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Studies on the Substrate Specificity of Human and Pig Lecithin:Cholesterol Acyltransferase: Role of Low-Density Lipoproteins†

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Received December 11, 1985; Revised Manuscript Received April 25, 1986

ABSTRACT: The substrate properties of low-density lipoprotein (LDL) fractions from human and pig plasma and of lipoprotein *a* [Lp(a)] upon incubation with either pig or human lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) were investigated and compared with those of pig high-density lipoproteins (HDL) or human HDL-3. The cholesterol esterification using purified native pig LDL-1, human LDL, or Lp(a) as a substrate was approximately 36–42% that of pig HDL or human HDL-3, while cholesteryl ester formation with pig LDL-2 was 41–47%. No significant difference was found in the substrate activity between pig HDL and human HDL-3, and between human LDL and Lp(a), respectively. After depletion of pig LDL-1, pig LDL-2, and human LDL from apolipoprotein A-I (apoA-I), cholesteryl ester formation decreased to about 22–28% of the value found with pig HDL. Depletion of human LDL from apolipoprotein E (apoE) did not result in significantly different esterification rates in comparison to native LDL. Total removal of non-apoB proteins from human LDL resulted in esterification rates of approximately 10–15% that of HDL. Readdition of apoA-I to all these LDL fractions produced solely in apoA-I-depleted LDL fractions an increase of cholesteryl ester formation, whereas in those LDL fractions that were additionally depleted from apoE and/or from apoC polypeptides, a further decrease in the esterification rate occurred. When the phosphatidylcholine (PC)/free cholesterol (FC) ratio in LDL was raised by incubation with liposomes (PC/FC molar ratio 3.5:1 to 4.5:1) in the presence of human lipid transfer protein, the substrate properties of phosphatidylcholine-enriched LDL were nearly equivalent to those of HDL. From our results we conclude (1) that native LDL or Lp(a) also in vivo may serve as substrate for LCAT and (2) that enrichment of LDL with phosphatidylcholine leads to a reorientation of surface lipids in LDL, thus allowing activator proteins and/or LCAT to interact with this substrate in a similar manner as with HDL.

The cholesteryl esters found in plasma are mainly produced by the action of lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43), an enzyme synthesized by the liver (Glomset, 1973, 1979). Initially, these cholesteryl esters are incorporated into high-density lipoproteins (HDL)[1] (Akanuma & Glomset, 1968) and transferred to or exchanged between low-density lipoproteins (LDL) and very low density lipoproteins (VLDL) (Nichols & Smith, 1965; Fielding & Fielding, 1980; Barter et al., 1982a,b). Together with triglyceride-rich particles and cholesteryl ester transfer protein (CETP) LCAT plays a major role in the interconversion of HDL-3 to HDL-2 (Dieplinger et al., 1985; Knipping et al., 1985). However, it was shown that LCAT interacts also directly with apoB-containing lipoproteins (Barter, 1983; Barter et al., 1984).

HDL are believed to serve as the main substrate for LCAT because of the content of apoA-I, a necessary cofactor for the LCAT reaction (Fielding et al., 1972). There exist, however, other cofactors, e.g., apoC-I (Soutar et al., 1975) or apoA-IV (Steinmetz & Utermann, 1984).

In previous work on the HDL-3/HDL-2 interconversion using pig plasma (Knipping et al., 1985) we observed a significant increase of cholesteryl esters in LDL during the LCAT reaction. This increase could not be explained by cholesteryl ester transfer from HDL to LDL, since pig plasma does not contain CETP (Barter et al., 1981).

In contrast to human LDL, pig LDL comprises two subclasses designated LDL-1 and LDL-2 (Janado & Martin,

† This work was supported by the Österreichischer Fonds zur Förderung der wissenschaftlichen Forschung Projekt P 5158 and P 5144 and the Österreichische Nationalbank.
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[1] Abbreviations: HDL, high-density lipoproteins; LDL, low-density lipoproteins; pig LDL-1, buoyant density fraction 1.020–1.063 g/mL; pig LDL-2, buoyant density fraction 1.063–1.090 g/mL; apoA-I, apolipoprotein A-I; apoC, apolipoprotein C; apoE, apolipoprotein E; apoB, apolipoprotein B; PC, phosphatidylcholine; FC, free cholesterol; CETP, cholesteryl ester transfer/exchange protein; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; SD, standard deviation.

0006-2960/86/0425-5242$01.50/0

1973), which differ in size, hydrated density, and chemical composition. Although both subclasses are physicochemically well characterized (Jackson et al., 1976; Jürgens et al., 1981; Nöthig-Laslo & Knipping, 1984; Herak et al., 1985), little is known about their origin or physiological function.

This study is devoted to the elucidation of the substrate specificity of pig and human LCAT with particular emphasis on the role of LDL.

Materials and Methods

Plasma Samples. Blood from individual fasting pigs was obtained from the slaughterhouse. Serum was prepared by low-speed centrifugation at 4 °C. For inactivation of LCAT, 5 mM sodium iodoacetate was added to whole blood. Human serum was prepared from the blood of normolipemic fasting volunteers.

Lipoprotein Separation. Lipoproteins were isolated by ultracentrifugation at 4 °C and 115000*g* for 48 h in a Sorvall OTD-75B ultracentrifuge. Pig VLDL were obtained at d <1.006 g/mL, pig LDL at d = 1.020–1.080 g/mL, and pig HDL at d = 1.080–1.21 g/mL. Pig LDL were further separated into LDL-1 and LDL-2 by the use of a TFT-41 swinging bucket rotor (Sorvall). Two milliliters of total pig LDL were adjusted to a density of 1.080 g/mL with solid NaBr and overlaid with 5 mL of a NaBr solution of a density of 1.050 g/mL. The tubes were filled with Tris-buffered 0.15 M saline and then centrifuged for 22 h at 200000*g*. In this way LDL-1 were well separated from LDL-2, and both fractions were obtained by aspiration.

Human "lipoprotein *a* negative" sera were used for isolation of LDL and HDL. LDL were isolated at d = 1.020–1.063 g/mL and further purified by overlaying 6 mL of an LDL solution of a density of 1.060 g/mL with a solution of density 1.020 g/mL following centrifugation under the same conditions as described above. Human HDL-3 were obtained at d = 1.10–1.21 g/mL. Lipoprotein *a* [Lp(a)] was prepared from strongly Lp(a)-positive human sera essentially as described by Kostner (1976). All fractions were dialyzed against 0.15 M NaCl containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 50 mg/L streptomycin and penicillin to avoid microbial growth. Fractions used for incubations were not older than 8 days.

Removal of Non-ApoB Proteins. Since the LDL fractions from pig and human plasma still contained small amounts of apoA-I, apoC, and apoE, in some cases pig and human LDL fractions were incubated with IgG fractions prepared from antisera against pig or human apoA-I (Knipping et al., 1975; Kostner & Alaupovic, 1972) and from antiserum against apoE (Kostner, 1982). ApoC polypeptides of human LDL were removed by incubation of 2 mL of 4% human LDL with equal volumes of phospholipid-poor lipofundin (10%) (Braun Melsungen AG) for 1 h at 37 °C (Zechner et al., 1986). After treatment with IgG or lipofundin all LDL fractions were again spun in the TFT-41 rotor under the same conditions as above. The same method was tried to remove the apoC polypeptides from pig LDL fractions, but without any success.

Cholesteryl Ester Transfer Protein (CETP). Human plasma, in which LCAT was inhibited by 5 mM sodium iodoacetate, was centrifuged at a density of 1.18 g/mL for 48 h at 160000*g* and 12 °C. The supernatant was discarded and the infranatant dialyzed against 10 mM Tris-HCl, pH 7.4, and 0.15 M NaCl and used as the source of CETP. Transfer activity was assayed as described previously (Barter & Jonas, 1979).

Purification of Human and Pig LCAT. Pig LCAT was isolated from fresh pig plasma and purified approximately 20000-fold as described earlier (Knipping, 1985). Briefly, pig plasma was ultracentrifuged at a density of 1.21 g/mL for 48 h and the clear middle zone immediately subjected to phenyl-Sepharose chromatography. This step was repeated twice with smaller columns. The LCAT-active fractions were dialyzed against 10 mM potassium phosphate buffer, pH 6.8, in the presence of 1 mM sodium deoxycholate for at least 24 h. LCAT was purified by chromatography over hydroxylapatite using 10 mM potassium phosphate, pH 6.8, as eluent.

Human LCAT was prepared by a combined procedure of Kostner (1974), Chen and Albers (1981), and Mahadevan and Soloff (1983). Briefly, 400 mL of fresh human plasma containing 1.4 mM of Ellman's reagent was ultracentrifuged at a density of 1.24 g/mL for 48 h, at 4 °C and 160000*g*. The intermediate fraction was aspirated and sequentially chromatographed on columns containing phenyl-Sepharose, DEAE-cellulose, and hydroxylapatite. In a final step, the hydroxylapatite fraction containing LCAT activity was chromatographed over TSK phenyl-Sepharose using the HPLC system of LKB (Bromma, Sweden). LCAT eluted at a NaCl concentration of 50 mM. The final purification was between 15000- and 18000-fold and the yield 5–7%.

Enzymes from both pig and human plasma showed a single band in SDS- or urea-containing polyacrylamide gel electrophoresis with an apparent molecular weight of 69000 and 68000, respectively. They did not react immunochemically with antibodies against human or pig apoA-I, albumin, apoC, or human apoD.

Preparation of Liposomes. Sonified small liposomes were prepared from egg phosphatidylcholine (PC) and cholesterol (FC) (Sigma, Taufkirchen, BRD). PC and FC dissolved in benzene were evaporated to dryness under a stream of nitrogen. One milliliter of 10 mM Tris-HCl buffer, pH 7.4, was added and the mixture sonicated for 30 min at 25 °C by use of a Labsonic sonifier. The mixture was centrifuged for 30 min at 15000 rpm to remove aggregates. The PC/FC molar ratios in the liposomes ranged from 3.5:1 to 8.4:1.

Isolation of ApoA-I from Pig and Human Plasma. Human apoA-I was isolated according to Kostner and Alaupovic (1972), and pig apoA-I was prepared essentially as described by Knipping et al. (1975). The purity of apoA-I from both species was checked by polyacrylamide gel electrophoresis in the presence of either urea or SDS. Furthermore, purity was tested by immunodiffusion in 1% agarose gels using antibodies against human or pig lipoproteins and apoproteins.

Assay for Determination of LCAT Activity. Lipoprotein fractions or liposomes corresponding to 90 nmol of FC were incubated with 200 nCi of [^{3}H]cholesterol (Amersham International, Amersham, England) in the absence or presence of 20 μg/mL human or pig apoA-I and in the presence of 4% bovine albumin, for 1 h at 37 °C. Fifty microliters of either pig or human LCAT and 10 μL of 1 M mercaptoethanol were added and the mixtures further incubated for 1 h. For each lipoprotein or liposome fraction, blanks were made by omitting LCAT from the incubation mixture. The total incubation volume was 1 mL. After incubation the samples were immediately frozen and lyophilized, and the lipids were extracted with chloroform/methanol (2:1 v/v) and separated by thin-layer chromatography on silica gel 60 plates (Merck, Darmstadt, BRD) using petroleum ether/diethyl ether/acetic acid (70:30:1) for development. Free cholesterol and esterified cholesterol were visualized by iodine vapor, cut out, and mixed with 10 mL of Ready Solv HP/b (Beckman Instruments, Vienna), and the radioactivity was measured in a scintillation counter. The blanks showed less than 1.0% of total radioac-

Table I: Chemical Composition of Pig and Human Lipoprotein Fractions[a]

lipoproteins	PR	FC	CE	PL	TG	PL/FC (M/M)
pig						
HDL (n = 12)	45.4 (2.7)	2.1 (0.6)	25.7 (1.6)	25.7 (1.5)	1.1 (0.5)	6.09
LDL-1 (n = 7)	23.7 (3.2)	8.5 (0.5)	46.0 (2.8)	19.3 (1.5)	2.5 (1.1)	1.14
LDL-2 (n = 7)	30.5 (3.5)	6.8 (0.6)	41.5 (3.9)	17.7 (1.1)	3.5 (1.5)	1.31
LDL-1 - A-I (n = 4)	23.2 (2.3)	8.4 (0.7)	46.5 (2.5)	19.5 (1.2)	2.4 (1.0)	1.17
LDL-2 - A-I (n = 4)	30.0 (2.5)	7.1 (0.7)	41.7 (2.3)	18.1 (1.5)	3.1 (1.5)	1.28
PCe-LDL - A-I (n = 1)	19.3	2.7	41.3	34.5	2.2	6.42
PCe-LDL - A-I (n = 1)	17.9	4.5	35.0	42.1	1.0	4.70
human						
HDL-3 (n = 4)	51.0 (3.1)	2.7 (0.5)	15.4 (1.1)	26.4 (1.8)	4.5 (1.2)	4.91
Lp(a) (n = 2)	34.7 (2.0)	7.3 (0.5)	36.5 (2.2)	18.3 (1.4)	3.2 (1.1)	1.26
LDL (n = 7)	23.4 (2.4)	9.0 (0.8)	41.7 (2.1)	20.6 (1.3)	5.3 (1.4)	1.15
LDL - A-I (n = 4)	22.9 (2.1)	8.9 (0.4)	42.2 (2.5)	21.0 (1.1)	5.0 (1.3)	1.18
LDL - C (n = 4)	22.2 (2.3)	9.1 (0.6)	42.4 (2.9)	21.2 (1.5)	5.1 (1.6)	1.17
LDL - E (n = 4)	23.1 (1.7)	9.1 (0.5)	42.0 (2.0)	20.6 (0.9)	5.2 (1.1)	1.14
LDL - E/A-I (n = 4)	22.8 (1.8)	9.0 (0.4)	42.9 (1.8)	20.2 (1.0)	5.1 (1.2)	1.13
LDL - E/A/C (n = 4)	21.9 (2.2)	9.2 (0.8)	42.8 (2.8)	21.1 (1.3)	4.9 (1.3)	1.15
PCe-LDL - A-I (n = 1)	22.5	3.7	40.4	28.9	4.5	3.84
PCe-LDL - A-I (n = 1)	21.8	3.9	39.4	30.2	4.7	3.88

[a] LDL fractions that were specifically depleted from non-apoB protein are designated by the removal apoprotein. The values are given in percent (SD) of total lipoprotein mass. Abbreviations: PR = protein; FC = free cholesterol; CE = cholesteryl esters; PL = phospholipids; TG = triacylglycerols.

tivity in the cholesteryl ester fraction and were subtracted from the samples. LCAT activity is expressed as nanomoles of cholesteryl esters formed per milliliter of enzyme solution per hour.

Preparation of Phosphatidylcholine-Enriched LDL (PCe-LDL). To 3 mL of human or pig LDL (containing about 20 mg of phospholipids) was added 90 mg of liposome PC (PC/FC molar ratio 3.5:1 to 4.5:1), and the mixture was incubated in the presence of CETP up to 24 h at 37 °C. LDL were separated from liposomes and CETP by heparin–Sepharose chromatography (Pharmacia, Uppsala, Sweden). The column (1 × 5 cm) was equilibrated with 10 mM Tris-HCl, pH 7.4, containing 50 mM NaCl. The incubation mixture was applied and the column washed with the same buffer. Excess liposomes were eluted by changing the NaCl concentration of the Tris buffer to 115 mM. LDL were eluted with 650 mM NaCl. The fraction was dialyzed against 10 mM Tris-HCl, pH 7.4, and contaminating apoA-I was removed by immunoadsorption as described above. Then LDL were reisolated by gradient ultracentrifugation in the swinging bucket TFT-41 rotor.

Chemical and Immunochemical Analyses. Free cholesterol and esterified cholesterol were determined with enzymatic test kits from E. Merck (Darmstadt, BRD). Triacylglycerols and phospholipids were assayed enzymatically (Biomerieux, Carbonniere les Bains, France). Protein was measured according to Lowry et al. (1951) using bovine serum albumin as a standard. SDS gel electrophoresis and polyacrylamide gel electrophoresis in the presence of urea were performed according to Weber and Osborn (1969) and Davis (1964), respectively. Non-apoB protein in LDL was determined according to Egusa et al. (1983).

Immunoquantitation of apolipoproteins was performed either by radial immunodiffusion or by Laurell electrophoresis using monospecific antibodies to human and pig apolipoproteins (Kostner & Holasek, 1972; Pilger et al., 1983; Knipping et al., 1984). For Laurell electrophoresis 7 × 7 cm plates and 6 mL of 1% agarose in Tris–barbital buffer, pH 8.2, were used. The antiserum content in the gel ranged from 0.5% to 4% (v/v). Five-microliter aliquots of samples were applied. Electrophoresis was performed for 4–6 h at 5 V/cm. Gels were washed, dried, and stained with 1% Coomassie blue. The antigen concentration was determined by comparing the rocket area with that of several standard dilutions. The sensitivity of this method was approximately 2 ng/sample. The LDL preparations were applied at a concentration of 3–5% (w/v), thus allowing us to determine contaminations of non-apoB proteins in the order of 0.01%.

Electron Microscopy. Negative-stain electron microscopy was performed on a Phillips EM 300, using 0.1–0.2 mg/mL LDL solutions in 0.15 M ammonium acetate (pH 7.4). Photographs were taken at a magnification of 204000×. Mean diameters were obtained by measuring 300 particles.

Results

Incubation of Lipoprotein Fractions with Pig and Human LCAT. In the first set of experiments we studied the influence of purified LCAT, derived from pig and human plasma, on pig HDL, human HDL-3, pig LDL-1, pig LDL-2, human LDL, and Lp(a). Since apoC proteins are known to modulate the LCAT activity (Soutar et al., 1975; Albers et al., 1979), in some cases human LDL were depleted from apoC polypeptides by incubation with phospholipid-poor lipofundin (Zechner et al., 1986) prior to incubation with LCAT. Removal of apoC from pig LDL by incubation with lipofundin was also tried but was incomplete. Therefore, only the results with apoC-depleted human LDL are presented. The chemical composition of all lipoprotein fractions used in this study is shown in Table I. The apolipoprotein content of pig and human LDL fractions was determined from solutions containing 30–50 mg/mL LDL and is depicted in Table II. Lp(a) contained in addition to apoB and apo(a) small amounts of apoA and apoC proteins (2–5% of the protein mass). These were not quantified because of lack of material.

Constant amounts of lipoprotein–FC were incubated with equal units of either pig or human LCAT at 37 °C for 1 h in the presence of albumin. Cholesteryl ester formation in pig HDL was 66 and 63 nmol/(mL·h) for pig and human LCAT; corresponding values for human HDL-3 were 68 and 66 nmol/mL·h) (Table III).

In contrast, all LDL fractions and Lp(a) showed a lower conversion of free cholesterol to cholesteryl esters. Pig LDL-1, human LDL, and Lp(a) gave similar esterification rates of approximately 38–42% that obtained with pig HDL or 36–40% that of human HDL-3. LDL-2 led to a production of slightly but significantly ($p < 0.05$) higher cholesteryl ester values of about 43–47% and 41–44% compared to pig HDL or human HDL-3, respectively. No difference was found between native

Table II: Apolipoprotein Content of Pig and Human LDL Fractions before and after Treatment with Lipofundin and/or with Specific Antibodies[a]

fractions	% non-apoB protein[b]	apolipoprotein content in % of non-apoB protein				
		apoA-I	apoA-II	apoC	apoE	albumin
pig						
LDL-1 (n = 4)	5.4 (2.0)	10.3 (2.1)		78.7 (5.4)	7.8 (2.4)	3.2 (1.6)
LDL-2 (n = 4)	3.2 (1.5)	12.8 (2.5)		76.5 (6.3)	6.8 (2.1)	3.9 (1.8)
LDL-1 - A-I (n = 4)	4.4 (1.8)	<0.10		nd	nd	nd
LDL-2 - A-I (n = 4)	2.4 (0.8)	<0.10		nd	nd	nd
PCe-LDL[c] (n = 1)	8.4	12.8		74.9	9.3	3.0
PCe-LDL[c] (n = 1)	7.2	12.1		76.3	8.1	3.5
human						
LDL (n = 4)	4.2 (1.8)	8.7 (2.4)	3.9 (1.1)	73.0 (6.7)	8.3 (2.3)	4.8 (2.1)
LDL - A-I (n = 4)	3.5 (0.9)	<0.10	nd	nd	nd	nd
LDL - C (n = 4)	1.0 (0.2)	10.5 (2.8)	<0.40	<0.40	49.8 (6.8)	39.7 (7.7)
LDL - E (n = 4)	3.6 (1.5)	nd[e]	nd	nd	<0.10	nd
LDL - E/A-I (n = 4)	3.0 (0.8)	<0.12	nd	nd	<0.12	nd
LDL - E/A/C (n = 4)	0.5 (0.1)	<0.70	<0.70	<0.70	<0.70	100.0[d]
PCe-LDL[c] (n = 1)	6.5	10.3	4.1	74.1	9.5	2.0
PCe-LDL[c] (n = 1)	7.0	9.8	3.7	73.9	10.1	2.5

[a] LDL fractions that were specifically depleted from non-apoB protein are designated by the removed apoprotein. The values in parentheses = SD values. [b] Lowry protein in the supernatant after isopropanol treatment (Egusa et al., 1983) in percent of total LDL protein. [c] Determined before removal of apoA-I. [d] We assumed that all residual protein consisted of albumin, since no other proteins could be detected. [e] nd = not detectable.

Table III: Cholesteryl Ester Formation during Incubation of Different Substrates with either Pig or Human LCAT[a]

lipoproteins	pig LCAT		human LCAT	
	nmol of CE/(mL·h) (SD)	%	nmol of CE/(mL·h) (SD)	%
human HDL-3 (n = 4)	68 (7.3)	100	66 (6.4)	100
pig HDL (n = 4)	66 (6.8)	97	63 (6.5)	95
pig LDL-1 (n = 7)	25 (3.6)	38	24 (3.8)	36
pig LDL-2 (n = 7)	30 (4.0)	44	29 (4.2)	44
human LDL (n = 4)	25 (3.3)	37	26 (3.9)	39
human LDL-C[b] (n = 4)	26 (3.7)	38	25 (3.5)	38
Lp(a) (n = 2)	27 (2.8)	40	26 (2.2)	39

[a] Ninety nanomoles of lipoprotein-free cholesterol was incubated in the presence of 4% albumin and 10 mM β-mercaptoethanol with 50 μL of either pig or human LCAT for 1 h at 37 °C in a total volume of 1 mL. Cholesteryl ester (CE) formation was measured in triplicate as described under Materials and Methods. Values are represented as mean nanomoles of CE formed per milliliter of enzyme solution per hour (SD). [b] Specifically depleted from apoC polypeptides by treatment with lipofundin.

and apoC-depleted human LDL fractions. All LDL fractions of this experiment still contained small amounts of apoA-I and apoE, residing on the LDL particles, as checked by immunoelectrophoresis. ApoA-IV, also known as an LCAT activator (Steinmetz & Utermann, 1984), could not be detected by SDS gel electrophoresis on the LDL fractions from both species.

Incubation of LDL-1 and LDL-2 with Differing Amounts of ApoA-I. In order to investigate whether the small but significant difference in cholesteryl ester formation between LDL-1 and LDL-2 was due to a different content in apoA-I on these particles, the amount of apoA-I in LDL was determined by SDS gel electrophoresis as well as by radial immunodiffusion using purified pig apoA-I as a standard. The native LDL subclasses were then incubated for 1 h without and with additionally added pig apoA-I prior to incubation with pig LCAT. For the control the same was performed with liposomes of PC/FC molar ratio 8.4:1. The results of these experiments are plotted in Figure 1 as a function of the total amount of apoA-I in the incubation mixture.

The esterification rate in liposomes without any apoA-I was approximately 0.2–0.6%/h [3–8 nmol/(mL·h) in our assay]. When increasing amounts of purified pig apoA-I were added to the liposomes, maximal cholesteryl ester formation was

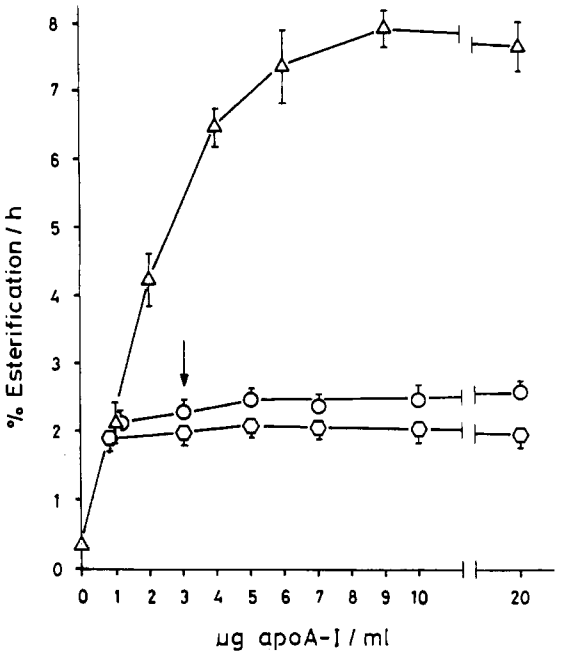

FIGURE 1: Action of pig LCAT on liposomes and pig LDL fractions in the presence and absence of additionally added apoA-I. Incubation of 90 nmol of FC of liposomes (Δ), native LDL-1 (○), and native LDL-2 (○) with pig LCAT was performed as described in Figure 3. The arrow designates the addition of purified pig apoA-I to native LDL fractions. Results represent means (±SD) from three different preparations.

achieved at about 5–10 μg/mL apoA-I. Cholesteryl ester formation using native LDL-1 (containing 0.8 μg of apoA-I/mL of assay volume, residing on the LDL particle as revealed by immunoelectrophoresis) or native LDL-2 (containing 1.2 μg/mL apoA-I) was in a similar range to that of liposomes, where the same amount of apoA-I has been added. However, only a minimal further increase of cholesteryl ester formation was obtained when purified apoA-I was added to the LDL fractions, corresponding to about 33% of the maximal value observed with the liposomes. Despite of the addition of apoA-I, the slight but significant difference in cholesteryl ester formation between LDL-1 and LDL-2 was still present.

Incubation of Pig and Human LCAT with LDL Fractions Depleted Solely from ApoA-I or Depleted from ApoA-I, ApoC, and ApoE. LDL-1 and LDL-2 were depleted from

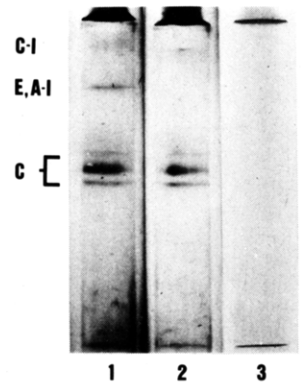

FIGURE 2: Polyacrylamide gel electrophoresis (10%) of human LDL fractions in the presence of 8 M urea: (1) native LDL; (2) apoA-I- and apoE-depleted LDL; (3) LDL depleted from non-apoB protein. Tetramethylurea- (TMU-) soluble proteins of 200 μg of apoLDL were applied. Gels were stained with Coomassie blue 250.

apoA-I by immunoadsorption. Human LDL were liberated either from apoA-I only or in some cases additionally from apoE and/or apoC. The specific removal of non-apoB proteins from LDL was checked by Laurell electrophoresis and by polyacrylamide gel electrophoresis (Figure 2). These LDL fractions, and as control pig HDL, were incubated with equal units of pig or human LCAT in the presence or absence of 20 μg/mL pig or human apoA-I and in the presence of albumin. Human HDL-3 was omitted in this experiment, since no significant differences in the substrate properties between human HDL-3 and pig HDL were found.

Similar results were obtained with the enzymes from both species as shown in Figure 3. Cholesteryl ester formation in pig HDL was 72 and 60 nmol/(mL·h) for pig and human LCAT, respectively. When LDL-1 and LDL-2 without any apoA-I served as substrates, only about 22% and 28% esterification of that of HDL were obtained, regardless of whether pig or human LCAT was used. Human LDL, liberated solely from apoA-I or from apoA-I and apoE, gave similar results as apoA-I-depleted pig LDL-1. The values obtained for human apoE depleted LDL were not significantly different from that of native LDL. In contrast, human LDL, depleted from all non-apoB proteins, produced the lowest esterification, namely, only about 10–15% that of HDL. Under addition of 20 μg/mL either pig or human apoA-I to apoA-I-depleted LDL fractions, the esterification rate of pig LDL-1 and human LDL increased from about 22% to 30% and that of LDL-2 from about 28% to 35% of the values obtained for HDL. In contrast, addition of apoA-I to LDL fractions, from which additionally apoE and/or apoC had been removed, resulted in a further decrease of the esterification rate. This finding was reproducible for all four experiments performed.

Incubation of Pig or Human LCAT with Pig or Human PCe-LDL. In earlier studies (Knipping, 1985) using liposomes of different PC/FC molar ratios, we found that similar esterification rates were produced by incubation with either pig or human LCAT when liposomes with PC/FC molar ratios from 3:1 up to 8.4:1 were used. In contrast, the esterification rates decreased significantly when liposomes with PC/FC molar ratios <3:1 served as substrates.

When we calculated the PC/FC molar ratios of the isolated lipoprotein fractions, we found an average PC/FC molar ratio of 6.09 ± 2.1 for pig HDL (n = 12), 4.91 ± 1.4 for human HDL-3, 1.14 ± 0.1 for LDL-1 (n = 7), 1.31 ± 0.15 for LDL-2 (n = 7), and 1.15 ± 0.19 for human LDL (n = 7). Thus, our LDL fractions resembled in their properties liposomes with PC/FC molar ratios <3:1. Our working hypothesis now was that if the PC/FC molar ratio in LDL is increased to >3:1, we should obtain similar esterification values as with HDL. We therefore prepared pig or human PCe-LDL from native pig LDL-1 and native human LDL as described under Materials and Methods. Before incubation with LCAT, apoA-I was removed from the particles by immunoadsorption. The chemical composition of pig and human PCe-LDL is shown in Table I. Whereas the cholesteryl ester/protein ratio was nearly unchanged in comparison to native LDL, the FC/protein ratio decreased and the PC/protein ratio increased. The PC/FC molar ratio of four different PCe-LDL ranged from 3.8:1 to 6.4:1.

Electron microscopy of native human LDL and of PCe-LDL (PC/FC molar ratio 3.84) revealed that both preparations were homogeneous, indicating that PCe-LDL was totally liberated from liposomes by heparin–Sepharose column chromatography and gradient ultracentrifugation. Enrichment of LDL with phosphatidylcholine led to an increase in diameter from 25.4 ± 1.56 (LDL) to 27.3 ± 1.47 nm (PCe-LDL).

When pig or human PCe-LDL were incubated with either pig or human LCAT in the absence of apoA-I, cholesteryl ester

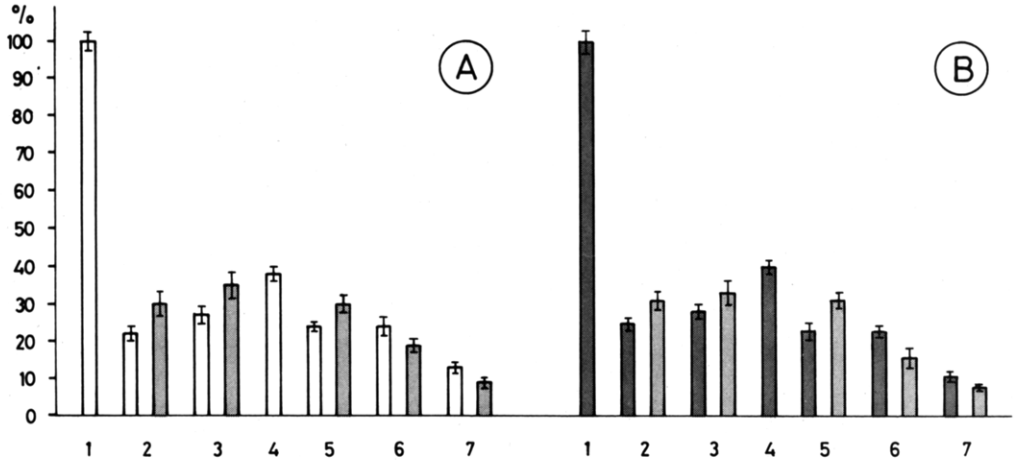

FIGURE 3: Action of pig (A) and human LCAT (B) on various pig and human LDL fractions: LDL fractions were specifically deprived from non-apoB protein by treatment with IgG of different antisera and/or phospholipid-poor lipofundin. Lipoprotein-free cholesterol (90 nmol) was incubated in the presence of 4% albumin with 10 μL of pig LCAT (A) or 20 μL of human LCAT (B) for 1 h at 37 °C and the LCAT activity assayed. Values are expressed as percent of esterification obtained with pig HDL. (1) Pig HDL; (2) apoA-I-depleted pig LDL-1; (3) apoA-I-depleted pig LDL-2; (4) apoE-depleted human LDL; (5) apoA-I-depleted human LDL; (6) apoA-I- and apoE-depleted human LDL; (7) human LDL, depleted from non-apoB protein. Grey bars: the same fractions plus pig (A) or human (B) apo-A-I (final concentration, 20 μg/mL). Results represent means (±SD) of four different preparations.

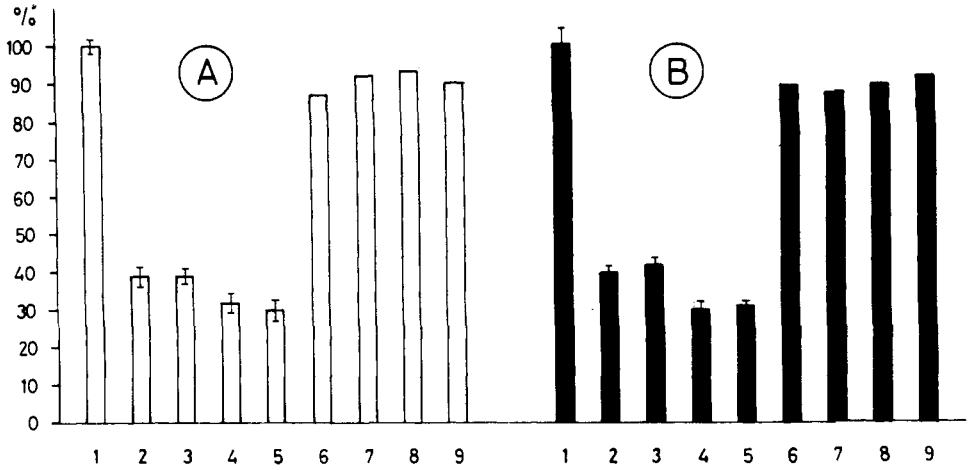

FIGURE 4: Action of pig (A) and human (B) LCAT on native and phosphatidylcholine-enriched LDL fractions. Pig or human PCe-LDL were prepared by incubation of pig LDL-1 or human LDL with liposomes (PC/FC molar ratio 3.5:1 to 4.5:1) and human CETP up to 24 h. Incubation of lipoprotein fractions with pig and human LCAT was performed as described in Figure 3. Values are expressed as percent of esterification obtained with pig HDL. (1) Pig HDL; (2) native pig LDL-1; (3) native human LDL; (4) apoA-I-depleted pig PCe-LDL; (5) apoA-I-depleted human PCe-LDL [bars 1–5 represent means (±SD) of four different preparations]; (6, 7) human PCe-LDL from two different preparations (PC/FC molar ratio 3.8:1) in the presence of 20 μg of apoA-I/mL of assay solution; (8) pig PCe-LDL (PC/FC molar ratio 4.7:1) plus 20 μg of apoA-I/mL; (9) pig PCe-LDL (PC/FC molar ratio 6.4:1) plus 20 μg of apoA-I/mL (bars 6–9 are values of single experiments).

formation was about 30% that of HDL. Addition of 20 μg/mL either pig or human apoA-I significantly increased the esterification rate up to 85–92% that of HDL (Figure 4).

Discussion

By incubating pig HDL or human HDL-3 with pig or human LCAT, we found virtually no significant difference in the substrate properties of both fractions. Therefore, for simplicity we omitted human HDL-3 in later experiments. Pig LDL-1, LDL-2, human LDL, and Lp(a) were also found to serve as substrates for LCAT from both species, since between 36% and 47% of the esterification rates observed with pig HDL or human HDL-3 were obtained. These data are in accordance with those published recently by Rajaram and Barter (1985). In unseparated human plasma, the authors found 56% of the total esterified [^{3}H]cholesterol in HDL, 33% in LDL, and 11% in VLDL. Upon incubation of [^{3}H]cholesterol-labeled human lipoproteins with purified human LCAT for 3 h, the authors could demonstrate that in the absence of CETP 73% of the esterified [^{3}H]cholesterol is found in HDL, 25% in LDL, and only 1% in VLDL. In other words, owing to the action of LCAT, cholesteryl ester formation in human LDL was about 34% that of HDL. This is quite comparable to our study, where cholesteryl ester formation in pig LDL-1 and human LDL was 38–42% that of HDL.

In this study the substrate properties of human LDL did not change, regardless of whether or not apoC polypeptides were removed prior to incubation with LCAT. Except apoC-I these polypeptides are known to have no or—as in the case of apoC-II—even an inhibitory effect on the LCAT reaction with liposomes as substrates (Soutar et al., 1975; Albers et al., 1979; Knipping, 1985). At least in pig LDL we would have expected a change in cholesteryl ester formation, since pig lipoproteins lack apoC-III polypeptides (Knipping et al., 1984) and the removal of apoC-II, the most prominent non-apoB protein on pig LDL, might have led to an increase of cholesteryl ester formation in LDL upon incubation with LCAT. But in contrast to human LDL, we were unable to deplete pig LDL from apoC by incubation with lipofundin. This finding may indicate that pig apoC-II is differently bound to pig LDL in comparison to the corresponding peptide in human LDL.

The substrate specificity of human LDL and pig LDL-1 was comparable. This is not astonishing, since both fractions have many characteristics in common (Jackson et al., 1976; Jürgens et al., 1981; Herak et al., 1984). In contrast, pig LDL-2 produced slightly, but significantly, higher esterification rates than LDL-1. Our opinion that this difference in esterification rates of LDL-1 and LDL-2 was due to a differing content of apoA-I in these fractions could not be confirmed, since addition of purified apoA-I to both LDL fractions following incubation with LCAT did not abolish the difference in cholesteryl ester formation (Figure 1).

Comparing human LDL and Lp(a), we found no difference in substrate specificity: at identical free cholesterol concentrations in the incubation mixture the cholesteryl ester formation in LDL or Lp(a) was the same, suggesting that there is no influence of the apoprotein *a* on the LCAT reaction.

To gain further insight into the role of apolipoproteins on the LCAT reaction, the LDL fractions were specifically liberated either solely from apoA-I or in the case of human LDL additionally from apoE and/or apoC. ApoA-I-depleted or apoA-I- and apoE-depleted LDL fractions still exhibited 22–28% of the esterification rate obtained for HDL. ApoA-I-depleted LDL-2 showed slightly but not significantly higher esterification rates than apoA-I-depleted LDL-1. Removal of apoE from human LDL did not significantly change the esterification in comparison to native human LDL. In a recent abstract by Steinmetz et al. (1985) apoE and apoE isoforms were reported to activate LCAT with an efficiency of only 15–19% that of apoA-I. Since apoE-depleted LDL still contained small amounts of apoA-I, an effect on the esterification rate due to the removal of apoE might not be apparent.

In contrast, human LDL, depleted from all non-apoB proteins, produced much lower values of esterification, reaching only about 10–15% that of HDL. The only difference of this substrate to apoA-I- and apoE-depleted LDL fractions was the additional removal of apoC polypeptides. On the other hand, the specific removal of apoC polypeptides alone did not measurably affect the cholesteryl ester formation in human LDL. It is therefore feasible that the total removal of all non-apoB proteins from the LDL surface alters the particle in a way that is unable to interact with LCAT.

However, it was interesting to note that addition of apoA-I to these LDL fractions showed a positive effect on the LCAT reaction only if the LDL fractions have not been depleted previously from apoA-I together with either apoE and/or apoC

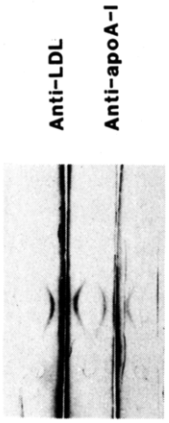

FIGURE 5: Immunoelectrophoresis of human PCe-LDL after incubation with 20 μg of apoA-I. Holes contain PCe-LDL. The plate was stained with Sudan black B.

polypeptides. An additional removal of appoproteins from the LDL surface may have altered the binding sites for apoA-I on the LDL particle in such a way that the added apoA-I was hindered from adopting its original conformation on the LDL particle, although it was bound to it as checked by immunoelectrophoresis. This possibly led to the inability of apoA-I to activate LCAT.

While these studies did not prove why HDL are better substrates than LDL, we presumed that the difference of the PC/FC molar ratio in HDL and LDL may play a role. Pig LDL-1 or human LDL were enriched in phosphatidylcholine, resulting in PC/FC molar ratios of 3.8:1 to 6.4:1. Addition of apoA-I to apoA-I-depleted PCe-LDL increased the cholesteryl ester formation upon incubation with LCAT to a value very similar to that of HDL. The increase in the esterification rate seemed to be due to the altered surface structure of LDL. However, by immunoelectrophoresis using anti-apoA-I or anti-apoB it was demonstrated that upon addition of apoA-I to PCe-LDL both antigens comigrated, forming only precipitin lines at the β-position (Figure 5). This strongly suggests that apoA-I was directly bound to PCe-LDL. This finding makes it very unlikely that lipids, especially phosphatidylcholine, became associated to added apoA-I, forming apoA-I/PC vesicles, which then could have served as substrates for LCAT. As in previous studies using liposomes with PC/FC molar ratio >3:1 (Knipping, 1985), the esterification rate was nearly equal for all PCe-LDL, regardless of whether PC/FC ratios of 3.8:1 or 6.4:1 were used.

Considering that normally one LDL particle has about 10 times as much phospholipid as one HDL particle, the question now is, why an increase of the PC/FC molar ratio in LDL plays a role in the LCAT reaction. HDL and LDL have a similar overall structure (Morrisett et al., 1975) with an outer monolayer of phospholipids surrounding a hydrophobic core. From ^{13}C NMR studies it is known that some of the free cholesterol of HDL is in the core (Avila et al., 1978), but much of it is located in an environment that could be next to protein (Hauser & Kostner, 1979). Yeagle et al. (1982) suggested that there may be sterol–protein interaction in HDL that is absent in LDL. The same authors proposed that much of the free cholesterol in LDL is not in the surface layer of the phospholipids. Additionally about one-fifth of the LDL phospholipids seems to be immobilized by the intact apoB of LDL (Yeagle et al., 1977). One may speculate that the phospholipids and/or free cholesterol in LDL are probably not sufficiently accessible for LCAT or, more probably, for cofactor proteins e.g., apoA-I. Enrichment of phospholipids in LDL may result in a reorientation of the surface lipids in LDL by changing the rigidity of the surface, thus allowing apoA-I or LCAT to interact better with this substrate. This latter theory would also fit with the results obtained for liposomes with PC/FC molar ratios <3:1 (Knipping, 1985). To solve these questions, a more detailed analysis on the mechanism of the interaction of LCAT with its substrate and of the activation reaction will be required.

In conclusion, we can say that native LDL are substrates for LCAT. Although these results were obtained with isolated LDL fractions, we found that by incubation of LCAT active pig serum LDL are substrates for LCAT, even in presence of HDL (unpublished results). This finding is also in accordance with the situation in pig plasma, where no or only minimal cholesteryl ester transfer activity is demonstrable, and therefore could explain the great amount of cholesteryl esters in LDL. The idea that LCAT does not act only on HDL but also on LDL and that this reaction is also of physiological relevance in human plasma is supported by the work of Carlson and Holmquist (1985a,b) on fish-eye disease. Their reports gave evidence that in such patients LCAT acts only on apoB-containing lipoproteins.

ACKNOWLEDGMENTS

We dedicate this article to Professor Dr. Anton Holasek, who has stimulated our investigations through many discussions, on the occasion of his 65th birthday. We are grateful to R. Moser and B. Hammer for excellent technical assistance.

Registry No. LCAT, 9031-14-5.

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Alteration of pK_a of the Bacteriorhodopsin Protonated Schiff Base. A Study with Model Compounds†

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ABSTRACT: Factors influencing the pK_a value of retinal protonated Schiff base ($RSBH^+$) are examined by using fluorinated alcohols and series of retinals bearing nonconjugated positive charges along the polyene. It is shown that the effective pK_a of $RSBH^+$ is increased by a solvent, forming a strong hydrogen bond, that stabilizes the anion but weakly interacts with the Schiff-base proton. A positive charge in the vicinity of the Schiff-base linkage markedly reduces the effective pK_a. The effect is significantly enhanced in fluorinated alcohols in which positive charges are weakly solvated. It is suggested that drastic pK_a reduction might take place during bacteriorhodopsin (bR) photocycle either by elimination of hydrogen-bonding stabilization or by a positive charge approaching the Schiff-base linkage. Weak solvation of the positively charged Schiff-base nitrogen (relative to ethanol solution) and strong solvation with its counterion lead to a red shift in the absorption maximum of retinal protonated Schiff base up to ca. 2400 cm^{-1} in hexafluoro-2-propanol relative to ethanol. This mechanism of introducing red shift in the absorption maximum of $RSBH^+$ might play a role in determining part of the opsin shift found in bR and the red shift observed in the transformation from the bR_{570} to K_{610} intermediate following light absorption. Nonconjugated positive charges shift the absorption maximum of $RSBH^+$. Their influence is further enhanced with fluorinated alcohols as solvents.

The purple membrane of *Halobacterium halobium* functions as a light-driven proton pump due to its pigment bacteriorhodopsin, a substance comprised of a retinal chromophore bound covalently at an ϵ-aminolysine residue of a protein via a protonated Schiff base [see Stoeckenius et al. (1979), Ottolenghi (1980), and Birge (1981) for reviews]. The pigment was found to exist in two forms: a light-adapted form absorbing at 570 nm (bR_{570})[1] with an *all-trans*-retinal chromophore and a dark-adapted modification absorbing at 560

† This work was supported by the Fund for Basic Research (Administered by Israeli Academy of Sciences and Humanities), by Minerva Foundation, Munich, and by Lillian Schermer Trust. M.S. is Incumbent of the Morris and Ida Wolf Career Development Chair.

[1] Abbreviations: bR_{570}, bacteriorhodopsin, the subscript denoting the wavelength of maximum absorption; HFIP, hexafluoro-2-propanol; RSB, retinal Schiff base; $RSBH^+$, retinal protonated Schiff base; TFE, trifluoroethanol; FT-IR, Fourier transform infrared spectroscopy.

0006-2960/86/0425-5249$01.50/0