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Research Article

Analyses of Carbohydrates of Apolipoprotein E in VLDL and IDL+LDL of Rat Serum*

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Abstract: Carbohydrate compositions of apolipoprotein E (apo E) isolated from rat serum very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL) + low density lipoproteins (LDL) were analyzed and compared. Rat serum VLDL and IDL+LDL were isolated by a discontinuous density gradient ultracentrifugation for 7.9×10^7g.min. Following delipidization, apolipoproteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Apo E polypeptides were eluted by diffusion in 100 mM PBS buffer (pH 7) containing 0.05% SDS. Sugars were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) and subjected to high performance liquid chromatography (HPLC) analysis. N-acetylneuraminic acids of apo E were derivatized with 4,5-methylenedioxy-1,2-phenylene-diamine dihydrochloride (DMB). Detection of the derivatives was performed with fluorometric HPLC analysis. Mannose, galactosamine, galactose, fucose and N-acetylneuraminic acid contents of apo E were similar in both VLDL and IDL+LDL, while glucosamine content was higher in IDL+LDL ($P<0.01$). The present data indicate that the carbohydrate composition of apo E varies according to the density of lipoprotein particles containing the apo E. This heterogeneity might result from organ-specific glycosylation in glycan chains of apo E due to the fact that the majority of peripheral tissues are capable of synthesizing apo E and that they may contribute up to 40% of total apo E synthesis.

Key Words: Lipoprotein, apolipoprotein E, carbohydrate sequence.

Rat Serum VLDL ve IDL+LDL Apolipoprotein E'lerinin Karbohidrat Analizleri

Özet: Rat serum VLDL ve IDL+LDL'inden izole edilen apo E'nin karbohidrat kompozisyonu analiz edildi ve karşılaştırıldı. Rat serum VLDL ve IDL+LDL fraksiyonları kesintili, dansite dereceli ultrasantrifügasyon ile 7.9×10^7g.min.'de izole edildi. Lipitten arındırılma işleminden sonra apolipoproteinler SDS-PAGE analizine tabi tutuldu. Apo E polipeptidleri, %0.05 SDS içeren 100 mM PBS tamponu (pH 7) ile diffüzyonla elde edildi. Nötral şekerlerin 1-fenil-3-metil -5-pirazolon (PMP) ile işaretleme işleminden sonra HPLC analizleri gerçekleştirildi. Apo E'de bulunan N-asetilnöyraminik asitler 1,2-diamino-4,5-metilendioksibenzen (DMB) ile işaretlendi. İşaretli şekerlerin tespiti florometrik HPLC analizleri ile yapıldı. Mannoz, galaktozamin, galaktoz, fukoz ve N-asetilnöyraminik asit konsantrasyonları hem VLDL ve hem de IDL+LDL fraksiyonlarında benzer olarak bulunurken glukozamin konsantrasyonu IDL+LDL fraksiyonunda en yüksek bulundu ($P<0.01$). Mevcut veriler, apo E'nin karbohidrat kompozisyonunun apo E içeren lipoprotein partiküllerinin dansitelerine göre değiştiğini ortaya koymaktadır. Bu heterojenite, periferal dokuların çoğunun total apo E sentezine %40'a varan bir katkı yapmaları nedeniyle apo E'nin glikan zincirlerindeki organ-spesifik glikozilasyondan ileri gelebilir.

Anahtar Sözcükler: Lipoprotein, apolipoprotein E, karbohidrat dizisi.

Introduction

Apolipoprotein E (apo E) is a glycoprotein with a molecular mass of 34,000 Da and is a component of several classes of plasma lipoproteins including very low density lipoproteins (VLDL), high density lipoproteins (HDL) and chylomicrons (1). Functionally, apo E plays a major role in the metabolism of plasma lipoproteins because of its ability to interact with lipoprotein receptors, low density lipoproteins (LDL) (apo B,E) (2) and remnant receptors (3) (LDL Receptor-Related Protein (LRP)) on the surface of a variety of cell types. All of the VLDL particles undergo hydrolysis by lipoprotein lipase

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(LPL) in extrahepatic tissue capillary beds and are transformed first into intermediate density lipoproteins (IDL) and then into LDL (4). When VLDL is exposed to LPL, most of the apo E appears to remain with partially degraded particles, IDL. Yet apo E is found in only minute amounts in LDL (5). It has been postulated that apo E is synthesized first as sialo-apo E and subsequently desialated in plasma to asialo-apo E before it can be effectively recognized and taken up by the liver (6).

The major objective of our study was to investigate whether or not the carbohydrate composition of apo E in VLDL undergoes any alteration as the VLDL is transformed into IDL and LDL by LPL. To this end, the present report compares monosaccharide compositions of apo E in VLDL ($d<1.006$) and IDL+LDL ($1.006<d<1.063$) of rat serum.

Materials and Methods

Materials

1-Phenyl-3-methyl-5-pyrazolone (PMP), also known as 3-methyl-1-phenyl-2-pyrazolin-5-one, was purchased from Aldrich Chem. Co. (Milwaukee, WI). 4,5-Methylenedioxy-1,2-phenylene-diamine dihydrochloride (DMB) was from BioChemika. Neu5Ac and Neu5Gc were obtained and other monosaccharides were purchased from Sigma Chem. Co. (St. Louis, MO). All other chemicals and reagents were of analytical grade and were commercially available.

Methods

Preparation of Lipoproteins

Male Wistar rats were starved for 18 h in order to prevent chylomicron secretion and allowed drinking water containing 20% sucrose to boost VLDL secretion. The rats were anesthetized with diethyl ether and bled out from the bifurcation point of the abdominal aorta. The blood was centrifuged for 15min at 1,500 rpm. The resulting serum (0.05mg/ml ampicilline added) was adjusted to density 1.10g/ml by adding solid KBr, 0.14g/ml. Then 9.5ml of serum was transferred to a polyallomer ultracentrifuge tube (36.2ml, OptiSeal, Beckman Instruments, Inc., U.S.A.). A discontinuous gradient was obtained by layering 9.5ml of d 1.063 NaCl/KBr solution on top of the serum followed by 9.5ml of d 1.019 solution. Finally, the tube was filled with d.1006 (NaCl/KBr) density solution (7). Ultracentrifugation was carried out in a fixed angle 70 Ti rotor of a Beckman L8-70M ultracentrifuge for 7.9×10^7g.min. at 10°C. Top 2-cm fraction (VLDL) and the 4-cm fraction underneath (IDL+LDL) were collected. Following delipidization with ice-cold diethyl ether, apolipoproteins were precipitated with 70% (v/w) trichloroacetic acid at room temperature. The resulting protein pellet was dissolved in 0.2ml of Laemmli sample buffer and pH was adjusted to 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to Laemmli's method (8).

To isolate the protein from the gel matrix, the protein bands were visualized by Coomassie blue staining. Bands were cut out and chopped finely with a razor blade. The apo E polypeptide was eluted by diffusion into 100 mM PBS buffer (pH 7) containing 0.05% SDS.

The apo E polypeptide was concentrated (and most of the SDS removed) by a collodion membrane (MWCO: 10,000Da) and all-glass collodion apparatus (Schleicher and Schuell, New Hampshire U.S.A). The resulting extract was freeze-dried and redissolved in 0.5ml of water.

PMP-Derivatization of Aldoses

Hydrolysis: Fifty microliters of sample was dissolved in 4M trifluoroacetic acid (TFA). The acid hydrolysis was carried out in a 1.5-ml screw-capped vial in a heating block maintained at 100°C for 4 h. The hydrolyzate was evaporated to dryness in vacuo using a Speed-Vac (Savant Instruments Inc., Holbrook, NY), followed by two cycles of addition of 100µl H_2O and ensuing evaporation (9).

PMP Labeling: PMP derivatization of aldoses was conducted by the method of Fu and O'Neill (10) with some modification. Into 60µl of sample or a mixture of aldoses in a 1.5-ml screw-capped vial were added 0.5 M methanolic solution (75µl) of PMP and 1.5M NaOH (15µl), and the mixture was kept for 2h at 70°C. After the reaction mixture was cooled to room temperature, 0.5M HCl (50µl) was added for neutralization. Chloroform (0.5ml) was added to the resultant solution, and the mixture was shaken vigorously. The organic phase (upper layer) was carefully removed and discarded. This extraction process was repeated twice and a portion of the resulting aqueous phase (lower layer) was injected into the HPLC column.

HPLC Analysis of PMP-Labeled Aldoses

Analysis of the PMP-labeled monosaccharides was carried out on a Gilson 715 HPLC system equipped with a Gilson 119 U.V. detector. A Shim-pack CLC-ODS (6.0x150) column was used for the separation of PMP-labeled carbohydrates. The column was eluted at 1ml/min, and A_{245nm}. of the effluent was monitored. Buffers A and B were 0.1M phosphate buffer (pH 7) containing 10 and 25% CH3CN, respectively. A linear gradient of 0 to 100% buffer B in 30min was used for the separation.

A mixture of monosaccharides, ca 1.5nmol each of fucose (Fuc), galactosamine (GalN), glucosamine (GlcN), glucose (Glc), galactose (Gal), and mannose (Man), was labeled with PMP. N-acetylmannosamine (ManNAc) was used as an internal standard and added (1.5nmol) to both the mixture and apo E samples after the TFA hydrolysis. An aliquot of 100µl of standard (containing 544-750pmol each monosaccharide) or apo E was analyzed by HPLC.

DMB-Derivatization of Sialic Acids

Hydrolysis of Sialic Acids: Apo E extract (10-20µl) placed in a screw-capped 1.5ml vial was mixed with 200µl of 2M acetic acid. The vial was closed and heated at 80°C for 3 h to hydrolyze the sample (11).

DMB-labeling: After the sample was cooled to room temperature, 200µl of DMB solution (7.0 mM), prepared by dissolving DMB dihydrochloride in 1.4 M acetic acid containing 0.75 M β-mercaptoethanol and 18 mM sodium hydrosulfite, was added and the mixture was heated at 50°C for 2.5h in the dark to develop the fluorescence (11). The reaction mixture was cooled in ice to stop the reaction. An aliquot of the resulting solution was injected into the column.

HPLC Analysis of DMB-Labeled Sialic Acids

A Gilson 715 HPLC system equipped with a Perkin-Elmer Fluorescence detector was employed. Detection of DMB-labeled sialic acids was performed with a Shim-pack CLC-ODS (6.0x150) column. It was operated at an emission wavelength of 448 nm and an excitation wavelength of 373nm. The mobile phase was a mixture of acetonitrile-methanol-water (9:7:84, v/v) (11). The flow rate was 1ml/min. The column temperature was ambient (ca. 20°C).

Standard solutions of N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) were prepared and the standards (Neu5Ac 100pmol, Neu5Gc 102pmol) were subjected to the procedure without hydrolysis. An aliquot of 40ml standard, containing 9.52pmol Neu5Ac and 9.71pmol Neu5Gc, or 50µl of apo E samples was analyzed by HPLC.

Results

Verification of Apolipoprotein E Isolated from VLDL (d<1.006) and IDL+LDL (1.006<d<1.063).

The purity of apo E preparations isolated by ultracentrifugation was analyzed by SDS-PAGE. Only a single apo E band was observed in both fractions (Figure 1). Thus the method for isolation of apo E in lipoprotein samples appears to be satisfactory.

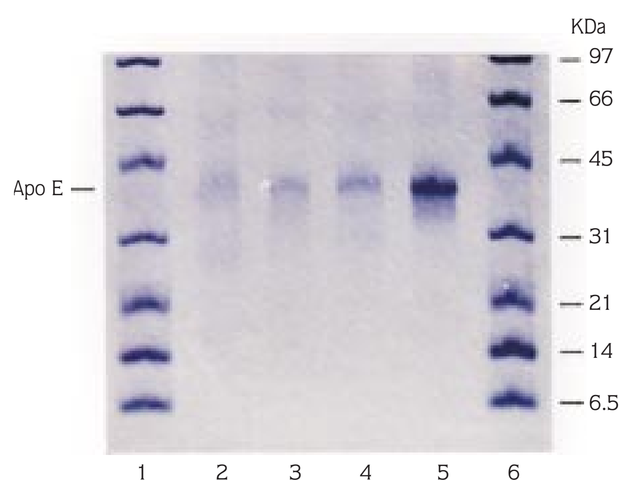

Figure 1. SDS-PAGE electrophoretogram of apo E isolated from VLDL and IDL+LDL of rat serum. Lanes: 1,6, standard proteins; 2,4, apo E from IDL+LDL; 3,5, apo E from VLDL.

Composition Analysis of Oligosaccharides in Apo E of Rat Serum VLDL (d<1.006) and IDL+LDL (1.006<d<1.063)

A mixture of seven monosaccharide standards, Man, GlcN, Fuc, GalN, Glc, Gal and ManNAc, was labeled with PMP and separated by reverse-phase HPLC. All seven PMP-labeled monosaccharides were baseline resolved. The peaks for all monosaccharides were sharp and symmetrical. ManNAc was included (after TFA hydrolysis) in the mixture as an internal standard for sample quantitation. HPLC analysis of PMP-labeled

monosaccharides of apo E in VLDL and IDL+LDL are shown in Figures 2 and 3 respectively.

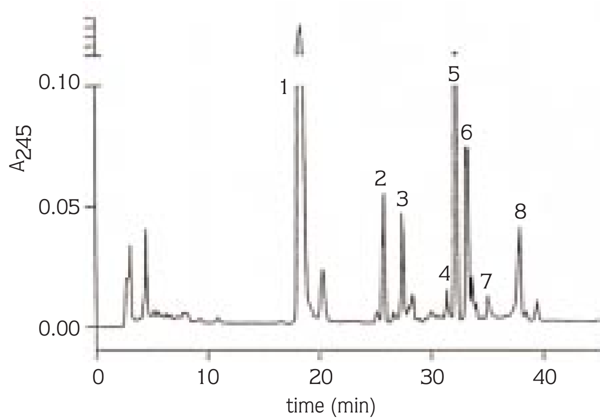

Figure 2. HPLC separation of PMP-labeled monosaccharides from apo E in VLDL of rat serum. Apo E was hydrolyzed with 4M TFA at 100°C for 4 h, PMP-Labeled, and analyzed by HPLC as described under Materials and Methods. Peaks: 1, PMP; 2, PMP-Man; 3, PMP-GlcN; 4, PMP-GalN; 5, PMP-Glc; 6, PMP-Gal; 7, PMP-Fuc; 8, PMP-ManNAc.

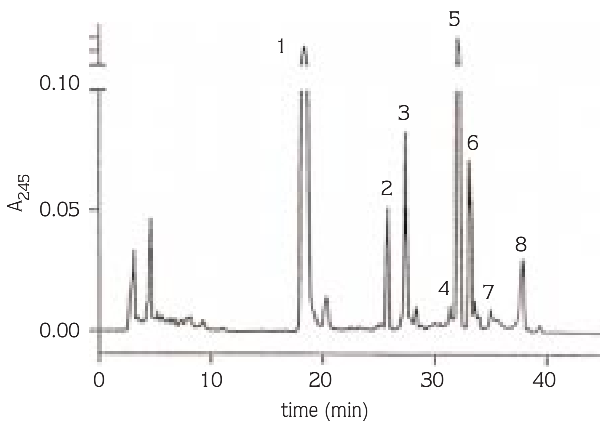

Figure 3. HPLC separation of PMP-labeled monosaccharides from apo E in IDL+LDL of rat serum. Apo E was hydrolyzed with 4M TFA at 100°C for 4 h, PMP-Labeled, and analyzed by HPLC. The peak assignment is the same as that in Fig.1.

Monosaccharide composition was determined in apo E of rat VLDL and IDL+LDL. Man, GlcN, GalN and Gal contents of apo E were similar in both fractions while contents of glucosamine was the highest in the denser fraction. For glucosamine content, a significant difference (p<0.01) was noted between VLDL and IDL+LDL. The carbohydrate compositions of apo E in the lipoprotein fractions are presented in the Table.

Table 1. % Composition of carbohydrate content of apo E in VLDL (d<1.006) and IDL+LDL (1.006<d<1.063) of rat serum.

	Percent Composition by pmol	
	Apo E (d<1.006)	Apo E (1.006<d<1.063)
Man	28±7.3	25.9±6.5
GlcN*	18.5±2.6	28.9±1.8
GalN	5.4±1.1	3±0.4
Gal	34.2±6.8	28.3±5.0
Fuc	12.6±4.0	11.5±3.5
Neu5Ac	1.2±0.2	2.3±0.5

* P<0.01
n:3

The sialic acids from apo E in VLDL or IDL+LDL were released by 2M acetic acid and analyzed and quantitated by HPLC as shown in Figures 4 and 5 respectively. The monosaccharide and sialic acid compositions of apo E are summarized in the Table. The percentages of sialic acids in apo E of both lipoprotein fractions were similar.

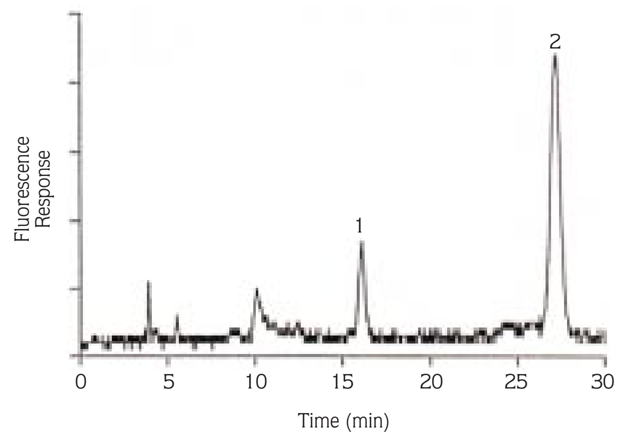

Figure 4. HPLC chromatogram of the DMB derivatives of neuraminic acids in apo E of rat VLDL. Apo E was treated as described in the text. Peaks: 1, DMB-Neu5Ac; 2, DMB or unknown product.

Discussion

The carbohydrate composition of apo E in VLDL was compared to that of apo E in IDL+LDL of rat serum. The carbohydrate composition of apo E in VLDL and IDL+LDL was found not to be identical. While Man, GlcN, GalN, Gal and sialic acid contents of apo E were similar in both fractions, for glucosamine content a significant difference (p<0.01) was noted between VLDL and IDL+LDL.

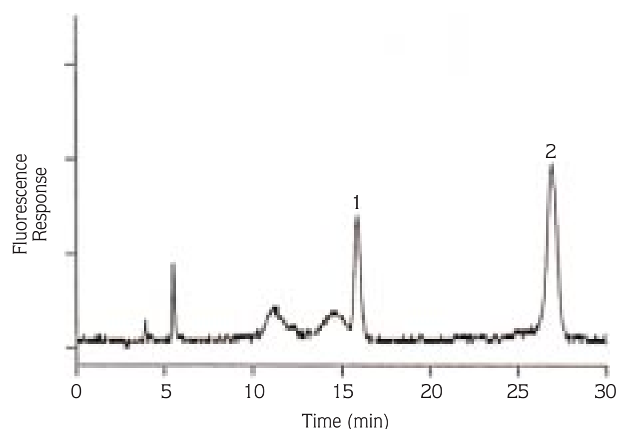

Figure 5. HPLC chromatogram of the DMB derivatives of neuraminic acids in apo E of rat IDL+LDL. Sialic acids from apo E were released by 2M acetic acid hydrolysis, DMB-labeled and analyzed by HPLC. The peak assignment is the same as that in Fig. 3.

It has been reported that apo E, the ligand for LDL and remnant uptake, is synthesized and secreted as sialo-apo E and subsequently undergoes extracellular desialylation in plasma to attain the major asialo-apo E isoprotein form observed in plasma before it can be effectively recognized and taken up by the liver (6). VLDL particles undergo hydrolysis by LPL and are transformed first into IDL and then into LDL (4). When VLDL is exposed to LPL, much of the apo E appears to be conserved within IDL. Yet apo E is found in only minute amounts in LDL. Most of the apo E is transferred to HDL prior to LDL formation (5). Therefore apo E in $1.006<d<1.063$ serum fraction (IDL+LDL) represents apo E in partially or fully degraded lipoprotein particles. The dwell time of the IDL+LDL in the circulation is higher than that of VLDL because the former turns over more slowly. This is also true for the apolipoprotein E residues in those lipoproteins. It was therefore expected that desialylation of apo E would occur as VLDL are transformed to IDL and LDL. However, considering the similarities in the contents of sialic acids in VLDL and IDL+LDL, this was not the case. It appears that desialylation of apo E might happen before IDL formation.

An earlier study has shown that apo E oligosaccharides of human VLDL contain galactose, mannose, glucose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid amounting together to 3-5.4% of the weight of apo E. Sialic acid and galactose were both major carbohydrates (12). However, in the present study, the sialic acid content of apo E in both lipoprotein fractions of rat serum was much lower compared to other monosaccharides. There are two possible reasons for this discrepancy. One possibility is species specific glycosylation of apo E. The another is apolipoprotein purification procedures.

Our compositional analysis as well as Jain and Quarfordt's work (12) indicate the presence of considerable amounts of Man, which is found almost solely in N-linked carbohydrates. In contrast to preceding data, incorporation of N-[H^3]acetylmannosamine and [H^3]mannose into macrophage apo E was not detected (13). Moreover, the carbohydrate attachment site of human plasma apo E was reported to be Thr 194 in monosialo- and disialo-apo E (14). It has also been demonstrated that no obvious sites of N-glycosylation are detectable in the amino acid sequence of rat apo E (15). Taken together, the preceding data indicate the presence of O-glycans in apo E. This controversy remains to be resolved.

Man, GalN, Gal, Fuc and Neu5Ac contents of apo E were similar in both fractions while GlcN content was higher in the denser fraction. The present data suggest that the carbohydrate content of apo E varies according to the density of lipoprotein particles containing the apo E. This heterogeneity might result from organ-specific glycosylation in glycan chains of apo E, because although the liver appears to be the major site of apo E biosynthesis, studies have shown that the majority of peripheral tissues including spleen, adrenal, kidney, testis, heart, lung, macrophages, brain and intestine are capable of synthesizing apo E (16-17) and that they may contribute between 20 and 40% of total apo E synthesis (17). Considering the fact that lipoproteins exchange apolipoproteins (low or intermediate molecular weight) with circulating apolipoproteins (18), the contribution of extrahepatic apo E with a distinct carbohydrate composition (possibly resulting from organ specific glycosylation) to IDL+LDL would therefore be conceivable.

Variations in the distribution and content of carbohydrate moieties of apo B-48 according to the relative density of the chylomicron particle was also reported (19). In view of the metabolic differences existing between chylomicron particles with different density (20), one may speculate that the heterogeneity with respect to distinct oligosaccharide chains might be related to the differences in their metabolism.

A physiological role for carbohydrates in lipoproteins has been proposed in the past, such as the regulation of their catabolism. It has been suggested that modification of LDL by removing or introducing sialic acid residues markedly enhanced or depressed binding and uptake of LDL by vascular smooth-muscle cells or fibroblasts (21). Desialylation of chylomicrons was reported to increase their rate of removal by the liver and the uptake was also accompanied by comparable increases in their metabolism (22). However, the functional significance of these carbohydrate moieties is far from conclusive.

This study, as far as the authors are aware, is the first to describe heterogeneity of the carbohydrate component of apo E. The determining factor for the amount of glucosamine in apo E appears to be the relative density of the lipoprotein particle containing the apo E. The reason for this and any possible physiological consequences remain unknown. Regardless of how or why glucosamine content varies in apo E, it is clear that the carbohydrate content of apo E of VLDL and IDL+LDL is heterogeneous and varies according to the relative density of the lipoprotein particle bearing the apo E.

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