J. Physiol. Biochem., 52 (1), 15-22, 1996
Revista española de Fisiología

Characterization and quantification of apolipoprotein E in the genetically hypercholesterolemic rat (RICO)

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(Received on 11 September, 1995)

L. E. CARDONA-SANCLEMENTE, F. SULTAN and S. GRIGLIO. *Characterization and quantification of apolipoprotein E in the genetically hypercholesterolemic rat (RICO).* J. Physiol. Biochem. (Rev. esp. Fisiol.), 52 (1), 15-22, 1996.

The levels of apolipoprotein E (apo E) in serum and in isolated lipoprotein fractions from genetically hypercholesterolemic rats (RICO) and their normocholesterolemic controls (SW), were evaluated by immunoblotting, and their polymorphism was analyzed by isoelectrofocusing. The present results confirm previous findings from a qualitative SDS-PAGE analysis. When measured by immunoblotting, total apo E increased by 35 % in the RICO rat plasma. In the different lipoprotein fractions, apo E in RICO rat increased in low density lipoproteins: LDL_1, LDL_2 (apo E rich HDL_1) and high density lipoproteins (HDL_2, HDL_3). Only in the chylomicron fraction did apo E decrease. The two-dimensional electrophoretic method demonstrated that four isoproteins designated E-1, E-2, E-3 and E-4 are present at a pI range from 5.36 to 5.56 in RICO rat as well as in control SW rat.

Key words: Hypercholesterolemic (RICO) rat, Apolipoprotein E (apo E), Lipoproteins, Isoelectrical focusing, Immunoblotting.

A relationship has been established between apolipoprotein E (apo E) phenotypes, serum total cholesterol and LDL cholesterol levels and progression of coronary atherosclerosis, but the mechanism(s) underlying this association is not known (6, 16, 17, 21). Apo E is a 34,000 dalton glycoprotein associated with lipoproteins including chylomicron remnants, VLDL, LDL and certain subclasses of HDL, chiefly in rats and fat-fed dogs (1, 12, 28).

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Human apo E polymorphism has been recognized by isoelectric focusing (26), and further clarified by using two dimensional electrophoresis (32, 33). The three major sialo apo E isoproteins (apo E-4, E-3 and E-2, from highest to lowest pI) detected in the human population are allelic products of a single locus (27). Support for the genetic basis of apo E polymorphism in rats was provided by using intact rats or cultured rat hepatocytes. Four unique isoelectric forms (designated E-1, E-2, E-3, and E-4 from acidic to basic, respectively) have been identified in rat plasma (18).

The main characteristics of the RICO rat (a rat with inherited cholesterolemia) have been described (2, 3, 13) by comparison to its normal heterozygote homologue (SW). Enhanced hepatic and intestinal cholesterogenesis and an increase in the mobile cholesterol pool, particularly in the plasma central compartment of the cholesterol system were observed (2). The RICO rat hypercholesterolemia stems mainly from an increased cholesterol content in the d > 1.006 lipoprotein fractions (LDL_1, LDL_2, HDL) without serious modifications in the composition of each type of lipoprotein as compared with the control SW. This hyperlipoproteinemia, demonstrable by qualitative SDS-PAGE analysis of the apoproteins in the plasma lipoproteins, was accompanied by an apo E enrichment in RICO versus the SW rat (3). In the present study, we have more precisely quantified the apo E differences between the RICO rat and control; we have further evaluated the apo E content in serum and isolated lipoprotein fractions by immunoblotting, and analyzed their polymorphism by isoelectric focusing. To investigate the apo E polymorphism in plasma and lipoprotein fractions of RICO rat, the isoelectric isoform points by two dimensional gel electrophoresis (2DE) have also been studied.

Materials and Methods

Animals and diet.– Male RICO and normocholesterolemic SW rats weighing 140-150 g were obtained from Ciba-Geigy (Basel, Switzerland). They were fed *ad libitum* for six weeks on a semi-purified diet containing 53 % saccharose, 23.0 % casein, 9.2 % lard, 5.0 % mineral mix, 4.0 % skimmed milk, 2.5 % yeast, 2.5 % vitamins, 0.8 % walnut oil, 0.2 % cystine and 0.05 % cholesterol. The rats were kept in wirebottomed cages in an air-conditioned animal house (22-23 °C) artificially lit between 07:00 and 19:00 h. Blood samples were obtained either from the caudal vein or by intra-aortic puncture under pentobarbital anesthesia starting at 9:00 h. Rats were treated in randomized blocks and the mean body weights (± SEM) were 355 ± 14 g and 354 ± 10 g in RICO and SW rats (n = 6) respectively. All procedures were approved by the Animal Care and Safety Committee and conformed to guidelines established by the Home Office.

Lipoprotein Separation.– Separation of lipoproteins was performed by ultracentrifugation using a shallow gradient. Briefly, rat plasma was adjusted to a density of 1.21 g/ml with solid KBr. An individual density gradient was then performed using different KBr density solutions (1.006, 1.020, 1.040, 1.063, 1.25 g/ml). Ultracentrifugal separation was accomplished by using a Beckman SW-41 swinging bucket 6 rotor run for 24 h at 40000 rpm and 15 °C (4, 5). The characterisation of the gradient was realized as described previously (3, 24). Twenty two fractions were collected in order to separate further the HDL fraction into HDL_2 and HDL_3 subfractions. Pooled fractions were collected corresponding to chylomicrons and VLDL (d < 1.006 g/ml: fractions 1 and 2), LDL, (LD_1 of density

between 1.006 and 1.040 g/ml: fractions 3 to 6), LDL_2 [apo E rich HDL_1] (LDL of density between 1.040 and 1.063 g/ml: fractions 7 to 11), HDL_2 (HDL of density between 1.063 and 1.1: fractions 12 to 14) and HDL_3 (HDL of density between 1.1 and 1.210 g/ml: fractions 15 to 18). Rat LDL_2 fraction obtained by this technique is essentially contaminated with HDL_1 (3). Chylomicrons were obtained separately from the other lipoproteins, from plasma of individual rats by overlaying 6 ml of plasma with 2 ml of 1.006 g/ml NaCl solution in each tube and centrifuging in a 50 Ti rotor in a L8-70 ultracentrifuge (Beckman) at 15000 rpm for 30 min at 15 °C. Chylomicrons were collected from the meniscus. Proteins were assayed by the Pierce BCA protein assay. Total and free cholesterol, triacylglycerol and phospholipid were determined by enzymatic methods using commercial kits (Biolyon and Boehringer).

Isoelectrical Focusing of rat apoproteins.– Two dimensional gel electrophoresis was performed similarly to the methods previously described (10, 19, 22) with some modifications. Urea (1.4 g) was dissolved in 1.3 ml water and 0.5 ml of a solution containing 30 g of acrylamide and 0.88 g of bisacrylamide. Ammonium persulfate (3 µl, 10 mg/ml) and 2.1 µl TEMED were used as polymerization catalysts. Gels were polymerized in 1.75 mm x 95 mm capillary tubes for 30 min in presence of 0.15 ml ampholytes (pH 5-7 and 4-6; Serva Heildelberg, FRG). After delipidation (acetone/alcohol 1/1, v/v), plasma or lipoprotein fractions were redissolved in 10 mM TRIS buffer, 8 M urea and DTT and placed on the gel. Solutions in the upper tray and the lower tray were 0.05 N NaOH and 0.025 N H_3PO_4 respectively. The gels were electrofocused at 500 V for the first 10 min, 1,000 V for the next 20 min, and 1,500 V for the last 1 h. After isoelectrofocusing, gels were placed for 5 min at room temperature in an equilibration solution containing 0.1 mol of TRIS (pH 6.8), 10 g of SDS and 25 ml of β-mercaptoethanol (per litter). The gels were then loaded on the top of an 8.3 cm x 10.2 x 0.75 mm thick polyacrylamide 15 % slab gel, and were electrophoresed until the dye front was 5 mm from the bottom of the gel (approximately 40 min under 30 mA at 4 °C and then stained in Coomassie Brilliant blue R-250 (Biorad). Then they were destained in 50 ml/l methanol and 75 ml/l acetic acid until a clear background was obtained. Fixation was made in acetic acid/methanol/water (5:50:45 v/v).

Preparation of rat apo E and anti-rat apo E immunoglobulin.– Rat apo E was isolated and purified as described by RIFICI *et al.* (19). Fifty Wistar rats (350-400 g) were given 10 % glucose in their drinking water for 7 days and their lipoproteins were separated from plasma by ultracentrifugation at d = 1.21 g/ml, for 48 h at 100,000 x g at 4 °C. After delipidation with ethanol/diethyl ether (1:3, v/v), a first purification was made on a Sephadex G150 column to eliminate apo B and apo C. The remaining apolipoproteins (apo A1, apo AIV and apo E) were applied to a Heparin-Sepharose column and eluted stepwise as described (19). Apo E was further purified by immunoaffinity with anti-rat apo A1 antibodies to eliminate contamination of apo E by apo A1. Purity was checked by SDS-PAGE and silver staining. Anti-rat apo A1 and anti-rat apo E antibodies were raised in rabbits. After an initial injection of either 100 µg of apo A1 or 150 µg of apo E mixed with Freund's complete adjuvant, four successive booster injections were made with the same amounts of apolipoproteins in Freund's incomplete adjuvant. The immunoglobulins were purified by two

successive 40 % ammonium precipitations.

Quantification of apo E.– Apo E was determined by the immunoblotting technic described by TOBIN *et al.* (25), adapted by GRIGLIO (unpublished results). Briefly, lipoprotein fractions (1 to 5 µg protein in 10 µl maximal volume) were transferred directly onto a nitrocellulose filter 0.45 µm (Schleicher and Schuell). The latter was first incubated with milk proteins overnight to prevent non-specific binding and washed with TS buffer (0.15 M NaCl, 50 mM Tris buffer, pH 7.4). The nitrocellulose filter was then incubated with anti-apo E antibody during 90 min at 20 °C, washed again with TS buffer and incubated with ^{125}I-labelled protein A for 1 h. This was followed by several more washing steps, the last being performed with TS buffer containing 0.2 M NaCl. The membranes were dried and exposed to Kodak X-Omat X-ray film at -80 °C for 18 h. Standard curves were obtained with purified rat apo E in the range from 67.2 ng to 1.075 µg to allow quantification of the apolipoprotein in the samples. The areas corresponding to the spots were cut from the membrane, and radioactivity was determined. A correlation of 0.96 was found between count number and apo E quantity.

Results

The plasma distribution of cholesterol, triacylglycerols, phospholipids and proteins in the various lipoprotein fractions in RICO and SW rats was comparable to data reported previously (3). In this study the HDL lipoprotein particles were further fractionated in order to detect the HDL_2 and HDL_3 sub-fractions (fig. 1). A two-dimensional electrophoretogram (pH 5-6) of rat chylomicron apoproteins

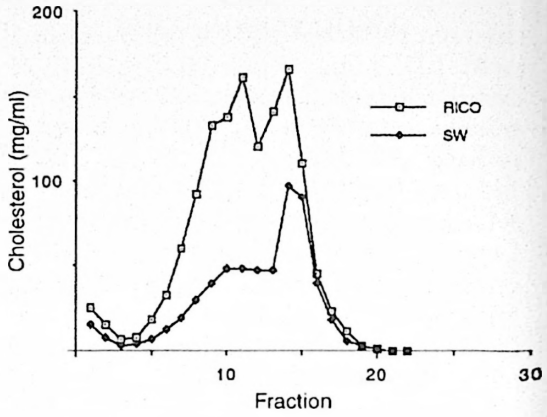

Fig. 1. *Tipical measured cholesterol profile from the 22 fractions of 0.5 ml collected from the top after centrifugation.*

was performed, which revealed the four apo E isoforms in SW and RICO rats (fig. 2). In a parallel study, the proteins from the gels were then transferred onto nitrocellulose films and incubated with anti-apo E antibody in order to confirm the presence and position of the isoforms. No differences in isoform pattern could be detected between the two strains of rats when VLDL, LDL and HDL fractions were compared (results not presented).

A typical agarose isoelectric focusing gel of RICO and SW rat plasma apo E revealed a charge heterogeneity. These plasma apo E isoproteins were designed E-4, E-3, E-2 and E-1, from highest to lowest pI in an apparent pH range of 5.56, 5.48, 5.41 and 5.36, respectively. No difference was observed between the two strains of rats. A comparable pattern of plasma apo E with pI values was reported by READON in normocholesterolemic rats (18). Table I lists apo E concentrations measured by immunoblotting in plasma and lipoprotein fractions, which represents a recovery of 66 % and 72 % of the mass directly measured in the plasma of RICO and SW rat, respectively. This is due to the cumulative effect of losses during ultracentrifugation and dialysis. As

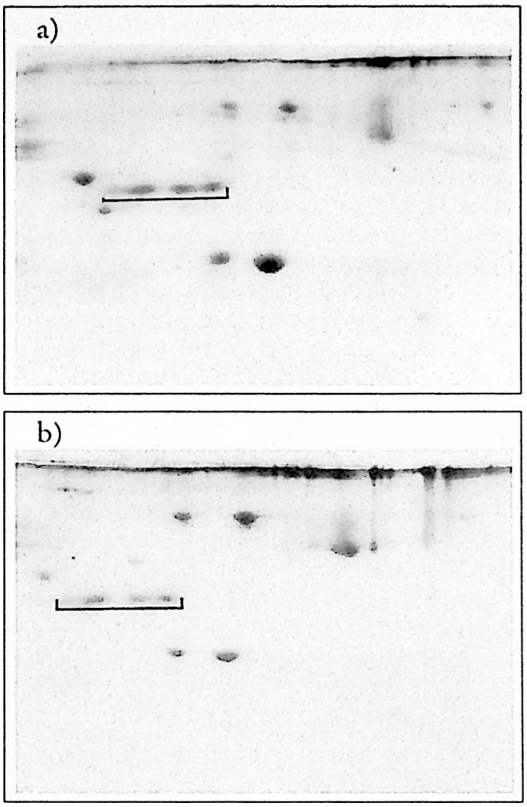

Fig. 2. *Two-dimensional electrophoretogram (pH 5-6) revealing the four isoforms of apo E in chylomicroms of RICO (a) and SW rats (b).*
Delipidated chylomicrons fraction was focused on two dimensional gel electrophoresis containing 2.5 % Ampholines.

shown here, there is a 35 % significant increase ($p < 0.001$) of plasma apo E in the RICO rat as compared to SW.

There were no significant differences between the concentrations of apo E in chylomicron, VLDL and HDL_3 fractions of RICO and SW rats. On the other hand, the main variation in apo E was measured in the LDL_2 and HDL_2 fractions, its level being significantly increased by 81 % ($p < 0.001$) and 117 % ($p < 0.005$), respectively. This variation relates to the increase in protein observed in these fractions in RICO rat as compared with SW rat.

Table I. *Concentration of apo E (mg/dl plasma) in plasma and lipoprotein fraction from RICO and SW rats.*
Values are means ± SEM from 6 rats in each group. Comparisons between RICO and SW rats were significant at ** $p < 0.001$ and * $p < 0.005$ levels (Student *t*-test). n.d., not detectable.

FRACTION	RICO rat	SW rat
Plasma	34.0 ± 1.3**	25.2 ± 1.4
Chylomicrons	0.38 ± 0.04	0.48 ± 0.05
Chylo + VLDL	0.84 ± 0.04	0.78 ± 0.05
VLDL	0.46 ± 0.06	0.39 ± 0.04
LDL_1	0.14 ± 0.04	n.d.
LDL_2	2.22 ± 0.09	1.02 ± 0.28
HDL_2	6.91 ± 0.24**	3.81 ± 0.28
HDL_3	6.45 ± 0.44	6.60 ± 0.52

Discussion

Apoprotein E is a major protein constituent of several cholesterol-enriched lipoproteins that accumulate in the plasma of rabbits, dogs, guinea pigs, rats and monkeys fed high levels of fat and cholesterol (7, 9, 11, 14, 30, 31). Previous studies indicate that, in rat, hypercholesterolemia induced by an atherogenic diet is mainly accompanied by an apo E increase in the lighter fractions (chylomicroms, VLDL, LDL) without changes in the HDL particles (29). Most apo E present in plasma is synthesized in mammalian liver, but the apo E gene is also expressed in many extrahepatic tissues (8), which is a major difference from the other apolipoproteins that only appear in liver and/or small intestine. Our present findings in the genetically hypercholesterolemic RICO rat differed from data obtained by dietary manipulations. Apo E significantly increases in the LDL_2 and HDL_2 fractions, with little or no significant variations in the chylomicron, VLDL and HDL_3 fractions. Previous studies on Wistar rats showed that apo A1 and apo E are constituents of the LDL_2 fraction (20),

which suggests that the LDL_2 fraction is largely made up of HDL_1 in the rat. In a preliminary estimation of the percentage distribution of apoproteins in the different lipoprotein fractions, an increase in apo E and a decrease in apo A1 were observed (3). In the present paper, this LDL_2 fraction is demonstrated in a quantitative way to be significantly (2-fold) enriched in apo E. The present level of our studies does not provide further information about the apo E increase in RICO rat, which could be due to a higher apo E synthesis, mainly hepatic, or to a decrease in catabolism of this LDL_2 (or apo E-rich HDL_1) fraction.

Little is known about dietary and genetic factors regulating apo E expression, synthesis and secretion in this strain of rat. Following addition of cholesterol (0.5 %) in the diet, a relative reduction of apo E in the RICO LDL_2 fraction was previously observed (3), whereas in normocholesterolemic rats under cholesterol diet, an increase in apo E level in all lipoprotein fractions has been observed (28). In most studied species, apo E exhibits charge heterogeneity. Apo E is secreted into plasma as a sialylated apoprotein, and neuroaminidase treatment indicates that sialic acid residues are partly responsible for the rat apo E polymorphism (28). In the present study, a charge heterogeneity was identified by two dimensional electrophoresis with four apo E isoforms found in RICO and control rats, in an apparent pI range from 5.56 to 5.36. The distribution of apo E isoforms was similar to previous reports obtained from normocholesterolemic rats (10). This distribution in RICO rat excluded a defect linked to the absence or large excess of any kind of isoforms.

The RICO rat is characterized by a hyperlipoproteinemia with an increase in the number of lipoprotein particles, except VLDL and chylomicrons (3, 13). The latter unmodified fractions do not contain increased apo E levels. By contrast, all the other fractions except HDL_3 have an increased content of apo E, which is significant for LDL_2 and HDL_2 fractions. Studies on the catabolic rate realized in RICO rats with heterologous or homologous VLDL fractions demonstrate that the rate of catabolism was significantly lower when RICO rat was donor rather than receptor (15). From this data, a possible explanation for LDL_2 and HDL_2 higher levels could be a defect in their catabolism. The lower lipoprotein lipase activity previously reported for the RICO rat suggests that an eventual modification of the VLDL cascade could be a contributing factor, but this needs further clarification.

In this paper, consideration of the apo E role in RICO rat has especially been focused on its concentration in the different lipoprotein fractions, but the possibility of the enrichment of one of the particular isoforms in one of the lipoprotein fractions cannot, at the moment, be excluded. Further studies are needed to identify the origin of the apo E increase in the higher HLD fractions (apo E-rich HDL_1 and HDL_2).

In summary, the hyperlipoproteinemic RICO rat is an interesting model for the comprehension of the different ways for a protective mechanism against atherosclerosis.

Acknowledgements

We extend our gratitude to Professor G. V. R. Born FRS who has consistently encouraged our work.

L. E. CARDONA-SANCLEMENTE, F. SULTAN y S. GRIGLIO. *Caracterización y cuantificación de la apolipoproteína E en la rata genéticanente hipercolesterolémica (RICO).* J. Physiol. Biochem. (Rev. esp. Fisiol.), 52 (1), 15-22, 1996.

Los niveles plasmáticos y lipoproteicos de la apolipoproteína E (apo E) de ratas homocigotas genéticamente hipercolesterolémicas (RICO) y de sus controles heterocigóticos normocolesterolémicos (SW), se evalúan y caracterizan por inmunocoagulación y por enfoque isoeléctrico. Los resultados obtenidos por análisis electroforético SDS-PAGE en ratas SW confirman estudios previos realizados en ratas Wistar normocolesterolémicas. En las ratas RICO se demuestra la presencia de las cuatro formas isoeléctricas con migraciones equivalentes a las isoformas heterocigóticas SW. Cuando la apolipoproteína E plasmática es evaluada por inmunocoagulación, se observa un aumento del 35 % en las ratas RICO respecto de las SW. En las diferentes fracciones lipoproteicas de las ratas RICO, la apo E está aumentada en las fracciones lipoproteicas de baja densidad LDL1 y LDL2 (fracción HDL1 rica en apo E) y en las fracciones de alta densidad (HDL_2 y HDL_3). Los niveles de la apo E se encuentran disminuidos solamente en la fracción de los quilomicrones. El método de electroforesis bidimensional demuestra que tanto en la rata homocigótica RICO como en la heterocigótica SW, están presentes cuatro isoformas denominadas E-1, E-2, E-3 y E-4 en un intervalo de 5.36 - 5.56 de punto isoeléctrico.

Palabras clave: Rata genéticamente hipercolesterolémica (RICO), Apo E, Lipoproteínas, Enfoque isoeléctrico, Inmunocoagulación.

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