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Decreased Activation of Lecithin : Cholesterol Acyltransferase by Glycated Apolipoprotein A-I

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Summary: Non-enzymatic glycation of plasma proteins may contribute to the excess risk of developing atherosclerosis in patients with diabetes mellitus. Glycated apolipoprotein A-I isolated from diabetic subjects was tested in vitro for its ability to activate lecithin : cholesterol acyltransferase, the principal cholesterol-esterifying enzyme in plasma. Activation by glycated apolipoprotein A-I was significantly lower at all concentrations than the activation by normal apolipoprotein A-I. Linear regression analysis of the kinetic data shows that the ratio app V_{max}/app K_m was significantly lower ($p < 0.01$) for glycated apolipoprotein A-I (0.29 nmol · l/h · μmol) than for normal apolipoprotein A-I (0.78 nmol · l/h · μmol). Because lecithin : cholesterol acyltransferase provides a driving force in reverse cholesterol transport by esterifying the cellular cholesterol removed by HDL, it is tempting to postulate that this abnormal activation may be associated with a reduction in reverse cholesterol transport and associated with the accelerated development of atherosclerosis in diabetic patients.

Introduction

Apolipoprotein A-I is the major protein constituent of human high density lipoproteins (HDL). The protein contains 243 amino acid residues and its primary structure is known (1). The principal physiological functions of apolipoprotein A-I are:

1) interaction with lipids in the surface of high density lipoprotein, thereby ensuring the structural cohesion of HDL particles, and

2) activation of the enzyme lecithin : cholesterol acyltransferase (2), which is responsible for the esterification of cholesterol in human plasma.

Several genetically determined variants of apolipoprotein A-I have been described in the human population and the effects of these mutations on the apolipoprotein functions have been tested. The variant ($Lys_{107\rightarrow 0}$) in which lysine at residue 107 is deleted have been proved to have a decreased association with high density lipoprotein (3) and a defective ability to activate lecithin: cholesterol acyltransferase (4). Another variant, ($Pro^{143} \rightarrow Arg$), in which proline-143 is substituted by arginine is a poorer activator of lecithin : cholesterol acyltransferase than is normal apolipoprotein A-I (5).

Post-translational modifications of apolipoprotein A-I may also alter the function of this apolipoprotein. We have previously reported that apolipoprotein A-I undergoes a non-enzymatic glycation in diabetic subjects (6) and that this modification alters its self-association and lipid binding properties in vitro (7). More recently, we have shown that the non-enzymatic glycation of apolipoprotein A-I diminishes its association with high density lipoprotein in vivo (8).

In this report we document that glycated apolipoprotein A-I purified from the plasma of diabetic subjects is deficient in its ability to activate lecithin : cholesterol acyltransferase in vitro.

Materials and Methods

Purification of apolipoprotein A-I and glycated apolipoprotein A-I

High density lipoprotein was isolated from pooled fresh non-diabetic and diabetic human plasma by sequential ultracentrifugation in the density range 1.063–1.20 kg/l (9). HDL was delipitated with ether: ethanol (3 + 1 by vol.) at 4 °C and the apolipoproteins were fractionated on a Sephadex G 150 column (10). Glycated apolipoprotein A-I was purified from the apolipoprotein A-I of diabetic patients by affinity chromatography in aminophenylboronic acid (Affi Gel 601) (7). All apolipoprotein preparations yielded a single band on SDS-polyacrylamide electrophoresis. Absence of other posttranslational modifications of apolipoprotein A-I and glycated apolipoprotein A-I was confirmed by amino acid analysis. Glycated apolipoprotein A-I contains 1–2 glucose molecules per apolipoprotein A-I molecule.

Purification of lecithin : cholesterol acyltransferase

Lecithin : cholesterol acyltransferase was prepared as described previously (11). Briefly, fresh human plasma was ultracentrifuged at d = 1.23 kg/l for 48 h at 45000 min^{-1} in a Ti 50 rotor at 10 °C. The clear middle zone between the floating lipoproteins and the sedimenting plasma proteins was aspirated, dialysed and chromatographed on a column containing DEAE cellulose. Fractions containing lecithin : cholesterol acyltransferase activity were precipitated with ammonium sulphate and butanol and chromatographed on a Sephadex G 100 column. The purity of this enzyme fraction was assessed by polyacrylamide gel electrophoresis in 10 g/l sodium dodecyl sulphate.

Substrates

Micellar, discoidal substrates containing 2843 µmol/l of 1-palmitoyl-2-oleoyl-*sn*-glycero 3-phosphorylcholine, 125 µmol/l of free cholesterol, 42 MBq/l of [^{3}H]cholesterol and various (3.5, 7.0, 14.0, 21.0, 28.9, and 35.0) µmol/l of apolipoprotein A-I or glycated apolipoprotein A-I, were prepared by the sodium cholate dialysis method (12).

The molar ratios in the micellar discoidal substrates were:

Apolipoprotein A-I in the complex (µmol/l)	Phospholipid :	Cholesterol :	Apolipoprotein
3.5	706	35	1
7.0	706	35	2
14.0	706	35	3
21.0	706	35	4
28.0	706	35	5
35.0	706	35	6

Assay of the lecithin : cholesterol acyltransferase-activating property of apolipoprotein A-I and glycated apolipoprotein A-I

Eighty µl of substrate, 20 µl of 2-mercaptoethanol, 10 µl of human serum albumin (50 g/l) and 140 µl of Tris-HCl 10 mmol/l, pH 7.4 were preincubated in a shaking water bath at 37 °C for 60 min. The reaction was initiated by addition of 50 µl of lecithin : cholesterol acyltransferase (0.30 g/l) and continued for 30 min under the same conditions. The reaction was stopped by the addition of 0.5 ml of methanol. The methanol-containing incubation mixture was extrated with 1 ml of hexane. Cholesteryl esters were separated from free cholesterol by silica gel column chromatography (elution with diethyl ether : hexane (1 + 6, by vol.).

The labelled cholesteryl esters were collected, mixed with 5 ml of scintillation cocktail and the radioactivity was measured in a liquid scintillation analyser, Packard 1600 TR. Blanks without enzyme were run in parallel. All the experiments were performed in duplicate. The precision of the method was 9.6%, as expressed by the coefficient of variation.

The activity of lecithin : cholesterol acyltransferase was calculated from the specific radioactivity of cholesterol and expressed as nmol of cholesterol ester formed per hour. Linear regression analysis was used to obtain the apparent (app) kinetic constants: app. V_{max}, app. K_m, and app. V_{max}/app. K_m.

Results

Apolipoprotein A-I and glycated apolipoprotein A-I were individually tested for their ability to activate lecithin : cholesterol acyltransferase in vitro.

Concentration-dependent, saturable activation of the enzyme reaction was observed for both the apolipoprotein A-I and the glycated apolipoprotein A-I. The results of the activator saturation experiments were analysed by the double reciprocal plot acc. to *Lineweaver-Burk* using normal *Michaelis-Menten* kinetics.

As shown in figure 1, the activation of esterification by glycated apolipoprotein A-I was significantly lower at all concentrations than that obtained by apolipoprotein A-I.

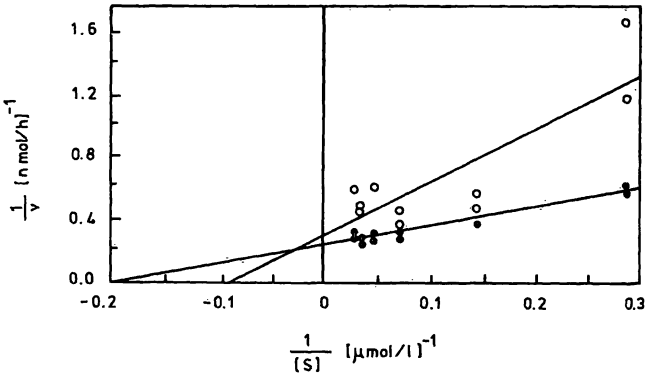

Fig. 1. *Lineweaver Burk* plots giving the reciprocal initial velocity of cholesterol esterification ($1/v_0$) as a function of the reciprocal substrate concentration, apolipoprotein A-I (o) and glycated apolipoprotein A-I (●) (1/[S]). Lecithin : cholesterol acyltransferase activity was assayed at 37 °C by measuring the formation of [^{3}H]cholesterol esters from different liposomes, containing various amounts of apolipoprotein A-I or glycated apolipoprotein A-I. The reaction was initiated by addition of 50 µl of lecithin : cholesterol acyltransferase and stopped after 30 min by adding 0.5 ml of methanol.

Regression equations:

Apolipoprotein A-I:

$$\frac{1}{v} = (3.462 \pm 0.524)\frac{1}{[S]} + 0.320$$

Glycated apolipoprotein A-I:

$$\frac{1}{v} = (1.279 \pm 0.019)\frac{1}{[S]} + 0.241$$

Tab. 1. Apparent kinetic constants for the micellar complexes

	Correlation coefficient	app. V_{max}	app. V_{max}/app. K_m	app. K_m
		nmol/h	nmol × l/h × μmol	μmol/l
Apolipoprotein A-I (n = 12)	0.95	4.14	0.78	5.29
Glycated apolipoprotein A-I (n = 11)	0.85	3.13	0.29	10.82

From linear regression analysis of kinetic data as shown in figure 1 app. V_{max} is the reciprocal of the y-intercept; app. K_m/app. V_{max} is the slope of the line; and app. K_m is calculated from the other two quantities. The ratio app. V_{max}/app. K_m was significantly lower for glycated apolipoprotein ($p < 0.01$) using the *Student*'s t-statistical test.

Table 1 gives the results from the linear regression analysis of the kinetic data: correlation coefficients, apparent V_{max}, apparent K_m and app. V_{max}/app. K_m quantities. The app. V_{max}/app. K_m of glycated apolipoprotein A-I differs significantly from that of normal apolipoprotein A-I ($p < 0.01$).

Discussion

The present study indicates that glycated apolipoprotein A-I isolated from diabetic subjects is deficient in activating lecithin : cholesterol acyltransferase in vitro.

Information is very limited on the lecithin : cholesterol acyltransferase activity in the plasma of patients with diabetes mellitus. *Fielding* and coworkers (13) described decreased activity of lecithin : cholesterol acyltransferase in diabetics, whereas *Schernthaner* et al. (14) found normal activity. *Gugliucci* et al. (15) reported that 10–15% glycation of lysine residues in apolipoprotein A-I produces a 40% decrease in lecithin : cholesterol acyltransferase activation, compared with the control. In the present study, differences in the reactivity of lecithin : cholesterol acyltransferase with glycated apolipoprotein A-I become measurable when the degree of glycation of lysine residues reached 3–5%.

An important consideration in studies of lecithin : cholesterol acyltransferase activity is the use of appropriate kinetic parameters to compare the reactivities of different substrates. In this report the ratio, apparent V_{max}/apparent K_m, was used to monitor the response of the enzyme to different substrates, after interaction of the enzyme with various concentrations of apolipoprotein A-I.

Studies using a series of native and synthetic fragments of apolipoprotein A-I support the concept that the interaction of the protein with lipids is of prime importance for this activating capacity of apolipoprotein A-I (11, 16, 17). Lecithin : cholesterol acyltransferase does not require apolipoprotein to bind to lipid interfaces; however, on a pure lipid surface the active site may not be accessible to the lipid substrates (18). When apolipoprotein is present, the access of substrates to the active site may be facilitated by a direct interaction of the enzyme with the protein cofactor, or by an activation of the lipid substrates via the protein cofactor. It is possible that the glycation of lysine may cause a disruption in the relationships of some portions of the apolipoprotein A-I structure. Studies of lecithin : cholesterol acyltransferase activation by synthetic peptides have led to the proposal that the major lecithin : cholesterol acyltransferase-activating domain of apolipoprotein A-I resides in the 22mer tandem repeats, located between residues 66 and 121 of the native apolipoprotein (19). Therefore, additional studies will be required in order to identify the preferential site of non-enzymatic glycation in apolipoprotein A-I, and to establish whether this involves amino acid residues that are specifically required in lecithin : cholesterol acyltransferase activation.

The significance in vivo of this decreased lecithin : cholesterol acyltransferase activation by glycated apolipoprotein A-I remains to be established. Because lecithin : cholesterol acyltransferase provides a driving force in reverse cholesterol transport by esterifying the cellular cholesterol removed by high density lipoproteins, it is possible that this abnormal activation may be associated with a reduction in reverse cholesterol transport and with the accelerated development of atherosclerosis in patients with diabetes mellitus.

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References

1. Brewer, H. B., Fairwell, T., La Rue, A., Houser, A. & Bronzert, T. J. (1978) The amino acid sequence of human apo A-I, an apolipoprotein isolated from high density lipoproteins. Biochem. Biophys. Res. Commun. *80*, 623–630.
2. Fielding, C. J., Shore, V. G. & Fielding, P. E. (1972) A protein cofactor of lecithin : cholesterol acyltransferase. Biochem. Biophys. Res. Commun. *46*, 1493–1498.
3. Ponsin, G., Gotto, A. M., Utermann, G. & Pownall, H. J. (1985) Abnormal interaction of the human apolipoprotein A-I variant (Lys 107 → 0) with high density lipoproteins. Biochem. Biophys. Res. Commun. *133*, 856–862.
4. Rall, S. C., Weisgraber, K. H., Mahley, R. W., Ogawa, Y., Fielding, C. I., Utermann, G., Haas, J., Steinmetz, A., Menzel, H. J. & Assmann, G. (1984) Abnormal lecithin : cholesterol acyltransferase activation by a human apolipoprotein A-I variant in which a single lysine residue is deleted. J. Biol. Chem. *259*, 10063–10070.
5. Utermann, G., Haas, I., Steinmetz, A., Paetzold, R., Rall, S. C., Weisgraber, K. H. & Mahley, R. M. (1984) Apolipoprotein A-I Giessen (Pro^{143} → Arg). A mutant that is defective in activating lecithin : cholesterol acyltransferase. Eur. J. Biochem. *144*, 325–331.
6. Calvo, C., Ponsin, G. & Berthezene, F. (1988) Characterization of the non enzymatic glycation of high density lipoprotein in diabetic patients. Diabete & Metab. *14*, 264–269.
7. Calvo, C., Talussot, C., Ponsin, G. & Berthezene, F. (1988) Nonenzymatic glycation of apolipoprotein A-I. Effects on its self-association and lipid binding properties. Biochem. Biophys. Res. Commun. *153*, 1060–1067.
8. Calvo, C. & Verdugo, C. (1992) Association in vivo of glycated apolipoprotein A-I with high density lipoproteins. Eur. J. Clin. Chem. Clin. Biochem. *30*, 3–5.
9. Havel, R. J., Eder, H. & Bragdon, I. H. (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest. *34*, 1345–1353.
10. Shepherd, J., Gotto, A. M., Jr. Taunton, O. D., Caslake, M. J. & Farich, E. (1977) The in vitro interaction of human apolipoprotein A-I and high density lipoproteins. Biochim. Biophys. Acta *489*, 486–501.
11. Soutar, A. K., Garner, C. W., Baker, H. N., Sparrow, J. T., Jackson, R. L., Gotto, A. M. & Smith, L. S. (1975) Effects of the human plasma apolipoprotein and phosphatidylcholine acyl donor on the activity of lecithin : cholesterol acyltransferase. Biochemistry *14*, 3057–3064.
12. Matz, C. E. & Jonas, A. (1982) Micellar complexes of human apolipoprotein A-I with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersions. J. Biol. Chem. *257*, 4535–4540.
13. Fielding, C. J., Reaven, G. M. & Fielding, P. E. (1982) Human noninsulin dependent diabetes: Identification of a defect in plasma cholesterol transport normalized in vivo by insulin and in vitro by selective immunoadsorption of apolipoprotein E. Proc. Natl. Acad. Sci. USA *79*, 6365–6369.
14. Schernthaner, G., Kostner, G. M., Dieplinger, H., Prager, R. & Mühlhauser, I. (1983) Apolipoproteins (A-I, A-II, B), Lp (a) lipoprotein and lecithin : Cholesterol acyltransferase activity in diabetes mellitus. Atherosclerosis *49*, 277–293.
15. Gugliucci, A. & Stahl, A. J. C. (1991) In vitro glycation of human apolipoprotein A-I reduces its efficiency in lecithin : cholesterol acyltransferase activation. Clin. Chim. Acta *204*, 37–42.
16. Pownall, H. J., Hu, A., Gotto, Jr. A. M., Albers, J. J. & Sparrow, J. T. (1980) Activation of lecithin : cholesterol acyltransferase by a synthetic model lipid-associating peptide. Proc. Natl. Acad. Sci. USA *77*, 3154–3158.
17. Pownall, H. J., Gotto, Jr. A. M. & Sparrow, J. T. (1984) Thermodynamics of lipid-protein association and the activation of lecithin : cholesterol acyltransferase by synthetic model apolipopeptides. Biochim. Biophys. Acta *793*, 149–156.
18. Jonas, A. (1991) Lecithin : cholesterol acyltransferase in the metabolism of high density lipoproteins. Biochim. Biophys. Acta *1084*, 205–220.
19. Anantharamaiah, G. M., Venkatachalapathi, Y. V., Brouillette, C. G. & Segrest, J. P. (1990) Use of synthetic peptide analogues to localize lecithin : cholesterol acyltransferase activating domain in apolipoprotein A-I. Arterioslcerosis *10*, 95–105.

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