The Degree of Sialylation of ApoC-III Is Altered by Diet

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SUMMARY

ApoC-III, an apolipoprotein that contains from 0 to 3 or more moles of sialic acid per mole of protein, plays important roles in lipoprotein metabolism. A high carbohydrate diet alters the relative proportions of the variously sialylated isoforms of ApoC-III in the plasma VLDL of man and of rat. Hepatic perfusion experiments in the rat indicate that the diet produces its affects on plasma VLDL by altering the hepatic output of ApoC-III. Diet is a relatively long-term stimulus to VLDL secretion. Our aim was to ascertain whether the changes in ApoC-III sialylation patterns could be reproduced by an acute stimulus to increased hepatic VLDL secretion, i.e., the perfusion of rat livers with media containing high levels of free fatty acid (FFA).

Male Sprague-Dawley rats (~250 g) were fed either Purina rat meal or a high carbohydrate formula for 3 wk. Livers were perfused with Krebs-Ringers bicarbonate, 3% bovine serum albumin containing either no added FFA, or 200 mg of added oleate. ApoB and ApoC-III$_3$ were measured by radioimmunoassay and lipids by enzymatic assays. Lipoproteins were isolated by preparative or zonal ultracentrifugation. The relative proportions of the various isoforms of ApoC-III were quantified by isoelectric focusing. The carbohydrate diet increased the plasma levels of triglycerides and ApoC-III$_3$ ~ twofold. The diet also increased the proportions of ApoC-III$_0$ relative to total ApoC-III from 33 to 42%, in plasma VLDL ($P < 0.02$). Perfusates of livers of carbohydrate-fed rats accumulated VLDL-TG and ApoC-III$_3$ at increased rates (93 versus 211, $P < 0.001$, and 12.7 versus 23.5, $P < 0.05$, μg/g/h, respectively) and the relative proportions of perfusate VLDL ApoC-III were altered: ApoC-III$_0$ rose from 37% to 59% and ApoC-III$_3$ fell from 60% to 38% ($P < 0.02$ for each). Perfusion of livers of control (meal-fed) animals with 200 mg oleate yielded twofold increases in the rates of accumulation of VLDL-TG over meal fed non-FFA perfused controls and changes in gross VLDL composition that resembled those produced by carbohydrate feeding, but ApoC-III$_3$ levels were not increased and the relative proportions of ApoC-III$_0$, ApoC-III$_{1,2}$ and ApoC-III$_3$ were not altered. Perfusion of livers of carbohydrate-fed rats with oleate produced fivefold and 2.5-fold increases in the rates of accumulation of VLDL-TG and ApoC-III$_3$, respectively. The proportion of ApoC-III isoforms were identical with those seen in the livers of carbohydrate-fed rats perfused without added oleate. Thus, the changes in ApoC-III sialylation were related more to diet than to the presence of added FFA, i.e., an acute stimulus did not reproduce the changes seen with diet. These data indicate that the sialylation of ApoC-III is under metabolic control.

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The group of apolipoproteins designated as ApoC is comprised of three distinctive proteins: ApoC-I, ApoC-II, and ApoC-III (also known as ApoR-ser, ApoR-glu, and ApoR-ala, respectively).[1] In human plasma, ApoC make up 60% of chylomicron protein, 50% of VLDL protein, and 10% of HDL protein.[1–3] ApoC-III has important metabolic functions in cell recognition[4,5] and in the modulation of lipoprotein lipase.[6,7] In man, ApoC-III consists of 79 amino acids and has a mol. wt. of ~9,000.[8] Each mole of ApoC-III also contains approximately 1 mole each of galactosamine and galactose. The oligosaccharide chain is linked to the threonine residue of the protein at position 74.[8] Variable amounts of sialic acid (from 0 to 4 or more mol/protein) may be present in the ApoC-III of human plasma lipoproteins.[3,9] The variably sialylated subspecies are distinguishable on eletrophoresis or isoelectric focusing in polyacrylamide gels and are designated as ApoC-III$_0$, ApoC-III$_1$, etc. Although information on the structures and functions of the ApoC-III of animals is not nearly as complete as it is for the human apoprotein, analogous variously sialylated apoproteins also have been identified in the lipoproteins of some animal species, including the rat.[10–13]

Several lines of evidence indicate that the pattern of sialy-

lation of ApoC-III is affected by metabolic factors. In man, a high carbohydrate, fat-free diet increases the proportions of ApoC-III_0 in plasma VLDL relative to the more sialylated forms.[14] ApoC-III_0 are relatively increased also in rats fed a high carbohydrate (CHO) diet[15] and in the Zucker fatty rat,[16,17] a strain which is affected by hereditary obesity and hyperlipidemia.[18] Similar changes in the sialylation pattern of ApoC-III are present in lipoproteins isolated from hepatic perfusates of these rats, suggesting that the diet and the obesity have affected the sialylation of ApoC-III in the liver.

Both the feeding of high CHO diets and the presence of obesity are long-term in vivo perturbations which result in increased rates of secretion of triglycerides (TG) and VLDL particles by the liver.[16,17] Our aim was to ascertain whether an acute increase in hepatic VLDL secretion also would produce changes in the pattern of sialylation of ApoC-III. One method for raising the VLDL-TG secretion rate of livers acutely is the perfusion of livers with high concentrations of free fatty acids (FFA).[19]

METHODS

Animals and perfusions. Adult male Sprague-Dawley rats weighing 300–350 g (Elridge Laboratory Animals, Barnhart, Missouri) were fed either rat chow (Rat meal, Ralston Purina, Inc., St. Louis, Missouri) (controls) or a commercially prepared high CHO diet (68% of mass CHO, 8% vegetable oil, 18% vitamin-free casein, 2% brewer's yeast, 4% salt mixture USP No. 2 plus vitamins, High Carbohydrate Diet, ICN Life Sciences Group, Cleveland, Ohio). Diets and water were given ad libitum for 3 wk and food was removed 4–5 h before the start of the study. Blood for analyses was obtained from the inferior venae cavae of animals under ether anesthesia.

Liver perfusions were done in a recycling system as described previously.[15] Livers were removed and cleared of blood by perfusing them with 150 ml of Krebs-Ringer bicarbonate buffer (KRB) at 37°C, pH 7.4. Livers were then mounted in a Miller-type perfusion apparatus (J. R. Thebeau, Boston, Massachusetts)[20] containing a "lung" as described by Hamilton et al.[21] Perfusates consisted of 100 ml of KRB, containing 3 g fatty acid free bovine serum albumin prepared from BSA fraction V, (Sigma Chemical Co., St. Louis, Missouri) by charcoal extraction[22] and dialyzed extensively against 0.9% sodium chloride and KRB, 300 mg of glucose, and 90 mg of amino acid mixture (Sigma Kit LAA-21, Sigma Chemical Co., St. Louis, Missouri). The viability of the livers was assessed by the rates of secretion of bile (0.3–0.5 ml/h), by the flow rates of the perfusate (50–80 ml/min) and by repeated measurements of the pH during the course of the perfusion (pH changed by less than 0.15 of a unit). Perfusions lasted up to 180 min. During this period, a linear increase in TG concentration in the perfusate was obtained. In addition, the validity of this perfusion procedure has been examined recently with respect to P_{O_2} changes in the perfusate as well as by electron microscopy of livers after perfusion.[15] FFA were infused as oleate (Sigma Chemical Co., St. Louis, Missouri) complexed to BSA.[19] Twenty milliliters of a 10% oleate-BSA solution (in KRB) were infused over 3 h.[23] FFA levels of perfusates were determined chemically.[24]

Plasma VLDL + LDL were analyzed by zonal ultracentrifugation performed in a linear NaBr density gradient in the density range of 1.00–1.30 g/ml.[25] Centrifugation was performed at 42,000 rpm at 15°C for 140 min using a Ti-14 zonal rotor and a Beckman Model L265B ultracentrifuge. Perfusate VLDL were isolated at a solution density of 1.019 in a 50.2 rotor spun at 40,000 rpm for 20 h at 4°C (solid KBr was added and density was checked with a densitometer, perfusates also were made 1mM in EDTA). Perfusate VLDL were "washed" under identical conditions by ultracentrifugation.

Protein was measured according to Lowry, et al.[26] or as modified by Bensadoun and Weinstein,[27] using bovine serum albumin as standard. Triglycerides, and free and total cholesterol were measured enzymatically using Boehringer Triglyceride Kit 126012 and Cholesterol Kit 124087 (Boehringer Mannheim Biochemicals, Indianapolis, Indiana). Results for cholesterol esters are expressed in terms of cholesterol oleate. Phospholipids were measured by the method of Bartlett.[28]

ApoB and ApoC-III were measured by previously described radioimmunoassays.[15,29] The latter assay quantifies virtually all of ApoC-III_3, but measures $<5\%$ of ApoC-III_0 and only $\sim 25\%$ of ApoC-$III_{1,2}$. Thus, the reported changes in ApoC-III_3 should be regarded as relative rather than absolute. The relative proportions of ApoC-III subspecies were quantified by isoelectric focusing in polyacrylamide gels. For this purpose, "washed" VLDL were dialyzed against 1 mM EDTA pH 8.2, lyophilized, delipidated with ether ethanol (3:2, v/v), solubilized in 8 M urea, and subjected to isoelectric focusing as previously described.[30] Bands are identified according to Gidez et al.[10] After staining with Coomassie Blue, gels were scanned at 550 nm using a Gilford 2400 S spectrophotometer equipped with a linear transporter. Areas were integrated using a Hewlett-Packard 9864A digitizer. The validity of quantifying apoproteins by measuring their dye uptakes after isoelectric focusing has been reported from this laboratory.[15]

RESULTS

Plasma TG levels of meal-fed control and CHO-fed rats were 109 ± 30 mg/dl (N = 16) and 261 ± 128 (N = 11), respectively ($P < 0.001$). Zonal ultracentrifugation (Figure 1)

FIGURE 1. Zonal ultracentrifugal profiles of rat plasma VLDL and LDL. The small amount of LDL present in rat plasma is obvious. The increased amount of VLDL in the high carbohydrate diet fed rats is clearly shown. In each case, 18 ml of plasma were ultracentrifuged.

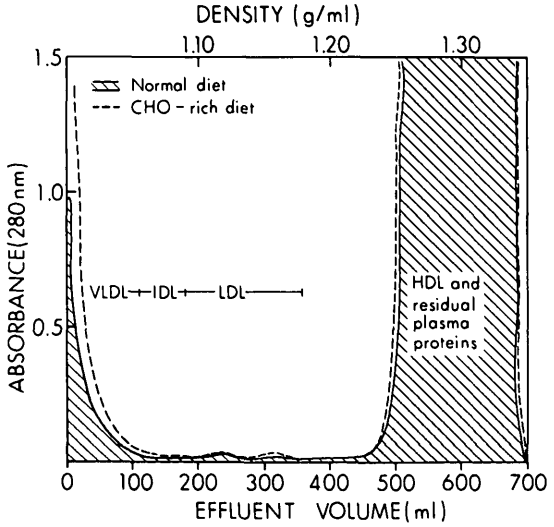


TABLE 1
Effect of high carbohydrate diet and perfusion with FFA on the rates of accumulation of ApoC-III_3 in hepatic perfusates

	Total perfusate ApoC-III_3 (μg/g/h)
Control	12.7 ± 5.7 (4)
Carbohydrate	23.5 ± 8.6* (4)
Control + FFA	11.4 ± 2.8 (4)
Carbohydrate + FFA	27.6 ± 8.6* (3)

FFA perfusates contained ~500 μeq/L of FFA, control levels were ~40 μeq/L. Apoprotein levels in plasma and perfusates were measured by radioimmunoassay. Results are means ± 1 SD. Number of perfusates is in parentheses. Comparisons are between controls (top line) and the three experimental groups.
* $P < 0.05$.

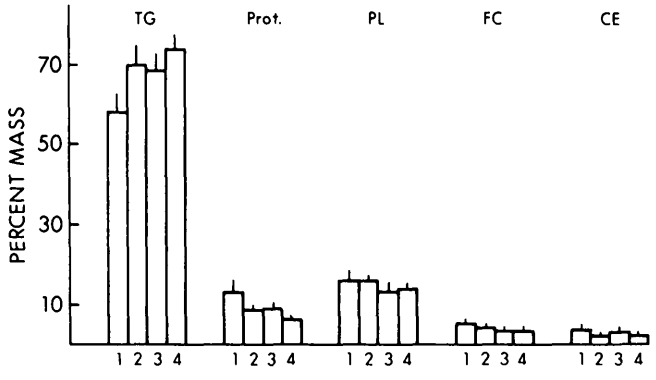
demonstrated a rise in VLDL with little change in LDL. Apparent plasma levels of ApoC-III_3 of control and CHO-fed rats were 11.1 ± 4.5 and 17.4 ± 5.6 ($P < 0.01$), respectively. In addition, the proportions of the variously sialylated forms of ApoC-III in plasma VLDL were altered: ApoC-III_0 rose from 33 ± 3% to 42 ± 5% and ApoC-III_3 fell from 54 ± 6% to 47 ± 5% (N = 10 rats per group, $P < 0.02$). ApoC-$III_{1,2}$ was not significantly changed (7 ± 2% versus 11 ± 3%).

Rates of accumulation of lipids and apoproteins in perfusates were linear over 3 h.[15,16,32] More ApoC-III_3 accumulated in the perfusates of livers taken from CHO-fed than in control perfusates (Table 1). The rates of accumulation of VLDL-TG rose about twofold, and rates of accumulation for VLDL-protein rose by ~30%. VLDL-ApoB stayed constant (3.7 versus 3.8 μg/g/h). Thus, perfusate VLDL compositions changed: VLDL-TG, as percent of VLDL mass, rose and VLDL-protein fell (Figure 2). VLDL-ApoB fell from 21% to 16% of VLDL protein and conversely, VLDL-nonApoB protein rose from 79% to 84% of VLDL-protein. The relative proportions of perfusate VLDL-ApoC-III also were altered by the high CHO diet. ApoC-III_0 rose by 22 percentage points and ApoC-III_3 fell by 22 percentage points (Table 3, Figure 3).

To ascertain whether an acute stimulus, which increased the secretion of VLDL-TG, also would affect the sialylation of ApoC-III, the livers of rats fed the control diet were perfused with 200 mg (780 μmol) of oleate over 3 h. FFA levels in perfusates without added oleate were ~40 μmol/L, whereas the infusion of oleate raised the FFA levels of perfusates to ~500 μmol/L during the 3 h of perfusions. Net uptakes of FFA under these two conditions were <1 μmol/g liver/h and ~16 μmol/g liver/h, respectively. Mean rates of accumulation of ApoC-III_3 in whole perfusates were not significantly different from controls (Table 1), but mean rates of accumulation of VLDL-TG and VLDL-protein were increased to about the same levels seen in the carbohydrate-no FFA perfusions (Table 2). Compared with non-FFA controls, % VLDL-TG rose and % VLDL-protein fell (Figure 2). VLDL-non-ApoB was 88% of VLDL-protein (versus 79% for controls). Note that rates of accumulation of VLDL and VLDL compositions were nearly identical for FFA-perfused controls and non-FFA perfused CHO fed rats, yet the relative proportions of the ApoC-III isoforms in VLDL differed between these two groups. Instead, VLDL isoforms were nearly identical for FFA and non-FFA controls (Table 3, Figure 3).

Finally, the livers of CHO-fed rats also were perfused with 200 mg of oleate over 3 h. The livers of these rats also took up ~16 μmol of FFA/g/h. Mean rates of accumulation of VLDL-TG and VLDL-protein were greatly increased (Table 2) over non-FFA controls, and rates for ApoC-III_3 also were increased (Table 1). Here too, VLDL composition was altered: VLDL-TG was low compared with non-FFA controls (Figure 2). Non-ApoB represented ~85% of VLDL-protein. In contrast with FFA-perfused controls, the relative proportions of ApoC-III resembled those of the CHO-fed animals perfused in the absence of oleate (Table 3, Figure 3). Thus, the relative proportions of ApoC-III isoforms were altered by diet but not by perfusion with FFA.

FIGURE 2. Compositions of VLDL isolated from rat hepatic perfusates by preparative ultracentrifugation at d < 1.019. 1 = normal meal-fed controls, and 2 = carbohydrate-fed rat; for both, livers were perfused with low FFA media (<40 μmol/L). 3 = meal-fed rat and 4 = carbohydrate-fed rat; for both, livers were perfused with 200 mg oleate over 3 h.



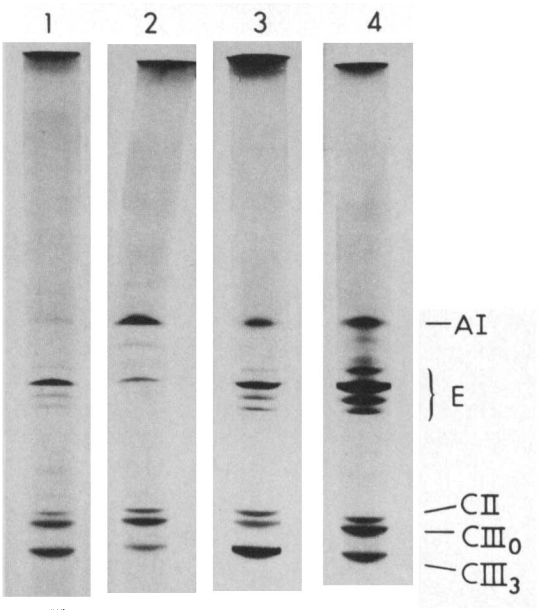


FIGURE 3. Isoelectric focusing of hepatic VLDL apoproteins. VLDL were isolated by ultracentrifugation at d < 1.019. 1 = meal-fed control, 2 = carbohydrate-fed rat; for both, livers were perfused with low FFA media (<40 μmol/L). 3 = meal-fed control and 4 = carbohydrate-fed rat; for both, livers were perfused with 200 mg of oleate over 3 h.

DISCUSSION

High CHO diets and perfusion with FFA both increased the output of hepatic VLDL (Table 2). Separately and in combination, these two perturbations induced the accumulation in

TABLE 2
Effects of diet and of perfusion with FFA on the rates of accumulation of VLDL lipids and apoproteins in hepatic perfusates

	VLDL-TG	VLDL-protein	VLDL-ApoB
	(μg/g/h)		
Control (8)	93 ± 64	18 ± 7	3.7 ± 1.8
Carbohydrate (5)	211 ± 54†	24 ± 3*	3.8 ± 1.2
Control + FFA (4)	208 ± 81*	28 ± 11	3.3 ± 1.9
Carbohydrate + FFA (5)	442 ± 55‡	36 ± 2‡	5.4 ± 1.0*

Results are means ± 1 SD. Number of perfusions is in parentheses. Comparisons are between controls (top line) and the three experimental groups.
* $P < 0.05$. † $P < 0.02$. ‡ $P < 0.001$.

perfusates of larger VLDL particles with altered lipid and apoprotein compositions. However, the perfusates of rats fed the high CHO diet whether perfused with or without FFA accumulated more ApoC-III than did perfusates of control meal-fed rats (Table 1), and the proportion of ApoC-III_0 in perfusate VLDL of CHO fed rats were increased relative to total ApoC-III (Table 3). These results confirm and extend the data reported previously for CHO-fed Sprague-Dawley rats[15] and for the Zucker fatty hereditarily obese hyperlipidemic rats.[16] In contrast, when hepatic VLDL secretion was increased by adding FFA to the perfusates, the accumulation of ApoC-III and the relative proportions of the ApoC-III isoforms in VLDL were not altered compared withe appropriately fed counterparts. Thus, the sialylation of ApoC-III was related more to the pretreatment of the animal than to absolute rates of accumulation of hepatic VLDL-TG in perfusates.

Based on the work of Nestruck and Rubinstein,[31] we initially proposed[15] that the degree of sialylation of ApoC-III may be related inversely to the residence time of VLDL in the Golgi apparatus of the liver. The data provided by the carbohydrate-fed Sprague-Dawley and Zucker fatty rats seemed to support that proposal, because in both of those conditions, VLDL production rates appeared to be increased and the degree of ApoC-III sialylation was decreased.[15,16] However, in the present experiments increases in VLDL secretory rates (and changes in VLDL composition) produced by FFA infusion were not accompanied by altered ApoC-III sialylation, effectively ruling out that possibility.

TABLE 3
Effects of high carbohydrate diet and perfusion with FFA on the proportions of perfusate VLDL-ApoC-III

	Perfusate		
Treatment	ApoC-III_0	ApoC-$III_{1,2}$	ApoC-III_3
	(% area)		
Control (5)	37 ± 7	3 ± 1	60 ± 4
Carbohydrate (4)	59* ± 8	3 ± 2	38* ± 6
Control FFA (6)	34 ± 6	7 ± 3	59 ± 8
Carbohydrate + FFA (4)	52* ± 7	5 ± 4	43* ± 4

VLDL ($d < 1.019$) were isolated and "washed" by ultracentrifugation and subjected to isoelectric focusing. Gels were scanned photometrically and areas under curves were integrated. Results are means ± 1 SD of proportions of individual ApoC-III isoform dye uptake areas relative to total ApoC-III dye uptake. Numbers of perfusions are in parentheses.
* $P < 0.02$.

Alterations in the relative proportions of the isoforms of ApoC-III in VLDL also could be produced during intrahepatic VLDL assembly, if different lipids had selective affinities for the variously sialylated forms of ApoC-III. If that were true, alterations of the isoforms of VLDL-ApoC-III would merely reflect changes in the lipid components of VLDL (perhaps particularly surface lipids). Data are not available to settle this point, but we believe this to be an unlikely explanation for two reasons. First, the lipid compositions of VLDL are likely to have been different under each of the four experimental conditions employed here, because high carbohydrate diets and perfusion with FFA each produce changes in different directions and combinations of dietary change and perfusion conditions would probably produce still different combinations.[32] Yet, the sialylation of ApoC-III appeared to be related to diet alone. Second, treatment with estrogens also increases hepatic VLDL output and alters the sialylation of ApoC-III, but the pattern of sialylation is altered in a different direction;[33] streptozotocin-induced diabetes mellitus in the rat also alters the degree of sialylation of ApoC-III in still another direction.[34] Therefore, it seems to us more likely that the perturbations which alter ApoC-III sialylation produce their effects by affecting the biochemical processes of sialylation. Obviously, more work needs to be done to settle this question.

Since the metabolic roles of sialic acid on ApoC-III are not known, the consequences of varying degrees of sialylation for the metabolism of lipoproteins after their secretion into plasma are not clear. However, the fact that sialylation seems to be under metabolic regulation, not only in rat but also in man,[14] implies that this may be a significant event in the metabolism of lipoproteins which warrants further study.

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