanisms by which diabetes mellitus accelerate this are multifactorial, but may include perturbations in lipoprotein metabolism bought about by the post-translational non-enzymatic glycation of the plasma lipoprotein proteins [2–4].

Curtiss and Witztum demonstrated that a number of apoproteins were glycated in hyperglycaemic subjects with diabetes [5], the majority of which were associated with very low density lipoproteins (VLDL). It is plausible to associate the hypertriglyceridaemia observed in subjects with diabetes with an impaired VLDL catabolism due to protein glycation. However, whether or not glycated apoproteins modify the catabolic behaviour of lipoproteins in hyperglycaemic diabetic patients remains to be demonstrated, particularly for the triglyceride rich VLDL.

The aim of this study was to determine whether apoprotein glycation had the potential to interfere with VLDL-triglyceride catabolism. To examine this, normal rat VLDL was glycated in vitro. The rate of endogenously radiolabelled VLDL-triglyceride and VLDL particle (exogenously radiolabelled apoprotein B) clearance was then determined in vivo. In addition, the ability of glycated VLDL-triglyceride to act as substrate for the endothelial lipases (lipoprotein lipase and hepatic lipase) was also examined. We report here that glycated VLDL is not catabolized as efficiently as native VLDL.

Materials and methods

Radiolabelling of rat VLDL-triglyceride

To each ml of ethanolic [9,10-^{3}H(N)]-oleic acid (19 mg/ml, 250 μCi/ml), 1.1 ml of 0.27 mol/l KOH was added and mixed thoroughly. The ethanol was evaporated at 70 °C under a constant stream of high purity nitrogen. Whilst mixing, 4 ml of 26% bovine serum albumin was added to the fatty acids. The oleic acid-albumin complex was then immediately injected into rats to radiolabel the VLDL-triglyceride.

Male Wistar rats of body weight 300–350 g were starved for at least 14 h. Water was available ad libitum. Rats were bled under pentobarbital anaesthesia (65 mg/kg body weight) from the abdominal aorta, 40 min after injection of 100 μCi of the [^{3}H]-oleic acid complex into the femoral vein. Blood was collected into tubes containing EDTA (1 mol/l) and plasma was separated after low speed centrifugation. Phenylmethylsulphonylfluoride and sodium azide were added to plasma to give a final concentration of 0.02 mol/l and 0.002 mol/l respectively. VLDL was isolated at density 1.006 g/ml by ultracentrifugation (Beckman model L8-70, Toronto, Ontario, Canada), using a Beckman 70.1Ti rotor at 108000 *g* for 18 h at 20 °C. The VLDL preparation was washed by recentrifugation through a NaCl solution of density 1.006 g/ml. We established in previous studies that more than 98% of the ^{3}H label was associated with the triglyceride of VLDL [6].

In some experiments the VLDL B apoprotein was also radiolabelled with ^{131}I according to the modified method of McFarlane et al. [7]. This was done after the glycation procedure. Free ^{131}I was removed by passage through a Sephadex G50 (fine) column, followed by extensive dialysis against 0.15 mmol/l NaCl. The distribution of the ^{131}I label in the VLDL lipids and protein components was assessed according to established procedures [7–8].

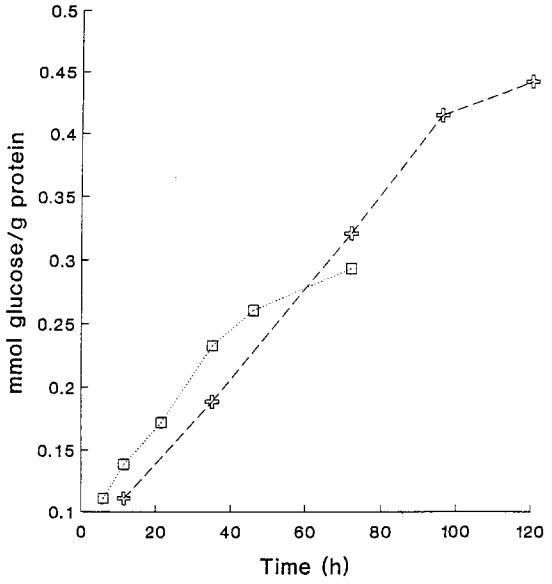

Fig. 1. Glucose incorporation into VLDL-protein with time. Radioactive glucose was incubated with VLDL in the absence (✣) and presence (□) of reducing agent. Results are the mean of two experiments

Glycation of VLDL

Radiolabelled VLDL (700 µg-1000 µg) sterilized by passage through a 0.22 µm low protein binding filter (Millex-GS, Millipore, Boston, Mass., USA) was non-enzymatically glycated in the presence of a sterilized 200 mmol/l D-glucose solution (final volume 3 ml). The assay solution contained 130 nmol/l sodium azide. Glycation was done at 37 °C for 72 h, in a shaking water bath (60 cycles/min), unless stated otherwise. A second VLDL fraction was glycated under identical conditions but with the inclusion of a reducing agent (sodium cyanoborohydride, $NaCNBH_3$), 200 mmol/l). A third VLDL fraction was incubated with 0.15 mol/l NaCl for an equivalent period of time and is referred to as the control VLDL fraction. The VLDL's were kept under a nitrogen atmosphere and in the dark during the glycation procedure. At the end of the 72 h incubation period, the VLDL fractions were collected and dialysed separately against 2 litres of 0.15 mol/l NaCl, pH 7.4 for a minimum of 16 h at 4 °C. The VLDL fractions were then used immediately for the lipolysis or turnover studies.

The rate of VLDL-protein glycation was determined by examining the incorporation of radiolabelled D-glucose-^{3}H (0.1775 µCi/mmol) vs time (270 µCi/mg protein). D-glucose-3-^{3}H was purified according to the method of Schleicher et al. [4]. Aliquots of 200 µl of the VLDL incubation mixture were taken at selected time intervals. VLDL-protein was precipitated and washed as described by Witztum et al. [9]. The amount of radioactive glucose which was incorporated into total VLDL protein was determined by directly counting the radioactivity in the precipitate (LKB model 1219 liquid scintilation counter, Toronto, Ontario, Canada). Glucose incorporation into the various apoproteins was determined by separating the apoproteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (3–10% polyacrylamide gradient gels, Isolab Inc. Cleveland, Ohio, USA), and measuring the amount of radioactivity associated with each of the proteins. VLDL was lyophilized and delipidated according to the method of Herbert et al. [10]. To obtain a clearer separation of the apoprotein isoforms, apoprotein B100, B95 and B48 were separated by 3% SDS-PAGE [11] and soluble proteins (apoproteins E and C) by isoelectric gel focussing electrophoresis (IEF) [12].

VLDL-triglyceride lipolysis studies with rat post-heparin plasma

The ability of glycated and non-glycated rat VLDL-triglyceride to be lipolyzed by lipases in rat post-heparin plasma (PHP) was examined. It is not possible to calculate K_m and V_{max} for a lipoprotein substrate and with an impure enzyme. Therefore, we measured the rate of lipolysis at various VLDL concentrations and used an approach analagous to traditional Michaelis-Menten kinetics to calculate the half maximal rate of VLDL-triglyceride lipolysis, termed "apparent K_m" and the point at which post-heparin plasma was saturated with substrate, termed "apparent V_{max}". In a typical VLDL-triglyceride lipolysis study the assay mixture contained 50 µl of a 3.5 in 5 dilution (in 0.15 mol/l NaCl) of PHP, 300 µl of VLDL-triglyceride (1 mmol/l triglyceride), 25 µl of heat inactivated rat serum and 325 µl of 0.15 mol/l NaCl. Incubation was done at 37 °C in a shaking water bath for 7.5 min. At the end of the incubation time the assay was stopped by the addition of 3.5 ml of isopropanol:hexane:0.5 mol/l H_2SO_4 (40:10:1). Then 2.1 ml of hexane followed by 1.4 ml of 0.05 mol/l H_2SO_4 were added. The tubes were capped and shaken vigorously for 10 min. After low speed centrifugation, 2.1 ml of the upper phase was transferred to tubes containing 1.05 ml of 0.1 mol/l NaOH in ethylene glycol. The tubes were capped, shaken vigorously for 10 min and centrifuged. 700 µl of the upper phase was transferred into vials containing scintillant. Recovery was monitored using an internal standard which was added to the PHP at the start of the assay (^{14}C palmitic acid, 0.1 µCi/ml PHP). Radioactivity was counted in an LKB (model 1219) liquid scintillation counter in dual label mode with auto quench correction. To determine the contribution of lipoprotein lipase (LPL) and hepatic lipase (HL) to total lipolysis, HL was determined in a second tube which contained the same components in the incubation medium except that the rat serum was omitted and the 0.15 mol/l NaCl was replaced with 350 µl of 3 mol/l NaCl, to give a final salt concentration of 1 mol/l. Under these conditions LPL is not active [13]. LPL was calculated as the difference between total lipase activity and HL activity. For some experiments the concentration of VLDL-triglyceride, PHP or time of incubation may have differed. These are described in the text.

In vivo kinetic studies

VLDL-triglyceride kinetics were determined by injecting VLDL-triglyceride[^{3}H-oleate] into the femoral vein of 330–370 g rats (under pentobarbital anaesthesia) and monitoring the decline in plasma triglyceride radioactivity. The mass of VLDL triglyceride injected was never more than 5% of the recipients total plasma triglyceride pool. At 1–2 min intervals 400 µl blood samples were drawn for up to 14 min, from an indwelling polyethylene cannula (PE-50, internal diameter 0.58 mm) in the opposite femoral vein. After each blood sampling the cannula was flushed with 0.15 ml of 0.15 mol/l NaCl. Plasma was collected after centrifugation at 3000 rev/min for 10 min at 4 °C. Radiolabelled triglyceride was extracted from 0.2 ml

Table 1. The half-life for [^{3}H]-triglyceride of normal and glycated VLDL

		Half-life (T1/2) (min)
Normal VLDL-TG	(10)	6.88 ± 0.45
Reductively glycated VLDL-triglyceride	(9)	12.36 ± 1.19[a]
Non-reductively glycated VLDL-triglyceride	(7)	11.31 ± 1.36[a]

Endogenously radiolabelled VLDL was glycated in the presence or absence of a reducing agent. The VLDL's were injected into normal recipient rats and the clearance rate determined. *(n)* = number of recipient animals. Half-life is min ± SEM. [a] denotes significance vs control at $p < 0.001$

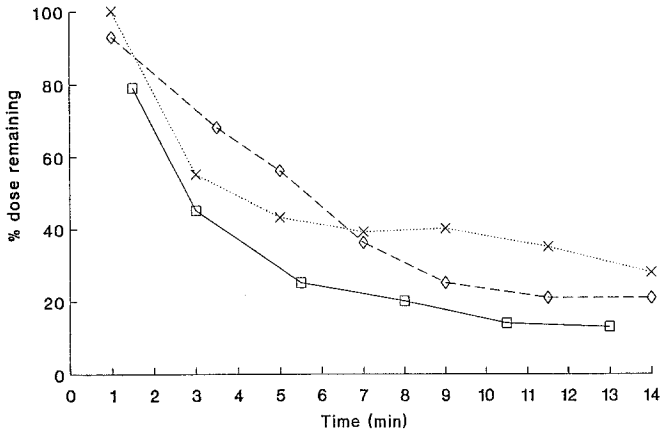

Fig. 2. Typical clearance of control VLDL-triglyceride (□), reductively glycated VLDL-triglyceride (×) and non-reductively glycated VLDL-triglyceride (◇) in normal rats

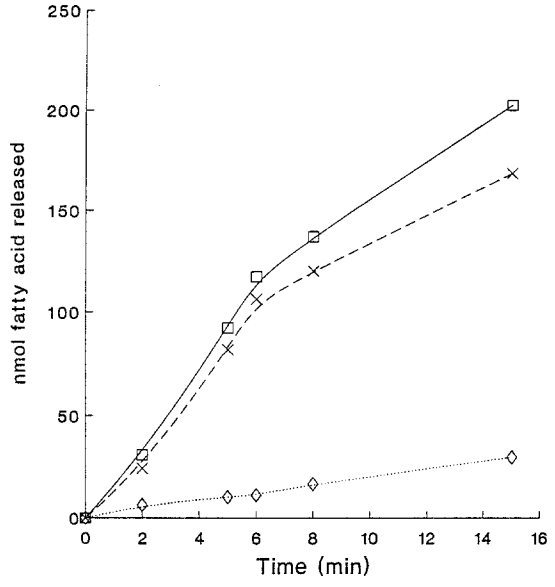

Fig. 3. Hydrolysis profile of normal VLDL-triglyceride lipolyzed with post-heparin plasma from a normal rat with time. Total activity (□) and hepatic lipase activity (◇) were measured. Lipoprotein lipase activity (×) was determined by the difference between the total and hepatic lipase activities

plasma with 4.8 ml of isopropanol, in the presence of 2 g heat reactivated Zeolite (Sigma, St. Louis, Mo, USA). Preliminary investigations showed that under these conditions, over 97% of the label in the isopropanol was in the triglyceride. Then 2.5 ml of the isopropanol extract was dried under nitrogen and radioactivity counted in scintillant, using an LKB liquid scintillation counter (model 1219) with auto quench correction. Clearance of radiolabelled VLDL-triglyceride was monoexponential in nature. Half-life of triglyceride was calculated after exponential regression analysis. The kinetics of VLDL-apoprotein B and VLDL-triglyceride were simultaneously determined in recipient rats by monitoring the rate of decline in plasma radioactivity of double labelled VLDL (^{3}H-triglyceride and ^{131}I-apoprotein B). Eight 300 µl blood samples were taken over a 30 min period. Apoprotein B was specifically precipitated directly from plasma as previously described [14]. Triglycerides were extracted prior to counting. Radioactivity associated with either protein or lipid was determined using standard channels ratio mode, in an LKB liquid scintillation counter with auto quench correction. The mean residence time (MRT) of triglyceride and apoprotein B were determined in preference to half-life, because over a 30 min period the clearance patterns were of a dual exponential nature (at least with respect to the control VLDL).

Statistical analysis

Each batch of PHP contained a different amount of lipase activity, and so the calculated "apparent K_m" and "apparent V_{max}" were not normally distributed. Therefore, the non-parametric Mann-Whitney test was used to compare the VLDL-triglyceride lipolysis studies. The in vivo kinetic data was compared by analysis of variance.

Results

Rate of lipoprotein glycation

The rate of VLDL-protein glycation was determined by monitoring the incorporation of [^{3}H]-glucose. Figure 1 shows glucose incorporation in the presence or absence of a reducing agent ($NaCNBH_3$, 200 mol/l). Glycation was linear for at least 72 h and not influenced by cyanoborohydride.

In vivo VLDL-triglyceride kinetics

Initially, we wanted to determine whether glycation of VLDL protein had the potential to interfere with removal of triglyceride in vivo. VLDL-triglyceride from normal rats was endogenously radiolabelled with [^{3}H]-oleate prior to glycation. The labelled VLDL-triglyceride was then injected into normal recipients and the rate of decline in plasma triglyceride radioactivity was used to determine the half-life. Figure 2 shows an example of the clearance of VLDL-triglyceride profile obtained when the different VLDL preparations were injected into normal recipient rats. Table 1 shows that triglyceride of VLDL that had been glycated with or without reducing agent remained in the plasma compartment about twice as long as that of normal VLDL.

In vitro lipolysis studies

An impaired removal of VLDL-triglyceride may be due to two possible mechanisms. The first is that the triglyceride of glycated VLDL particles is less able to be lipolysed by endothelial triglyceride lipases. Second, VLDL or their remnants (which are in the greater part depleted of triglyceride) may be cleared less readily by receptor and/or non-receptor mediated processes. To test the first of these, we did a study analogous to those used to obtain Michaelis-Menten kinetics. The concentration of VLDL-triglyceride at which rates of lipolysis were half maximal ("apparent K_m") as well as the concentration at which lipolysis was maximized ("apparent V_{max}") for PHP- LPL and HL from normal rats were determined.

The VLDL-triglyceride lipase study was first characterized prior to "apparent K_m and V_{max}" determinations. Figure 3 shows a typical curve of the time course of hydro-

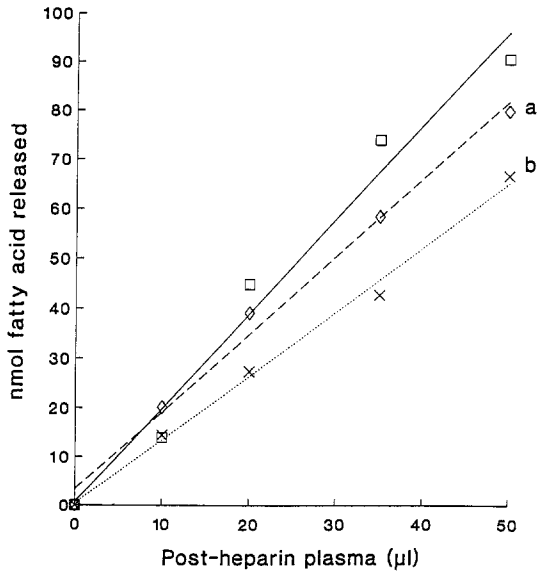

Fig. 4. Mean fatty acids released for normal VLDL-triglyceride (□), reductively glycated VLDL (×) and non-reductively glycated VLDL (◇) with increasing concentration of post-heparin plasma. Results are the mean of four experiments. Significance at $p < 0.025$ and $p < 0.010$ vs normal VLDL-triglyceride denoted by (a) and (b) respectively

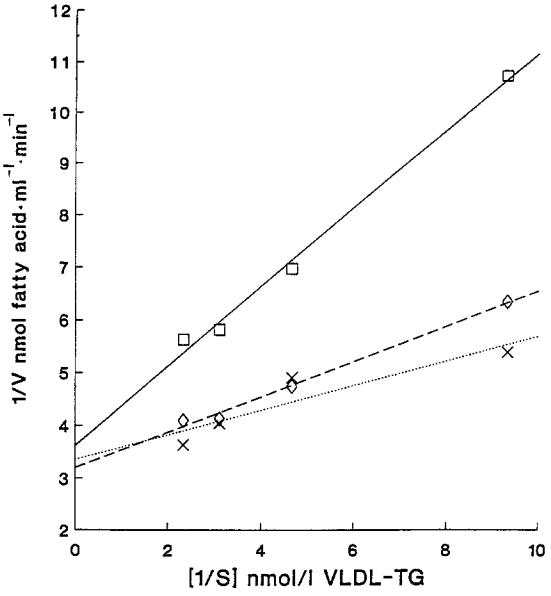

Fig. 5. Linweaver-Burk plots obtained for normal VLDL-triglyceride (□), reductively glycated VLDL (×) and non-reductively glycated VLDL (◇) against post-heparin plasma (PHP) from one normal rat. 1/V = (rate of product released per ml PHP per min)$^{-1}$. 1/S = (concentration of substrate)$^{-1}$

lysis, obtained by incubating normal VLDL-triglyceride with PHP-LPL and -HL. Activities were linear until 6 min at 37 °C, but thereafter LPL diminished at a slow rate. An identical qualitative profile was obtained with both reductive and non-reducitve glycated VLDL.

VLDL-triglyceride at constant concentration was incubated with increasing concentrations of PHP-lipases. Lipolysis was found to be linear over a fivefold increase in the PHP concentration irrespective of the type of VLDL-triglyceride used as substrate (Fig. 4). The rate at which glycated and normal VLDL-triglyceride were hydrolyzed differed however, as shown in Table 2. Glycated VLDL-triglyceride, irrespective of whether this was prepared in the presence or absence of a reducing agent was lipolyzed by PHP-LPL at a slower rate (measured as the rate of product released). On the other hand, VLDL-triglyceride glycated in the absence of $NaCNBH_3$ exhibited a quicker rate of HL mediated hydrolysis, than did normal control VLDL-triglyceride.

Increasing concentrations of VLDL-triglyceride were lipolyzed with PHP. Figure 5 is an example of Linweaver-Burk plots for normal and glycated VLDL against PHP-LPL. Table 3 a lists the K_m data obtained for different enzyme sources. The LPL "apparent K_m" was significantly higher for glycated VLDL than for non-glycated VLDL. This was independent of the two different glycation procedures. On the other hand, there was no significant trend for the "apparent V_{max}" of LPL and the different VLDL-triglyceride preparations (Table 3 b). In addition, there was no detectable difference in the "apparent K_m" for glycated VLDL and PHP-HL. In some instances the glycated particles appeared to be resistant to the activity of this enzyme whereas on other occassions the glycation appeared to enhance the rate of hydrolysis.

In vivo kinetics of VLDL-triglyceride and VLDL-apoprotein B

The delayed clearance of glycated VLDL-triglyceride may, in addition to perturbations in the lipolytic cascade, be due to slower particle removal mechanisms. In order to test this, the triglyceride of VLDL, (both normal and glycated) was endogenously labelled. Subsequently, the protein component was labelled with ^{131}I. The radiolabelled VLDL was then injected into recipient rats and the decline in plasma radioactivity of the two isotopes determined. Apoprotein B [^{131}I] radioactivity clearance was

Table 2. The rates of lipolysis of normal and glycated VLDL-triglyceride

Substrate (VLDL-TG)	Normal		Reductively glycated		Non-reductively glycated	
Enzyme activity	LPL	HL	LPL	HL	LPL	HL
Post-heparin plasma batch no.	(nmol/min)		(% of normal lipolysis rate)			
1	1.726	0.157	41	123	49	240
2	2.738	0.260	74	94	71	206
3	1.077	0.219	83	65	81	140
4	1.231	0.278	73	70	79	143
Mean ± SEM			68 ± 9[a]	88 ± 13	70 ± 7[a]	163 ± 21[a]

VLDL-triglyceride was incubated with increasing amounts of post-heparin plasma from normal rats. Lipoprotein lipase (LPL) activity was determined as the total hydrolase component less the hepatic lipase (HL) activity. The rate of normal VLDL-triglyceride lipolysis is given as the slope of product released (fatty acid). The rate of lipolysis of reductively glycated VLDL and non-reductively glycated VLDL for each source of post-heparin plasma is expressed as a percentage of the rate obtained for normal VLDL. [a] denotes significance at $p < 0.05$

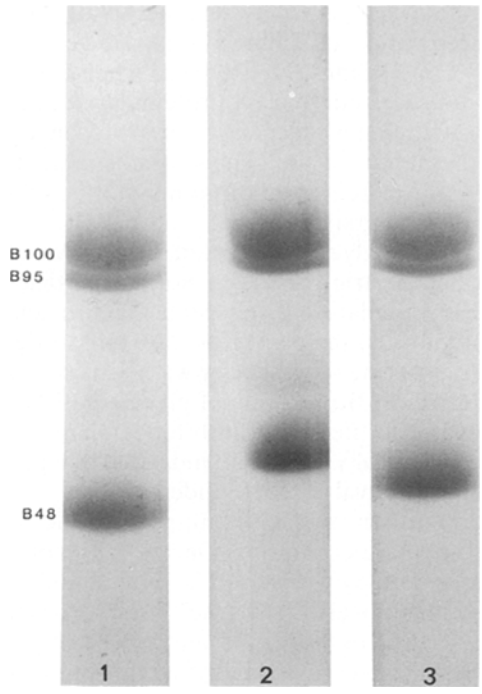

Fig. 6. Lanes 1–3 show apoprotein B for normal VLDL, reductively glycated VLDL and non-reductively glycated VLDL respectively, separated by sodium dodecyl sulphate polyacrylamide tube gel electrophoresis (3% acrylamide)

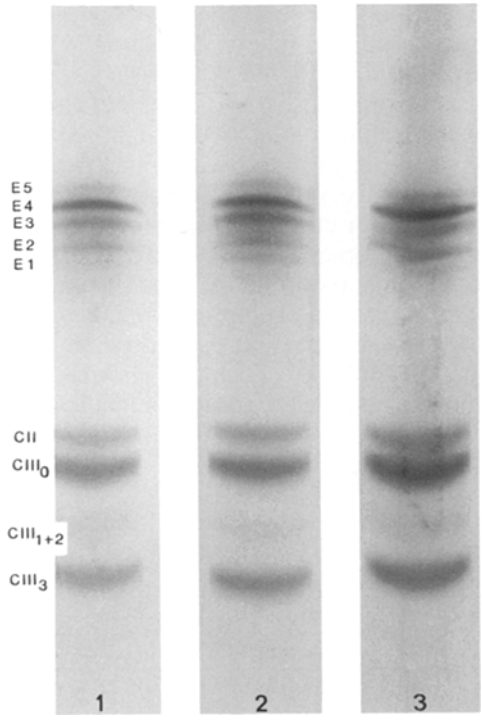

Fig. 7. Isoelectric pattern of VLDL apoproteins in 7% acrylamide tube gels. Lanes 1–3 represent apoproteins from normal VLDL, reductively glycated VLDL and non-reductively glycated VLDL respectively

used as a measure of particle removal. Table 4 lists the MRT of triglyceride and apoprotein B for the three types of VLDL. The MRT of triglyceride and apoprotein B for reductively glycated VLDL were found to be much greater than that of normal VLDL. Similarly the MRT's of non-reductively glycated VLDL tended to be longer than those of normal VLDL. Correlation of the VLDL-triglyceride MRT with apoprotein B MRT showed that for normal VLDL, triglyceride removal was independent of particle clearance (correlation coefficient $r = 0.12$). On the other hand, both types of glycated VLDL exhibited a triglyceride clearance which correlated with apoprotein B removal ($r = 0.95$ and $r = 0.72$ for reductively glycated VLDL and non-reductively glycated VLDL respectively).

Glucose incorporation into VLDL apoproteins

Apoproteins regulate the catabolism of lipoproteins and so to determine how the glycation procedure may potentially alter these, we incubated VLDL with [^{3}H]-glucose, separated the various VLDL apoproteins by SDS-PAGE and IEF and measured the amount of radioactive glucose incorporated into each apoprotein. Total apoproteins were first separated on 3–10% polyacrylamide gels. No difference in banding patterns was observed between treatments. VLDL-glycated in the presence of a reducing agent had 27% of the radioactivity associated with apoprotein B whilst VLDL glycated in the absence of reducing agent had 40% of radioactivity associated with apoprotein B. The majority of the [^{3}H]-glucose was found associated with the soluble protein bands (E's and C's). To resolve the distribution of glucose radioactivity amongst the apoproteins in greater detail, isoforms of apoprotein B and apoproteins E and C were separated by 3% SDS-PAGE (Fig. 6) and by IEF (Fig. 7), respectively. Radioactivity was distributed equivalently between the

Table 3 a. The "apparent K_m" for post-heparin plasma lipoprotein lipase and hepatic lipase with normal and glycated VLDL-triglyceride

Substrate (VLDL-TG)	Normal		Reductively glycated		Non-reductively glycated	
Enzyme activity	LPL (1)	HL (2)	LPL (3)	HL (4)	LPL (5)	HL (6)
Post-heparin plasma batch no.	apparent K_m (nmol/l)		(% K_m for enzyme with normal VLDL-TG)			
			(3)/(1)	(4)/(2)	(5)/(1)	(6)/(2)
1	154	383	149	80	# #	47
2	170	538	165	80	394	81
3	119	382	1062	193	–	–
4	254	302	60	67	–	–
5	2	275	5350	103	3850	79
6	87	203	322	133	187	155
7	88	169	299	118	67	269
8	52	384	463	116	202	109
Mean ± SEM			984 ± 633[a]	111 ± 14	1882 ± 1114[a]	123 ± 33

Control and glycated VLDL were incubated at increasing concentrations with post-heparin plasma lipases. "Apparent K_m" for lipoprotein lipase (LPL) and hepatic lipase (HL) was determined by analogy to Michaelis-Menten kinetics (Fig. 5). The "apparent K_m" for glycated VLDL is shown as a percentage of that obtained for the normal VLDL. [a] denotes significance at $p < 0.05$ vs normal VLDL-triglyceride. # # denotes that "an apparent K_m" could not be derived

Table 3 b. The "apparent V_{max}" for post-heparin plasma lipoprotein lipase and hepatic lipase with normal and glycated VLDL-triglyceride

Substrate (VLDL-TG)	Normal		Reductively glycated		Non-reductively glycated	
Post-heparin plasma batch no.	LPL (1)	HL (2)	LPL (3)	HL (4)	LPL (5)	HL (6)
	apparent V_{max} (nmol/min)		(% V_{max} for enzmye with normal VLDL-TG)			
			(3)/(1)	(4)/(2)	(5)/(1)	(6)/(2)
1	912	684	79	78	##	63
2	866	984	106	71	218	72
3	345	415	266	63	–	–
4	674	340	42	75	–	–
5	289	407	55	135	125	107
6	306	321	57	140	108	155
7	436	247	86	155	66	221
8	271	311	111	126	115	116
Mean ± SEM			100 ± 25	105 ± 13	126 ± 25	122 ± 24

Control and glycated VLDL were incubated at increasing concentrations with post-heparin plasma lipases. "Apparent V_{max}" for lipoprotein lipase (LPL) and hepatic lipase (HL) was determined by analogy to Michaelis-Menten kinetics (Fig. 5). The "apparent V_{max}" for glycated VLDL is shown as a percentage of that obtained for the normal VLDL. ## denotes that "an apparent V_{max}" could not be derived

apoprotein B isoforms of reductively glycated VLDL. On the other hand, VLDL apoprotein B glycated in the absence of $NaCNBH_3$ had 49% of radioactivity with B48. The apoprotein B100 and B95 contained 37% and 24% of the total apoprotein B radioactivity. The two main soluble apoproteins namely E and C were associated with essentially an equivalent proportion of the radioactivity (50% each for reductively glycated VLDL and (46%):(54%) (E:C) for non-reductively glycated VLDL).

Discussion

A characteristic feature of subjects with diabetes is their elevated plasma VLDL-triglyceride. In hyperglycaemic diabetic patients, the majority of glycated apoproteins are associated with VLDL [5], and so in this study we investigated whether VLDL glycation altered its catabolism in a manner which could accentuate the hypertriglyceridaemia.

Glycation was done in vitro as opposed to isolating VLDL from hyperglycaemic donors, in order to avoid other changes in the physical and/or chemical nature of the lipoproteins. A reducing agent ($NaCNBH_3$) was used for one of the glycation procedures to rapidly convert the unstable Schiff base form of the glucose-amine adduct. In the presence of reducing agents a glucitollysine adduct is produced [15], by contrast to ketoamine and hemiketal forms in the absence of reducing agents [16]. Curtiss and Witztum, using monoclonal antibodies which recognize glucitollysine have demonstrated low levels of this type of residue naturally occurring in normal and diabetic individuals [16], though the significance of this is unknown. Nevertheless, we felt that it would be of value to determine whether there were any qualitative differences in terms of the catabolic indices between the Schiff base form (non-reduced) and ketoamine form (reduced) of glycated VLDL. Caution must be exercised when extrapolating kinetic data derived from lipoproteins glycated in vitro, and in rats, with that which may occur in vivo in humans, because the nature of the glucose-amine adducts and the mode of lipoprotein catabolism may differ substantially between the two species.

To establish whether glycation affected the removal of VLDL-triglyceride, the triglyceride of normal particles was endogenously radiolabelled, glycated in vitro and then injected into normal recipient rats. The rate of decline in plasma triglyceride radioactivity was then used as a measure of clearance. Both forms of glycated VLDL were found to have a half-life significantly longer than that of saline incubated control VLDL. This indicated that glycation impaired the removal of triglyceride in vivo.

A delay in lipoprotein triglyceride removal could be related to two separate mechanisms. One could involve impairment of triglyceride hydrolysis. The other could be related to a defect in removal of the particle itself. In order to determine if glycation of the VLDL altered the ability of VLDL-triglyceride to serve as a substrate for these lipases, we studied some of their kinetic characteristics. Lipase rich PHP was used as the source of lipoprotein and hepatic lipase. The lipoprotein lipase "apparent K_m" was found to be much higher for both types of glycated VLDL. No significant trend was established for the V_{max} determinations and so the data led us to conclude that the glycated particles were not as good substrates to the action of lipoprotein lipase.

VLDL-triglyceride may also remain in the plasma compartment for an extended period of time if the particle removal processes are impaired. To examine this we radiolabelled VLDL-triglyceride and VLDL-apoproteins with a different isotope for each, and injected these into normal recipients. The removal of radioactivity associated with triglyceride and apoprotein B from the plasma compartment was then calculated. Normal VLDL-triglyceride was removed in a manner that was independent of particle removal. This would be expected in a normal animal where the lipolytic cascade is of primary significance. In contrast, the data for glycated VLDL showed that in addition to VLDL-triglyceride, apoprotein B removal

Table 4. The mean residence time of normal and glycated VLDL apoprotein B and triglyceride in normal rats

		Mean residence time (min)	
		(Apoprotein B)	(Triglyceride)
Normal VLDL	(10)	16.87 ± 4.79	19.23 ± 4.79
Reductively glycated VLDL	(5)	137.31 ± 49.13[b]	213.19 ± 87.04[c]
Non-reductively glycated VLDL	(10)	46.98 ± 15.38	50.40 ± 18.52[a]

VLDL was radiolabelled for triglyceride and for apoprotein B with different isotopes. VLDL was glycated with glucose in the presence or absence of a reducing agent. Clearance of VLDL was monitored in normal recipient rats. *(n)* = number of recipient animals. N ± SEM. [a,b] and [c] denote significance vs normal VLDL at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively

(i.e., particle clearance) was delayed. In fact, the clearance of glycated VLDL-triglyceride correlated very strongly with the removal of the whole particle. We are unable to distinguish whether the delayed particle clearance merely reflects the impaired lipolysis of VLDL-triglyceride, or whether the glycation itself interferes with receptor and non-receptor removal mechanisms. In support of the latter, Turk and Skrabalo [17] demonstrated that VLDL glycated in vitro in a manner not disimilar to ours impaired high affinity binding and degradation by human skin fibroblasts. Furthermore, LDL glycation to an extent comparable to that occurring in diabetic patients (2–5% glycated lysine residues) decreased the lipoprotein clearance in guinea pigs by 5–25% [18] and also in man [19].

The sites of apoprotein glycation may differ depending on the methods employed [20]. In order to explore how the glycation of apoproteins may have related to the catabolism of VLDL-triglyceride, we determined the incorporation of radioactive glucose into each of the major apoproteins. Apoprotein B was substantially glycated though the soluble proteins (E's and C's) were found to contain the majority of the radioactive glucose. The receptor binding region of apoprotein E is glycatable [21] and so the very long plasma residency time of glycated VLDL may reflect an impairment in receptor mediated removal. One can predict that apoproteins AI, AII and C would have highly reactive groups for glycation [15]. We found that the C apoproteins contained about 35% of the total glucose-amine adducts for both types of glycated VLDL. We were unable to distinguish in this study which of the C isoforms were glycated, however, it may be that if CII/CIII bound glucose, then the lipoprotein lipase-VLDL interaction may be altered.

In conclusion, we have presented data which demonstrate that glycation of VLDL impairs removal of it's main lipid, triglyceride and delays clearance of the particle as a whole. Thus, glycation in vivo may be a contributing factor to the hypertriglyceridaemia observed in subjects with diabetes mellitus. Coupled with reports that glycated LDL are cleared by scavenger pathways and glycated HDL are cleared more rapidly, glycation may exacerbate the pathogenesis of vascular disease amongst diabetic patients.

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